

Microbial Plant Pathogens

Microbial Plant Pathogens

Detection and Management
in Seeds and Propagules

Volume 1

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WILEY Blackwell

This edition first published 2017 © 2017 John Wiley & Sons, Ltd.

Registered Office

John Wiley & Sons, Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

Editorial Offices

The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

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Library of Congress Cataloging-in-Publication Data

Names: Narayanasamy, P., 1937– author.

Title: Microbial plant pathogens : detection and management in seeds and propagules / P. Narayanasamy.

Description: Hoboken : John Wiley & Sons, 2017. | Includes bibliographical references and index.

Identifiers: LCCN 2016036177 (print) | LCCN 2016037345 (ebook) | ISBN 9781119195771 (cloth) |

ISBN 9781119195788 (pdf) | ISBN 9781119195795 (epub)

Subjects: LCSH: Seed-borne phytopathogens. | Seed-borne plant diseases.

Classification: LCC SB732.8 .N27 2017 (print) | LCC SB732.8 (ebook) | DDC 632/.3–dc23

LC record available at <https://lcn.loc.gov/2016036177>

A catalogue record for this book is available from the British Library.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Set in 10/12pt Warnock by SPi Global, Pondicherry, India

Dedicated to the memory of my parents for their love and affection

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Preface

Agricultural and horticultural crops are raised by using seeds or propagules whose health status has to be ensured for healthy and high-quality produce to satisfy the needs of human and animal populations. From time immemorial, seeds and propagules were obtained from selected plants growing in the wild environment, saved to grow plants of subsequent generations. In doing so, the improvement of quality parameters such as appearance, color, aroma, and taste was achieved by selecting plants with desired characteristics. However, due importance was not allocated to select plants with resistance to diseases caused by microbial plant pathogens, resulting in progressive increase in the susceptibility of plants to diseases and phenomenal crop losses which were considered responsible for dreadful famines and untold human suffering. Several diseases transmitted through seeds/propagules have been found to be highly destructive, with the potential to ruin the economy of certain countries. Such critical conditions were primarily ascribed to the failure to select disease-free seeds and propagules for future generations of crops.

Early detection and precise identification of the pathogen(s) present in seeds/propagules which are involved in a disease(s) occurring at a geographical location constitute the basic strategy for development of effective disease management systems suitable for various agroecosystems. Studies of pathogen biology, the infection process, and epidemiology of crop diseases have highlighted the weak links in the life cycles of microbial pathogens in order to disrupt pathogen development and the progress of disease under field conditions. The principles of crop disease management are essentially based on exclusion, eradication, and immunization and various disease management strategies emerge from these principles. The need to produce disease-free seeds and propagules to restrict the introduction of pathogens into fields/new locations and subsequent disease spread has been clearly indicated by different investigations. The role of quarantines and certification programs in excluding the introduction of new diseases into a country where the pathogen may be absent or less important has been well realized. The effectiveness of adoption of simple cultural practices in restricting disease incidence and further spread has been indicated in some pathosystems. The development of crop cultivars with built-in resistance to diseases, the most economical disease management strategy, has been achieved through traditional breeding methods or biotechnological approach and has been shown to be instrumental in keeping many diseases under check. Employing biological control agents is advantageous, since this strategy has been demonstrated to be effective not only in restricting the disease incidence and spread but also in preserving the ecological environments. The application of

chemicals, although more effective, must be restricted because of the accumulation of chemical residues in grains and other food materials as well as significant environmental pollution. The integration of compatible disease management strategies has been shown to provide additive effects, resulting in enhancement of effectiveness of the disease management systems; greater efforts have therefore been made to explore the possibility of developing integrated disease management systems for several economically important diseases that need to be tackled more effectively.

This book includes information distilled from over 2500 citations following an extensive literature search, with the aim of providing a comprehensive knowledge of various aspects of microbial plant pathogens transmitted via seeds and propagules, particularly pathogen detection and management of diseases caused by them. Graduate students, researchers, and teachers of Plant Pathology, Plant Protection, Microbiology, Plant Breeding and Genetics, Agriculture and Horticulture, and especially certification and quarantine personnel, will find the information presented in this book useful. Protocols appended at the end of relevant chapters form a unique feature of this book and may assist researchers in fine-tuning their projects.

P. Narayanasamy
Coimbatore, August 2016

Acknowledgement

The author gratefully expresses his sincere appreciation to his alma mater for being an unlimited source of inspiration in reaching his present position of being able to share his experience and thoughts with those who have contributed to the advancement of science in general and plant pathology in particular. He heartily thanks the staff and students of the Department of Plant Pathology, Tamil Nadu Agricultural University for their assistance in one way or another in improving the usefulness of this book to the audience. The efforts of the author to provide useful information on the selected subject matter in this book have become fruitful thanks to the enormous loving support of his wife Mrs N. Rajakumari whom he thanks profusely. With immense pleasure, the author conveys his appreciation to his family members Mr N. Kumar Perumal, Mrs Nirmala Suresh, Mr T. R. Suresh and Mr S.Varun Karthik for encouraging and uplifting his spirits with their affectionate, amiable attitude and deeds.

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Microbial Plant Pathogens

Microbial Plant Pathogens

Detection and Management
in Seeds and Propagules

Volume 2

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WILEY Blackwell

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Library of Congress Cataloging-in-Publication Data

Names: Narayanasamy, P., 1937– author.

Title: Microbial plant pathogens : detection and management in seeds and propagules / P. Narayanasamy.

Description: Hoboken : John Wiley & Sons, 2017. | Includes bibliographical references and index.

Identifiers: LCCN 2016036177 (print) | LCCN 2016037345 (ebook) | ISBN 9781119195771 (cloth) |

ISBN 9781119195788 (pdf) | ISBN 9781119195795 (epub)

Subjects: LCSH: Seed-borne phytopathogens. | Seed-borne plant diseases.

Classification: LCC SB732.8 .N27 2017 (print) | LCC SB732.8 (ebook) | DDC 632/.3–dc23

LC record available at <https://lcn.loc.gov/2016036177>

A catalogue record for this book is available from the British Library.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Set in 10/12pt Warnock by SPi Global, Pondicherry, India

10 9 8 7 6 5 4 3 2 1

Dedicated to the memory of my parents for their love and affection

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Preface

Agricultural and horticultural crops are raised by using seeds or propagules whose health status has to be ensured for healthy and high-quality produce to satisfy the needs of human and animal populations. From time immemorial, seeds and propagules were obtained from selected plants growing in the wild environment, saved to grow plants of subsequent generations. In doing so, the improvement of quality parameters such as appearance, color, aroma, and taste was achieved by selecting plants with desired characteristics. However, due importance was not allocated to select plants with resistance to diseases caused by microbial plant pathogens, resulting in progressive increase in the susceptibility of plants to diseases and phenomenal crop losses which were considered responsible for dreadful famines and untold human suffering. Several diseases transmitted through seeds/propagules have been found to be highly destructive, with the potential to ruin the economy of certain countries. Such critical conditions were primarily ascribed to the failure to select disease-free seeds and propagules for future generations of crops.

Early detection and precise identification of the pathogen(s) present in seeds/propagules which are involved in a disease(s) occurring at a geographical location constitute the basic strategy for development of effective disease management systems suitable for various agroecosystems. Studies of pathogen biology, the infection process, and epidemiology of crop diseases have highlighted the weak links in the life cycles of microbial pathogens in order to disrupt pathogen development and the progress of disease under field conditions. The principles of crop disease management are essentially based on exclusion, eradication, and immunization and various disease management strategies emerge from these principles. The need to produce disease-free seeds and propagules to restrict the introduction of pathogens into fields/new locations and subsequent disease spread has been clearly indicated by different investigations. The role of quarantines and certification programs in excluding the introduction of new diseases into a country where the pathogen may be absent or less important has been well realized. The effectiveness of adoption of simple cultural practices in restricting disease incidence and further spread has been indicated in some pathosystems. The development of crop cultivars with built-in resistance to diseases, the most economical disease management strategy, has been achieved through traditional breeding methods or biotechnological approach and has been shown to be instrumental in keeping many diseases under check. Employing biological control agents is advantageous, since this strategy has been demonstrated to be effective not only in restricting the disease incidence and spread but also in preserving the ecological environments. The application of

chemicals, although more effective, must be restricted because of the accumulation of chemical residues in grains and other food materials as well as significant environmental pollution. The integration of compatible disease management strategies has been shown to provide additive effects, resulting in enhancement of effectiveness of the disease management systems; greater efforts have therefore been made to explore the possibility of developing integrated disease management systems for several economically important diseases that need to be tackled more effectively.

This book includes information distilled from over 2500 citations following an extensive literature search, with the aim of providing a comprehensive knowledge of various aspects of microbial plant pathogens transmitted via seeds and propagules, particularly pathogen detection and management of diseases caused by them. Graduate students, researchers, and teachers of Plant Pathology, Plant Protection, Microbiology, Plant Breeding and Genetics, Agriculture and Horticulture, and especially certification and quarantine personnel, will find the information presented in this book useful. Protocols appended at the end of relevant chapters form a unique feature of this book and may assist researchers in fine-tuning their projects.

P. Narayanasamy
Coimbatore, August 2016

Acknowledgement

The author gratefully expresses his sincere appreciation to his alma mater for being an unlimited source of inspiration in reaching his present position of being able to share his experience and thoughts with those who have contributed to the advancement of science in general and plant pathology in particular. He heartily thanks the staff and students of the Department of Plant Pathology, Tamil Nadu Agricultural University for their assistance in one way or another in improving the usefulness of this book to the audience. The efforts of the author to provide useful information on the selected subject matter in this book have become fruitful thanks to the enormous loving support of his wife Mrs N. Rajakumari whom he thanks profusely. With immense pleasure, the author conveys his appreciation to his family members Mr N. Kumar Perumal, Mrs Nirmala Suresh, Mr T. R. Suresh and Mr S.Varun Karthik for encouraging and uplifting his spirits with their affectionate, amiable attitude and deeds.

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Volume 1

Pathogen Detection and Identification

1

Introduction

Plant diseases are caused by biotic and abiotic stresses. The microbial plant pathogens – oomycetes (fungus-like), fungi, bacteria, phytoplasmas, viruses, and viroids – form a major group of biotic stresses causing a variety of diseases in various crops grown in different agroecosystems, whereas abiotic stresses are due to adverse environmental conditions which may have an influence both on the plants and the pathogens. The environmental conditions have an important role in the buildup of inoculum and in predisposing the plants to infection by the pathogens to a different extent, depending on the geographical locations where the infected seeds/propagules are to be planted.

1.1 Concepts and Implications of Pathogen Infection of Seeds and Propagules

Use of clean seed and propagules (vegetatively propagated planting materials) is emphasized as the basic step for the commencement of implementation of strategies of crop disease management systems. Seedborne pathogens are not confined to the spermosphere alone and they may also be present in or on the surface of other organs or storage tissues of the plant. Seed pathology is considered a branch of plant pathology, devoted to the detection of microorganisms associated with seeds. That seeds are carriers of microbial plant pathogens, resulting in the infection of developing seedlings, has been known for a long time. The Danish Government Institute of Seed Pathology (DGISP) for developing countries was established in 1967 at Copenhagen by the concerted efforts of Dr Paul Neergaard, who is considered as the Father of Seed Pathology. The term “seed pathology” was introduced by him during investigations of seedborne pathogens and the diseases caused by them. As the Chairman of the Plant Disease Committee (PDC) of the International Seed Testing Association (ISTA), Dr Neergaard has contributed immensely to the standardization of methods for detection of seedborne fungi. He authored the book *Seed Pathology*, which contains voluminous information on the various aspects of seed pathology and is the main reference book for all plant pathologists. Seed pathology has developed into an important discipline within plant pathology. The propagules are the “seed” for the asexually propagated crops, and the importance of propagules carrying pathogens in the incidence and spread of crop diseases has been emphasized by several investigations.

The role of seed- and propagule-borne inoculum in crop disease epidemiology and the indispensability of using disease-free seeds and propagules for profitable crop cultivation are well realized. Several seed- and propagule-borne pathogens have been shown to be disseminated and perpetuated through various other means. Comprehensive studies are therefore essential to elucidate the host–pathogen interactions, leading to the incidence and spread of diseases caused by seed- and propagule-borne pathogens and consequent substantial losses in quantity and quality of the produce induced by them. Some seedborne pathogens may become soilborne when the infected seeds are planted, and the pathogen present in the soil can infect the plants systemically and infect different tissues en route to the seeds as in the case of *Rhizoctonia solani* causing rice sheath blight (Narayanasamy 2002). *Fusarium graminearum*, incitant of the Fusarium head blight (FHB) disease of wheat, develops on the infected plant debris left on the soil after harvest. The inoculum from the infested tissues reaches spikes at the time of anthesis through wind currents (Inch and Gilbert 2003). *Verticillium dahliae* is seedborne in spinach. The possibility of spinach seeds becoming soilborne and infecting lettuce plants was explored under microplot conditions. Verticillium wilt developed on lettuce following two or three plantings of *Verticillium*-infested spinach seeds/plant tissues. Polymerase chain reaction (PCR) assay was employed to detect and confirm the identity of *V. dahliae* in infected lettuce plants obtained from the microplots. In addition, transmission of a green fluorescent protein (GFP) -tagged mutant strain of *V. dahliae* from spinach to lettuce was demonstrated in greenhouse experiments. The results provided clear evidence that the transmission of *V. dahliae* introduced through infected spinach seed could be the source of inoculum for Verticillium wilt disease in lettuce (Short et al. 2015).

Seeds and propagules infected by microbial pathogens form the primary sources of inoculum from which they are disseminated by other modes of transmission such as soil, water, or wind. As the environmental conditions have significant effects on the pathogen and the crops, incorporation of epidemiological concepts and the effects of various disease management strategies have to be considered as components of seed pathology. As the propagules are the seed for the vegetatively propagated crops, the propagule-borne pathogens have to be managed by applying different strategies as in the case of seedborne pathogens. It is logical to widen the scope of research and applications pertaining to the microbial pathogens associated with the seeds and propagules, and to develop management systems for restricting the incidence and spread of the diseases caused by them.

1.2 Economic Importance of Seed- and Propagule-Borne Microbial Pathogens

Microbial pathogens have been demonstrated to be the causes of various crop diseases of great historical and economic importance. A major catastrophe due to potato late blight disease caused by *Phytophthora infestans* occurred during the 1840s, resulting in the infamous Irish potato famine. The acute food shortage was primarily responsible for the migration of about 1.5 million people from Ireland to other countries (Large 1940). A similar magnitude of human suffering due to another fungal pathogen, *Helminthosporium oryzae*, ruined the rice crops extensively in India. It has

been estimated that about 2 million persons perished due to starvation, because of the exorbitant cost of rice which was beyond the reach of the majority of Indians during that decade (Padmanabhan 1973). These two pathogens, transmitted through infected potato tubers and rice grains respectively, revealed the economic importance and social relevance of the pathogens which are either seed- or propagule-borne. *Fusarium* head blight (FHB) disease infecting wheat and barley is caused by different *Fusarium* spp. and still remains an enigma for all involved in cereal production. FHB disease is considered as a re-emerging disease of devastating impact on cereal grain production. A series of severe FHB epidemics that occurred in the USA and Canada during 1991–1996 led to unparalleled economic and sociological impact in the Northern Great Plains region. The combined direct and secondary economic losses between 1993 and 2001 were estimated to cost US \$7.7 billion in nine states of USA (Nganje et al. 2004; McMullen et al. 2012). In addition, the fungal pathogens involved in FHB disease produce different kinds of mycotoxins capable of causing ailments in human beings and animals if contaminated grains and feed are consumed. The quantitative and qualitative losses are very difficult to estimate precisely.

Rice bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* is widespread in all SE Asian countries. In Japan, rice crops in 300,000–400,000 ha were infected by BLB disease with yield losses ranging over 20–30% (Ou 1972). The estimated yield losses due to BLB disease in tropical Asia may vary over 2–74% depending on the location, season, weather, cultivar, and stage of crop growth at the time of infection. Yield loss induced at the kresak phase of the disease was found to be greater in India (Srivastava and Kapoor 1982; Reddy 1989). The bacterial pathogen *Xanthomonas axonopodis* pv. *citri* causing citrus canker disease was considered to have been introduced into the USA from Asian countries through infected budwood materials. Quarantine laws were imposed as soon as the first incidence of citrus canker was observed, and the massive eradication of 20 million citrus trees at a cost of US \$20 million effectively checked the spread of the disease (Schumann 1991). Tomato bacterial canker disease caused by *Clavibacter michiganensis* subsp. *michiganensis* is considered to be one of the most destructive bacterial diseases in tomatoes with the potential to cause heavy yield losses. In Ontario, Canada yield losses of up to 84% in commercial fields were recorded (Poysa 1993). In Michigan State, USA tomato bacterial canker disease accounted for as much as US \$300,000 in a single year for individual processing tomato growers (Hausbeck et al. 2000). Because of the potentially destructive nature of the pathogen, the Good Seed and Plant Practices Organization functioning in the Netherlands and France directed disease management strategies for preventing infection of tomato seeds and avoiding the use of infected seedlings (de León et al. 2011; Sen et al. 2015). Huanglongbing (HLB), also referred to as greening of citrus caused by *Candidatus Liberibacter asiaticus*, has been known to affect citrus production in East Asia for more than a century (Bové 2006); the disease is now found in threatening proportions in the USA and other countries. Tree decline, premature fruit drop and formation of small misshapened fruits lead to drastic production losses, incurring an estimated loss of US \$3.63 billion in revenue in Florida alone (Hodges and Spreen 2013; Wang and Trivedi 2013). The HLB disease can be spread through infected nursery stock and the Asian citrus psyllid *Diaphorina citri*, indicating the need for directing management strategies toward production of certified pathogen-free nursery stock, eradication of infected trees, and reduction of vector populations through chemical application (Gergerich et al. 2015).

The socioeconomic consequences of seedborne viruses such as *Bean common mosaic virus* (BCMV) and *Lettuce mosaic virus* (LMV) have been revealed by several investigations (Stewart and Reddick 1917; Zink et al. 1956; Grogan 1983). The number of seedborne viruses reported increased from 47 in 1969 (Bennett 1969), to 85 in 1974 (Phatak 1974) to 119 in 1981 (Mandahar 1981), and the number of viruses reported to be transmitted via seeds is increasing steadily with time. In addition, viruses are also known to be readily transmitted through propagules. Infected plants may remain asymptomatic because of long latent (incubation) periods required for symptom expression in the mother plants, especially in perennial fruit trees if infection occurs late in the growing season. Planting materials prepared from such plants carry the virus(es) and symptoms of infection may be expressed when they are planted later. These plants may establish poorly, exhibit decline, and the produce from such plants may be of poor quality; infection by viruses leads to both quantitative and qualitative losses (Narayanasamy 2011). *Plum pox virus* (PPV) causes one of the most devastating virus diseases (sharka) infecting stone fruits in all countries. Sharka disease, once limited to Europe for most of the twentieth century, has spread to Africa, South America, North America, and Asia (OEPP/EPPO 2004). Long-distance spread occurs primarily through grafting for producing propagation materials, and aphids may have an important role for local dispersal in some locations. Transmission of PPV via pollen also has been observed (Isac et al. 1998). The incidence of PPV reached such alarming proportions that the destruction of 648 ha of stone fruit orchards in Pennsylvania was necessary during the decade from when the disease incidence was first observed in 1999. The eradication cost was estimated at US \$53 million (Welliver 2012). On the other hand, the cost associated with sharka disease management in Europe was estimated to exceed 10 billion Euros prior to 2006 (Cambra et al. 2006).

Citrus tristeza virus (CTV), existing in the form of many strains, induces different kinds of symptoms depending on the virulence of the CTV strain, cultivar, and the scion/rootstock combinations. CTV spreads primarily through infected plants and budwood; secondary spread is through different aphid species, *Toxoptera citricida* being most efficient in transmitting the virus from infected to healthy plants. Citrus decline due to CTV has taken a heavy toll in several countries and the virus killed more than 100 million trees propagated widely on sour orange rootstock during the last 80 years in South America, the USA, Spain, and Israel (Moreno et al. 2008). When two or more CTV strains infect citrus plants simultaneously complex symptoms appear, making it difficult to establish the identity of the strain(s). The complexity increases further because of continuous recombinations occurring in nature, resulting in the appearance of new or more virulent strains (Mukhopadhyay 2011). Severe epidemics occurred in locations where the vector *T. citricida* was active and was found in large populations in Brazil and Venezuela (Michaud 1998). The economic impact of CTV on the citrus industry could be lessened considerably by strengthening quarantine and certification programs and using certified virus-tested budwood (Gergerich et al. 2015).

1.3 Nature of Seed- and Propagule-Borne Microbial Pathogens

Microbial pathogens infecting plants may be carried in and/or on the seeds and propagules in an active or dormant stage. These planting materials may or may not exhibit visually recognizable symptoms of pathogen infection. Under favorable environments,

the fungal pathogens may multiply and infect the emerging seedlings or become systemic and develop in the apical tissues of the plants without expressing symptoms of infection, as in the case of cereal smut diseases. Detection of the presence of the pathogens based on the symptoms alone will not be sufficient to establish the identity of the pathogens present in seeds and propagules. Several techniques – conventional methods, immunoassays, and molecular techniques – have been applied for the detection, identification, differentiation, and quantification of fungal pathogens rapidly and precisely (Chapter 2). The genetic diversity to determine the variability of isolates/strains in pathogenicity, survival ability, production of toxins, and sensitivity to environments and fungicides has to be assessed to develop effective systems of management of diseases caused by the fungal pathogens (Chapter 3). Fungal pathogens follow different paths of invasion to reach the seed tissues or storage organs. Monitoring the movement of pathogens from seed-to-plant and plant-to-seed is essential to break the vulnerable link in the life cycle of the fungal pathogens so that the progress of disease development may be arrested, resulting in the failure of infection/disease development (Chapter 4).

Bacterial pathogens with simpler structural characteristics compared to fungal pathogens require detection techniques based on their biochemical, physiological, immunological, and molecular properties for their detection, identification, differentiation, and quantification. Nucleic acid-based techniques have been shown to be more sensitive and reliable and provide precise results rapidly. The phytoplasmal pathogens defying all efforts to culture them in cell-free media have to be examined in planta only. Most of them are unable to become seedborne because they induce sterility in plants to different extents. Infection of vegetative propagules is the most important method of the transmission of phytoplasmas. They are also transmitted by natural biological vectors. Various diagnostic techniques have therefore been evaluated for their efficacy in detecting and differentiating different phytoplasmal pathogens present in the propagules of plants selected for propagation (Chapter 5). Biological properties of and the process of infection by bacterial pathogens are discussed, with a view to focusing attention on the vulnerable stages in the population buildup and formation of infection foci of bacterial pathogens to facilitate the application of effective disease management strategies. The sensitivity of bacterial pathogens to chemicals and adverse environmental conditions indicates the possibility of selecting suitable disease management strategies for suppressing the development of the diseases caused by these pathogens (Chapter 6).

Plant viruses and viroids have the simplest structure with no physiological activity of their own; they are therefore dependent on the synthetic machinery of the host plants for their replication. Both viruses and viroids are seed- and/or propagule-borne in several plant species while the viruses have, in addition, specific biological vectors for their dissemination to different healthy plants and in locations. On the other hand, viroids do not appear to have any proven vector for their dispersal. Various techniques based on the biological, immunological, and nucleic acid properties of viruses have been applied for the detection, identification, and differentiation of viruses and their strains with varying degrees of efficiency, depending on the sensitivity of the techniques employed. Nucleic acid-based techniques are preferred because of their high levels of sensitivity, rapidity, and reliability. Viroids with single-stranded RNA alone as their genome are detected and identified more frequently by employing nucleic acid-based techniques as compared to biological tests which require a longer time and more labor and space (Chapter 7). The process of infection of plants by viruses has been intensively studied using electron microscopy, immunoassays, and nucleic acid-based methods.

The movement of viruses from the point of inoculation to other tissues systemically and finally to seed tissues has been monitored. The investigations have indicated the obstacles to be overcome to target the viruses by using antiviral chemicals which cannot interact with the viruses without affecting plant tissues (Chapter 8).

1.4 Development of Crop Disease Management Systems

The management of crop diseases caused by seed- and propagule-borne microbial pathogens depends largely on the understanding of the effects of various factors – for example, susceptibility/resistance levels of crop cultivars, virulence of the pathogen species/strains, and the prevailing environmental conditions during crop seasons – on the incidence and severity of diseases caused by the pathogens. These factors favoring incidence and severity of diseases leading to epidemics are discussed in Chapter 9. All research efforts to understand the host–pathogen interactions have been made with the ultimate goal of containing the development and spread of crop diseases under various agroecosystems. Disease management strategies are based on a three-pronged thrust on the pathogens which are endowed with various armories to deactivate the defense mechanisms of the host plant species. The major principles of disease management systems are exclusion, eradication, and immunization.

The disease management strategies based on the principle of exclusion include the establishment of certification and quarantine programs and the use of disease-free seeds and propagules to prevent the introduction of pathogens from other countries or areas where the pathogen may not be present or not important (Chapter 10). Various cultural practices and physical and chemical techniques have been shown to reduce the pathogen inoculum, resulting in the reduction in the rate of pathogen population buildup and consequent reduction in incidence and severity of crop diseases (Chapter 11). The selection of crop genotypes for high yield potential has led to increased levels of susceptibility of cultivars to several disease(s). The genetic potential of the cultivars to tolerate/resist infection by microbial pathogens could be enhanced by adopting traditional breeding procedures. However, in the absence of dependable sources of resistance among wild relatives of the crop species, biotechnological methods have extended a helping hand to enhance the levels of resistance of cultivars by introgressing gene(s) from diverse sources of plants and animals; such methods are not possible through conventional breeding procedures (Chapter 12).

Crop disease management strategies aimed to adversely affect the pathogen development directly involve the use of biotic and abiotic biological control agents and/or chemicals. Fungi, bacteria, and viruses have been demonstrated to inhibit the pathogen development to varying degrees. The biocontrol potential of the biotic agents differed widely with species/strains which suppress the pathogen development through different mechanisms of action. As can be expected of biological materials, the effectiveness of most biological control agents varies widely because of the competition with other microorganisms as well as the pathogens for nutrients and space. In general, mixtures of strains/species of biological control agents have been found to be more effective than an individual species/strain. Abiotic agents such as manures, organic compounds of plant and animal origin, and inducers of resistance to crop diseases have been shown to have potential for reducing the incidence and spread of crop diseases. Use of biotic and abiotic biological control agents for crop disease management is an ecofriendly strategy

with no adverse effect on nontarget organisms, and is also compatible with existing cultural practices applied in different geographic locations. However, commercially available formulations are very few compared with the number of chemicals available in the market. The enhancement of resistance of crop cultivars using inducers/activators of resistance of crop cultivars against microbial pathogens appears to be a productive approach for containing crop diseases (Chapter 13).

Disease management using fungicides, bactericides, antibiotics, and antiviral chemicals is reported to be more effective than other disease management tactics which may be difficult to apply because of crop-imposed limitations or a lack of cost-effectiveness or impractical nature. Several kinds of chemicals have been used for seed/propagule treatment, soil application, and foliar spray, and different levels of effectiveness of chemical application have been demonstrated. The development of resistance to chemicals in fungal and bacterial pathogens has been observed as these pathogens are able to produce new strains that can tolerate the dosage of chemicals recommended for application, necessitating the replacement or withdrawal of such chemicals to which the pathogens have become less sensitive/resistant. In addition, the presence of residues of chemicals in treated grains, vegetables, and fruits and the environmental pollution remain strong impediments for the continued use of chemicals against plant pathogens. A constant search is underway for alternative strategies leading to a reduction in the quantum of chemicals applied or to replacement with other biological materials (Chapter 14).

Various strategies of crop disease management may be able to individually offer protection to plants, but often below the desired levels. Some efforts have been made to integrate strategies that are compatible with each other (Chapter 15), so that the combined application of two or more tactics may be more effective than when applied individually. It will be worthwhile intensifying such investigations in order to assess the advantages of integrating as many strategies as possible to derive more benefits to the growers and to provide the consumers with healthy food materials in future. No single management tactic is known to be effective under the varied ecological conditions in which various crops are cultivated. Choosing suitable strategies and integrating them into crop production systems is therefore essential. As management decisions are primarily based on economic consideration (benefit: cost ratio), some technologies have not been implemented due to a lack of cost-effectiveness, despite having been shown to be effective in suppressing disease development. The impact of adopting effective management systems on social systems and human welfare is discussed to indicate their sustainability over several years.

This book contains the latest information gathered from an extensive literature search that will assist researchers, teachers in departments of Plant Pathology, Microbiology, Molecular Biology and Biotechnology, Plant Physiology, and Plant Breeding and Genetics, as well as the personnel of Certification and Plant Quarantine programs. The protocols presented at the end of certain chapters as appendices will provide a useful start to investigations.

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2

Detection and Identification of Fungal Pathogens

Microbial plant pathogens – oomycetes, fungi, bacteria, viruses, and viroids – infect true seeds and propagules such as tubers, corms, setts, and cuttings. These become the primary sources of inoculum that may introduce the disease(s) into a new location or field where the incidence of the disease(s) may be limited or absent, and also carry the pathogens to the subsequent generations of crop plants. In order to prevent the establishment of new pathogens, early detection and precise identification of pathogens in seeds and propagules is an important component of integrated disease management systems. As specific restrictions are imposed by different governments to protect domestic crop production from pathogens likely to be introduced through imported seeds and propagules, sensitive detection and precise identification of the fungal pathogens is essential. Both conventional isolation-based methods and modern isolation-independent techniques have been applied with varying degrees of effectiveness and reliability. Quarantine and certification programs have been established in most countries around the world to examine different kinds of plants and plant materials for freedom from diseases and pests affecting the crop(s) concerned. Conventional methods employed for examining seeds and propagules include visual inspection for disease symptoms, microscopic examination, and culturing the fungal pathogens in appropriate culture media. Blotter test, grow-out test, and direct plating on agar media are applied to assess the extent of seed health. Bait tests have been found to be useful for the detection of fungal pathogens in the roots of susceptible crop plants. Isolation-independent immunoassays and nucleic acid (NA-) based procedures provide more precise, sensitive, reliable, and rapid results. Further, the results of these techniques are not influenced by the environmental conditions to which the test materials are exposed prior to testing (Narayanasamy 2001, 2011).

2.1 Detection and Differentiation of Fungal Pathogens in Seeds

Fungi associated with seeds may be pathogenic or they may be saprophytes developing on seed surface competing with the pathogens for space and nutrients. The saprophytic fungi may produce toxic compounds that may cause seed rots, resulting in reduction in germination and gappiness in the field. Fungi that infect seeds also reduce the seed germination and induce disease symptoms in roots and also in aerial plant organs. Various methods have been followed to detect and identify the fungal pathogens in seeds of different crops.

2.1.1 Conventional/Isolation-Dependent Methods

The Handbook on Seed Health Testing containing Worksheets has been published by the International Seed Testing Association (ISTA). The Worksheets have specific ISTA number with description of methods for detecting seedborne fungi, bacteria and viruses (ISTA 1994). In addition, the International Seed Health Initiative (ISHI) coordinates the production and supply of healthy seeds of vegetable crops. ISHI provides status reports which focus the efforts on the importance of different pathogens transmitted through seeds to the cooperating agencies (Meijerink 1997). The morphological characteristics are basically considered for the identification of the seedborne fungal pathogens which are classified into different phyla, class, families, genera, and species.

The morphologic (phenotypic) characteristics of the asexual and sexual spores and spore-bearing structures form the basis for classical taxonomy of oomycetes (fungus-like) and fungal pathogens for assigning them into various groups from kingdom down to species level (Agrios 2005) as detailed below.

Fungus-like microorganisms (oomycetes)

Kingdom: Protozoa

Phylum: Myxomycota: producing a plasmodium or plasmodium-like structure

Phylum: Plasmodiophoromycota (Plasmodiophoromycetes): endoparasitic slime molds

Order: Plasmodiophorales: plasmodia produced within cells of roots and stems of infected plants; obligately parasitic in nature

Genus: *Plasmodiophora* – *P. brassicae*, causing clubroot of cruciferous plants;

Spongospora – *S. subterranea*, causing potato powdery scab disease

Kingdom: Chromista (Stramenopiles)

Phylum: Oomycota with biflagellate zoospores

Class: Oomycetes: zoospores produced in sporangia

Order: Saprolegniales

Genus: *Aphanomyces* – *A. euteiches*, causing pea root rot disease

Order: Peronosporales

Family: Pythiaceae

Genus: *Phytophthora* – *P. infestans*, causing potato late blight disease

Family: Peronosporaceae

Genus: *Plasmopara* – *P. halstedii*, causing sunflower downy mildew disease;

Sclerospora – *S. graminicola*, causing pearl millet downy mildew disease;

Peronosclerospora – *S. sorghi*, causing sorghum downy mildew disease

True fungi

Kingdom: Fungi, producing achlorophyllous mycelium with cell walls containing glucans and chitin

Phylum: Chytridiomycota, producing zoospores with single posterior flagellum

Class: Chytridiomycetes, producing aseptate mycelium

Genus: *Synchytrium* – *S. endobioticum*, causing potato wart disease

Phylum: Ascomycota, producing sexual spores (ascospores) generally in groups of eight within an ascus enclosed in specialized structures known as ascocarps and asexual spores in pycnidia or acervuli

Class: Pyrenomycetes: filamentous ascomycetes

Order: Hypocreales

- Genus: *Gibberella* – *G. fujikuroi*, causing rice bakanae disease; *Claviceps* – *C. purpurea*, causing ergot diseases of grain crops; *C. sorghi*, causing sorghum ergot disease
 Class: Loculoascomycetes
 Order: Diaporthales
 Genus: *Diaporthe* – *D. phaseolorum*, causing soybean pod and stem rot disease; *Magnaporthe* – *M. grisea*, causing rice blast disease
 Order: Xylariales
 Genus: *Eutypa* – *E. lata*, causing grapevine canker disease
 Order: Dothidiales
 Genus: *Mycosphaerella* – *M. graminicola*, causing cereal leaf spot diseases; *Pyrenophora* – *P. teres*, causing barley net blotch disease; *Leptosphaeria* – *L. maculans*, causing cabbage black leg disease
 Class: Discomycetes
 Order: Helotiales
 Genus: *Sclerotinia* – *S. sclerotiorum*, causing canola stem rot disease
 Phylum: Basidiomycota, producing sexual spores known as basidiospores externally on a club-like one- or four-celled spore-bearing structure called basidium
 Order: Ustilaginales
 Genus: *Ustilago* – *U. maydis*, causing corn smut disease; *U. tritici*, causing wheat loose smut disease; *Sporisorium* – *S. sorghi*, causing sorghum kernel smut disease; *Tilletia* – *T. caries*, causing wheat bunt disease; *T. indica*, causing wheat Karnal bunt disease
 Order: Ceratobasidiales
 Genus: *Thanatephorus* – *T. cucumeris* (anamorph- *Rhizoctonia solani*), causing rice sheath blight disease
 Class: Deuteromycetes or Mitosporic fungi existing only in the asexual stage in their life cycle; conidia are formed on conidiophores separately or in specialized structures such as sporidia, synnemata, pycnidia, or acervuli
 Genus: *Alternaria* spp., *Bipolaris* spp., *Drechslera* spp., *Fusarium* spp., *Septoria* spp., and *Verticillium* spp. infecting seeds of several crops resulting in seed rot, seedling blight, anthracnose, head blight and scab diseases

2.1.1.1 Dry Seed Examination

During harvest, seeds may get admixtures of fungal structures such as ergot sclerotia, smutted kernels, discolored and shriveled seeds, and free insects. A dry examination is carried out using a stereoscope or an illuminated swinging-arm desk magnifier of $\times 2$ magnification. Usually samples of 400 or more seeds are subjected to examination under stereoscopic microscope for detection of fungal fructification such as pycnidia, acervuli, and smut sori. Seeds have to be incubated under high-humidity conditions to induce the fungal pathogens to sporulate, making it easier to detect their presence in the seeds (Narayanasamy 2002). Incidence and contamination of *Tilletia* species in conventionally produced, nonprocessed wheat seeds were assessed. Analysis of 151 samples of basic, certified, and commercial seed lots showed that 129 samples were contaminated with *Tilletia* spp. The teliospores had prominent gelatinous sheath together with conspicuous deep reticulation. The contamination level of basic and certified seed was about 1 teliospore per 10 seeds, whereas 4 of 16 commercial seed samples were contaminated with more than 900 teliospores per seed, higher than the permissible contamination level (Župunski et al. 2012). Infestation level of *Alternaria brassicola* in commercial batches of

cabbage seeds produced between 1984 and 2001 in Japan was investigated. A total of 100 seeds from each lot were incubated separately on agar at 25°C. *A. brassicola* was most frequently isolated (up to 94%) depending on seed lot. The pathogen was isolated even from seeds refrigerated for 17 years. *A. alternata* was also isolated, although less frequently (<5%) from cabbage seed lots (Kubota et al. 2006).

2.1.1.2 Histological and Cytological Methods

Histological examination of tissues may reveal the presence of pathogens in infected seeds. Wheat loose smut pathogen *Ustilago nuda* infects wheat seed embryos. The embryos are extracted in NaOH (5%) and then stained with trypan blue (Khanzada et al. 1980; Khanzada and Mathur 1988). Infection of wheat and barley seeds by *U. tritici* and *U. nuda* was detected by a modified method. Wheat and barley seeds were incubated in NaOH (10%) containing trypan blue (1 g L⁻¹) for 12–16 h. The embryos were then separated from the endosperm by passing the seeds through different sieves, followed by boiling in alkali solution for 15 min. After washing the embryos and boiling again in acetic acid or lactic acid (40–45%) for 1 min, the embryos were viewed under the microscope to note the presence of pathogen mycelium stained with trypan blue (Feodorova 1987). Treatment with NaOH (0.2%) also was reported to be useful for detecting infection by *Trichoconiella padwickii* in rice seeds. Change of color of infected portion of the seeds to black indicated the infection by *T. padwickii* (Dharam Singh and Maheshwari 2001). Latent infection of sunflower by *Plasmopara halstedii* was detected by light microscopic examination. Sections of hypocotyls of inoculated, but healthy-looking plants of compatible and incompatible host–pathotype combinations revealed the presence of vital hyphae and oospores. Plants with latent infection showed enhanced cell division activities which restricted entry of pathogen into the cortical parenchyma. Further, hypersensitive reactions inhibited further growth in pith parenchyma (Heller et al. 1997).

Quinoa, an ancient nutritious seed crop, is infected by the downy mildew pathogen *Peronospora farinosa* f.sp. *chenopodii* in the Andean highlands in Peru. Seed samples from different provinces were examined for the presence of oospores, using the light microscope. The presence of oospores was detected in ultrathin sections of fixed seeds of 19 samples. The oospores were located in the pericarp. Seeds of the infected samples were sown in the greenhouse at two levels of relative humidity. There was heavy sporulation and seedling infection in samples at higher humidity, compared to lower-humidity conditions. No correlation could be established between oospore density and seedling infection. The results suggested that the presence of fungal structures may not result in disease incidence in developing seedlings or plants (Danielsen et al. 2004). Sorghum kernel smut pathogen *Sporisorium sorghi* could be detected in hand-cut sections of apical meristem of sorghum plants growing from infected seeds. Sections stained with trypan blue revealed the presence of *S. sorghi* in apical meristem and nodes of young plants (Moharam et al. 2012). *Ustilago nuda* and *U. tritici*, causing loose smut disease in barley and wheat, respectively, do not induce any visible symptoms until heading stage. Reliable detection of the presence of the pathogens in infected plants in the early stages would be highly advantageous for both resistance breeding and development of effective seed treatments. Establishment of the loose smut pathogen in plants was detected by fluorescence microscopy. In hand-cut sections stained with fluorochrome Polonkophor®, fungal hyphae were observed to invade the shoot apical meristem and leaf primordia during the first days after the onset of germination. At the

first node stage, the ear and leaf primordia were generally extensively colonized. Both pathogens were observed in high density in the nodes of respective host plants (Wunderle et al. 2012).

2.1.1.3 Seed Washing Test

The seed washing or sedimentation test may be required for the detection of some fungal pathogens present in the seeds if dry seed examination, even after incubation, is not effective. Seed samples (50 seeds) infected by downy mildew or smut diseases are placed in test tubes containing distilled water (10 mL) and a few drops of (10–20) of 95% ethyl alcohol or a detergent, followed by agitation in a mechanical shaker for 10 min. The supernatant is then separated after centrifugation for 10 min at 3000 rpm. The pellet is resuspended in sterile water (2 mL). The fungal pathogens present in the seeds are identified by recording the morphological characteristics using a light microscope.

2.1.1.4 Blotter Test

The most common tests used for detection of seedborne fungi are the blotter test and its variants. Three layers of moistened absorbing (blotting) paper are used as substrate for 5–10 seeds, depending on the seed size, and placed equidistant from one another in a petridish. The plates with representative seed samples are incubated at $22 \pm 2^\circ\text{C}$, under near-ultraviolet (NUV) light with alternate cycles of 12 h of light and darkness for 7 days. The proliferating fungal colonies, if any, are observed for their morphological characteristics under the stereoscopic microscope, which form the basis of their identification. Pictorial representations of fungal pathogens infecting sorghum, pearl millet, chickpea, and peanut have been useful for the identification of the seedborne pathogens of these crops (Ahmed and Ravinder Reddy 1993). The standard blotter test involves surface sterilization for isolation of internally seedborne fungal pathogens. Seeds are immersed in a NaOCl solution containing 1% chlorine for 10 min or in 1.7% NaOCl for 1 min, followed by immersion in 70% ethanol for 1 min (ISTA 1996). Wetting the blotter paper with 0.1–0.2% 2,4-D (herbicide) solution retards germination of seeds, allowing the development of the fungal pathogens present inside the seeds. This procedure was successfully applied routinely for testing common bean and soybean seeds for the presence of seedborne pathogens (Dhingra et al. 1978). Detection of *Leptosphaeria maculans* (anamorph *Phoma lingam*) in crucifer seeds was accomplished by blotter test (Hewett 1977; Maguire and Gabrielson 1983). In a later investigation, 2,4-D was replaced with freezing of carrot seeds for detecting the presence of *Alternaria radicina* (Pryor et al. 1994).

In some cases the fungal pathogens may not produce spores, the characteristics of which are required for the identification of the fungi. When the results of blotter tests become unreliable, time-consuming inoculation tests have to be performed in such cases. Fenugreek seeds were incubated on moist filter papers at $28 \pm 2^\circ\text{C}$ and fungi belonging to 29 genera including *Aspergillus stellifer* and *Emericella vasicolor* were detected (El-Nagerabi 2002). Filter paper test was employed to test the efficiency of *Streptomyces* sp. to suppress the development of *Stenocarpella maydis* infecting maize seeds (Bressan and Figueiredo 2005). Blotter test was shown to be useful in detecting *Alternaria padwickii* in rice seeds, but less effective compared with isolation of pathogen in bean agar (BA) from rice seeds, followed by identification based on its morphological characteristics (Gutiérrez et al. 2010).

Deep-freezing blotter (DFB) method was developed for the detection of some seedborne pathogens and its efficiency was compared with standard blotter method (SBM),

since these methods were found to be ineffective for the detection of seedborne pathogens of fenugreek. An improved method of seed health testing was developed by soaking blotting paper in solutions of NaOH (0.3 M) and KOH (0.2 M), instead of using tap water for moistening blotting papers. The alkali treatments improved the effectiveness of detection of *Verticillium dahliae* to 6.5–7.5% from 0.3% in SBM and 1% in DFB methods. Furthermore, it was also possible to detect the presence of *Fusarium semitectum* and *Curvularia* sp. using NaOH, but not with SBM (Elwakil and Ghoneem 2002). In a further study using the alkaline blotter method (ABM), blotter disks were treated with NaOH (0.8%) or KOH (0.4%) and the treated disks induced the growth of *Cephalosporium* sp. and *Fusarium* sp. which were slow-growing. These pathogens could not be detected in peanut seeds by SBM and DFB procedures recommended by ISTA. The combination of DFB and SBM procedures enhanced the effectiveness of detection of slow-growing fungal pathogens of peanut by stimulating the development of these pathogens which may not be able to compete with saprophytic fungi associated with peanut seeds (Table 2.1; Elwakil et al. 2007; Section 2.3.1). The comparative effectiveness of seven incubation methods in detecting the fungal pathogens, *Alternaria alternata*, *A. zinnia*, *Cladosporium* spp. and *Fusarium* spp. in zinnia seeds was assessed. Deep freezing blotter test and blotter test with mannitol were more efficient in detecting these fungal pathogens in zinnia seeds compared to other treatments (Ska et al. 2012).

Table 2.1 Effect of alkali treatments on the colony diameter and sporulation of seedborne fungi associated with peanut seed lots* (Elwakil et al. 2007).

		NaOH (%)			KOH (%)		
Property	Control	0.4	0.8	1.0	0.2	0.4	0.8
<i>Cephalosporium</i> sp.							
pH	72	12.0	12.5	13.2	11.8	12.5	13.0
Growth diameter	2.8c	2.6f	4.5a	3.0d	3.2c	4.4a	4.0b
Sporulation	14.0f	15.6c	21.9b	15.3d	15.1e	22.5a	12.9g
<i>F. verticillioides</i>							
Growth diameter	7.4c	6.0d	9.0a	5.0e	6.0a	8.0b	4.0f
Sporulation	54.0e	59.2d	169.7a	56.4de	111.6c	162.0b	42.6f
<i>F. oxysporum</i>							
Growth diameter	6.5c	6.9b	9.0a	6.6c	5.7d	9.0a	5.8d
Sporulation	134.2c	129.5d	207.3a	125.5e	64.3f	191.3b	45.1g
<i>F. solani</i>							
Growth diameter	7.0d	8.0b	9.0a	5.8f	7.5c	9.0a	6.0e
Sporulation	74.2c	58.8e	80.6b	46.7f	69.6d	81.3a	42.9g
<i>Verticillium</i> sp.							
Growth diameter	5.4d	6.9b	8.0a	4.9e	6.7c	7.9a	3.9f
Sporulation	13.4f	27.0c	39.4a	14.4e	24.7d	35.9b	10.7g

* Growth diameter in centimetres measured on fungal colony grown in Czapek's agar amended with an alkali solution at different concentrations; values of means followed by the same letter in different treatments are not different significantly ($P = 0.05$) as per Duncan multiple range test (DMRT).

2.1.1.5 Direct Plating

The fungal pathogens infecting seeds may be isolated in pure culture, using general media such as potato dextrose agar (PDA) or specific media that differentially support the development of slow-growing fungal pathogens. Seedborne fungal pathogens such as *Phoma lingam* and *Ustilaginoidea virens* may develop well on simple water agar and PDA, respectively (Mangan 1983; Ladhalakshmi et al. 2012). On the other hand, detection of *Alternaria dauci* and *A. radicina*, present in carrot seed, plant debris, and other substrates, could be accomplished by using a selective medium based upon carrot leaf extract which promoted profuse sporulation by both pathogens. Glucose, sodium polypectate, and mineral salts were incorporated to enhance spore and mycelial production. Inclusion of streptomycin sulfate and metalaxyl (or mefenoxam) combined with either benomyl or thiophanate inhibited the growth and sporulation of other unwanted microorganisms. The production of unique dark mycelium formed by both pathogens indicated their presence. The sensitivity of *Alternaria dauci*-*radicina* selective medium (ADRSM) to detect *A. radicina* on carrot seeds was similar to other methods available. However, ADRSM was more sensitive than other media for detecting *A. dauci* on infected carrot seeds (Strandberg 2002).

A semiselective medium is required for the growth of *Fusarium oxysporum* f.sp. *gladioli*, infecting gladiolus. The macerated corm tissue was plated on the semiselective medium and incubated for 1 week. Although colonies of *F. oxysporum* could be identified in this medium, it was not possible to differentiate between pathogenic and nonpathogenic isolates which are morphologically similar (Roebroek et al. 1990). Seed samples (27) of lettuce cultivars grown most frequently in Lombardy (northwestern Italy) were severely affected by Fusarium wilt disease caused by *F. oxysporum* f.sp. *lactucae*. The pathogen was isolated on the Fusarium-selective medium using subsample of seeds (500–1500) belonging to the same seed lots used for sowing. Seeds were either unwashed or disinfected with 1% sodium hypochlorite. Nine of the 27 samples of seeds obtained from commercial seed lots were sown in fields infested by *F. oxysporum* f.sp. *lactucae*. Among the 16 isolates of the pathogen, only one was isolated from disinfected seeds. Three of the isolates were pathogenic on lettuce cultivars tested and the level of virulence was similar to those from other countries (USA and Taiwan). The results demonstrated that infected lettuce seeds could be a potential source of inoculum for lettuce wilt disease caused by *F. oxysporum* f.sp. *lactucae* in Italy (Garibaldi et al. 2000).

Mycosphaerella pinodes, incitant of leaf blight of garden pea, is difficult to isolate from infected seed, plant debris, and infested soil using commonly used media. A semiselective medium was developed for efficient isolation of the pathogen from infected plant tissues and soil. A starch-casein (SC) semiselective medium was developed by amendment of Czapek–Dox agar medium with soluble starch (30g), casein (2g), propamocarb ($25\mu\text{g mL}^{-1}$), atrazine ($300\mu\text{g mL}^{-1}$), streptomycin sulfate ($100\mu\text{g mL}^{-1}$) and neomycin sulfate ($200\mu\text{g mL}^{-1}$). This semiselective medium was useful for detecting *M. pinodes* in artificially and naturally infested soil, diseased seeds, leaves, and stem of peas (Chen et al. 2010). The extent of lamb's lettuce (*Valerianella olitorice*) seed infection by *Phoma valerianellae* was assessed by direct plating test. All six samples of lamb's lettuce seed were contaminated by *P. valerianellae* at levels of 0.6–15.0%. Surface disinfection of seeds did not eliminate contamination, but could reduce it (0.1–10.0%) (Pellegrino et al. 2010). *Sporisorium relianum* causal agent of head smut of sorghum and corn was isolated by plating to monitor changes in disease dynamics and virulence

of various isolates (Zhang et al. 2011). In the case of certain fungal pathogens, the addition of supplements to the medium has been reported to enhance their growth rate. In shake cultures in different media, addition of 0.3% peptone enhanced the growth of *Sporisorium sorghi* compared with unsupplemented medium. Of the media tested, mycelial production was highest in malt dextrose broth supplemented with peptone (Moharam et al. 2012). For the isolation of pathogens causing mold diseases of sorghum such as *Alternaria cochliobolus* and *Fusarium* species, dichloran chloramphenicol peptone (DCPA), dichloran chloramphenicol, rose Bengal (DRBC), and pentachloronitrobenzene (PCNB) and modified V8 juice (ModV8) agars were compared for their effectiveness in supporting the development of mold pathogens. Radial growth of the mold pathogens was more effectively promoted by DRBC and ModV8 agar media than DCPA and PCNB agar media (Funnell-Harris et al. 2013). In order to assess the extent of infection of cowpea seeds by fungal pathogens, farmer-produced seeds, foundation seeds, and seeds from seed-producing companies were tested by isolating the pathogens *Fusarium oxysporum* (69%), *Macrophomina phaseolina*, and *Pseudomonas syringae* pv. *phaseolicola* (48%) from cowpea seeds. Seed recycling in farmer-produced seed did not seem to increase the levels of seedborne pathogens. On the other hand, it is likely that distribution of cowpea seeds by companies may pose a potential threat, since they distribute seed over large areas (Biemond et al. 2013). Morphological characteristics of the fungi isolated were determined and compared with those already described. The morphological characters alone may be insufficient for precise identification of various isolates belonging to different *formae speciales*, races, or biotypes within a morphologic species.

2.1.2 Isolation-Independent Methods

It may not be possible to isolate some pathogens that are slow-growing or obligately parasitic in nature such as downy mildew pathogens. Isolation-independent methods are effective in detecting such pathogens which are internally or externally seedborne.

2.1.2.1 Grow-Out/Seedling Symptom Test

The grow-out test is performed in the greenhouse by planting a specified number of test seeds, preferably on sterile soil. Visual examination of emerging seedlings is carried out to determine the percentage of seeds germinated, the development of disease symptoms on seedlings, and the percentage of seedlings exhibiting disease symptoms. This test is useful for assessing field performance and estimating the number of infection loci/unit area. The grow-out test has to be conducted for seed lots to be distributed to farmers for commercial cultivation. Soybean seed health was assessed by grow-out test for infection by *Colletotrichum truncatum* (Dhingra et al. 1978). Infection by some pathogens may not be detected by using the blotter test or direct-plating test. Soybean seed infection by *Sclerotium* sp. could not be detected by blotter test or by plating the seeds on PDA; however, seed infection by this pathogen was detected by the grow-out test (Dhingra and Muchovej 1980). Obligate parasites cause downy mildew of sorghum and corn (maize). Grow-out tests are particularly useful in detecting these pathogens, as they have not been successfully isolated in any cell-free media so far. The requirements of a large greenhouse space and the long time to obtain results are the major limitations for wider application of this test.

2.1.2.2 Physical Methods

Seeds that are not fully filled due to pathogenic or physiological causes are lighter in weight and tend to float in water whose density may be increased by adding solutes such as sodium chloride (NaCl). Immersion of seed lots in sodium chloride solution (20%) is a simple procedure to remove poorly filled seeds and fungal structures such as sclerotia from the seed lot. The infected seeds and fungal structures will float on the surface of the salt solution and they can be either mechanically removed or filtered out. Large wheat consignments are generally inspected by visual examination. The reliability of results of the visual examination are significantly affected by inspector fatigue. This may result in harvest delays and missed wheat kernels infected by Karnal bunt pathogen *Tilletia indica*. A high-speed optical sorter was tested to determine if infected kernels could be removed from a 1800 g sample. The sorter could remove about 8% or more of the sample, while the reject portion contained 100% of the bunted kernels. Concentrating the bunted kernels in a smaller sample size might reduce sample inspection time and reduce inspection errors. This high-speed optical sorter could be employed for removing infected seeds rapidly and efficient processing of wheat consignments at a rate of up to 8.8 kg h^{-1} . The results indicated the potential of the optical sorter for wider application for removal of bunted grains from wheat consignments marked for food and feed (Dowell et al. 2002). A color image analysis using the MultiScan® 4.01 was employed to assess the extent of fungal colonization of wheat kernels inoculated with *Fusarium* sp. MS Excel® software was used for calculating the colonization by the fungal pathogen. This system was used for rapid evaluation of kernel colonization by fungal pathogen to monitor grain quality of food and feed (Wiwart and Korona 1998). Infection of wheat ears by *Fusarium* spp. is dangerous for both human and animal consumption. Hence, the ability to monitor symptom location and severity before the harvested product is further processed or stored might help determine whether the grain is fit for human/animal consumption for bioconservation or is completely unusable. Nondestructive detection of Fusarium head blight (FHB) could be achieved by image processing or spectroscopy. A combination of these two procedures in the form of spectral imaging was evaluated. Transmission spectra were recorded both in the visible and near-infrared range from FHB infected kernels. These images were analyzed using light absorption and the relation between two wavelength bands. The results indicated that near-infrared spectral images could perform much better than spectral images in the visible range. Kernels with more than 6000 pg of *Fusarium* DNA (as determined using TaqMan RT-PCR assay) could be reliably identified. It was possible to predict the presence of *Fusarium* DNA above 100 pg (Polder et al. 2005).

Optical measurements, both image analysis and near-infrared (near IR) spectroscopies have been utilized for detection of Fusarium-damaged kernel (FDK). In the imaging investigations, kernel morphology and color characteristics were primarily used to distinguish damaged kernels from healthy kernels. Identification of FDK in hard red winter wheat was possible at 95–97% accuracy with as few as two wavelengths (Delwiche and Gaines 2005). In a later investigation, the efficiency of high-speed optical sorting of wheat (*Triticum aestivum*) kernels was assessed for reduction of deoxynivalenol (DON) concentration. *Fusarium*-damaged wheat, cleaned of nonkernels and foreign materials (4.5 kg/sample, DON range $0.6\text{--}20 \text{ mg kg}^{-1}$) was fed into a high-speed bichromatic sorter. A wavelength filter pair combination of 675 and 1480 nm was selected for sorting. Visual measurement of the proportion of *Fusarium*-damaged kernels were collected on incoming and sorted seed (separate analysis of accepted and rejected portions), and

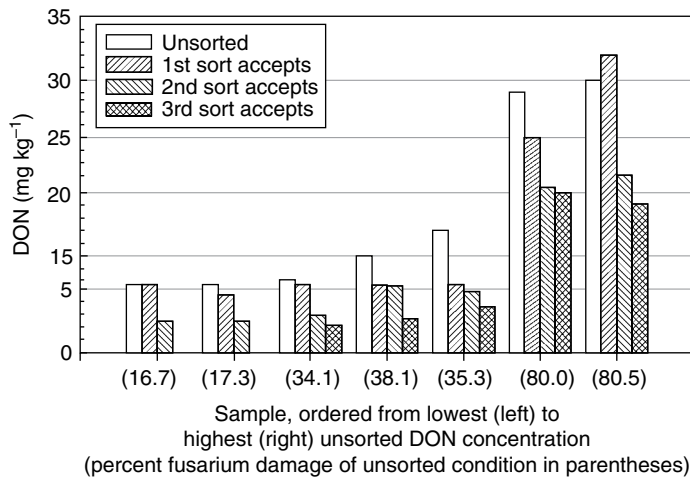


Figure 2.1 Sorting of wheat kernels damaged by *Fusarium* spp., using high-speed optical sorter (Scam Master II DE). Rapid separation of Fusarium damage kernels (FDK) with different concentrations of deoxynivalenol (DON). Source: Delwiche et al. (2005). Reproduced with permission of the American Phytopathological Society, MN, USA.

measurements of DON concentrations were also made. The results indicated that the fraction of DON contaminant level in the sorted wheat to that in the unsorted wheat ranged over 18–112% with an average of 51%. Multiple sorting was effective in producing wheat whose DON contamination was 16–69% of its original, unsorted value (Fig. 2.1; Delwiche et al. 2005). Wheat cultivars with differing levels of susceptibility to FHB were artificially inoculated with a spore suspension of the pathogen in field plots. A color (red, green, and blue) and a multispectral (red, infrared) camera system with real-time image analysis software were developed and compared to detect disease symptoms in the plots with different wheat cultivars. The chlorophyll defect of the infected wheat ears was classified against the image background by setting binarization thresholds, and black and white images were obtained. For both systems, a linear correlation was noted between the camera and the visually detected disease levels of wheat ears in plots. In the noninfected control plots without disease symptoms, the multispectral system accurately measured “no disease.” The multispectral system showed a superior calibration capacity. This system used only one calibration step before starting the measurements, whereas the color system required calibration for each variety (Dammer et al. 2011).

Effective and accurate disease evaluation methods are essential for successful identification of *Fusarium* head blight (FHB) -resistant sources and selection of resistant cultivars. In order to assess the relationships among different types of resistance to FHB, 363 US winter wheat accessions (74 soft and 289 hard) were repeatedly evaluated for *Fusarium*-damaged kernels (FDK) and deoxynivalenol (DON) concentration under greenhouse and field conditions. Single-kernel near-infrared (SKNIR) -estimated FDK and DON were compared with visually estimated FDK and gas-chromatography–mass-spectroscopy-estimated DON. Significant correlations were observed between percentages of symptomatic spikelets and visual FDK in greenhouse and field, although correlation coefficients were also observed between visually scored FDK (0.72, $P < 0.001$)

and SKNIR-estimated DON (0.68, $P < 0.001$). The results indicated that both visual scoring and SKNIR methods could be employed for estimation of FDK and DON for selection of resistant sources for breeding programs (Jin et al. 2014). The levels of fitness of saprophytic survival and pathogenicity on wheat spikes of 50 isolates of *Fusarium graminearum* isolated from wheat spikes, including 25 isolates each of the 15-acetyldeoxynivalenol (15-ADON) and 3-ADON genotype, were tested. The isolates were characterized and compared for 14 different attributes of saprophytic and pathogenic fitness on a susceptible cultivar. Isolates of the two genotypes could not be differentiated for most of these traits. Three principal components – ascospore production on corn stalks, total trichothecene amount in wheat kernels, and incidence of diseased spikelets up from the point of inoculation – accounted for 29.4, 18.9, and 10.8% of the variation among the isolates each, with isolates of both genotypes representing similar proportions (15-ADON/3-ADON, $n = 14/13$ and $11/12$). Within a corresponding population of *F. graminearum* causing wheat FHB disease in New York, isolates with a 3-ADON genotype did not possess any detectable advantage over isolates with a 15-ADON genotype in saprophytic fitness or in pathogenic fitness on a susceptible wheat cultivar, in contrast to the earlier view that 3-ADON isolates had greater levels of saprophytic and pathogenic fitness on wheat spikes (Spolti et al. 2014).

2.1.2.3 Chemical Methods

Pathogenic fungi cause serious deterioration of seed quality and are responsible for mycotoxicoses in humans and animals following consumption of contaminated grains. Production of characteristic volatiles and odor from infected cereal grains may be an early indication of deterioration of quality of grains. *Tilletia caries*, causal agent of wheat common bunt disease, induces the formation of odor from infected wheat grains. The volatiles produced by the pathogenic infection may be detected by an electronic nose more efficiently than a panel of grain assessors. The electronic nose was suggested to sense a different characteristic not related to common bunt odor (Börjesson and Johnson 1998). In a later study, the reliability and efficiency of electronic nose technology was assessed for its potential as an indicator of fungal activity and differentiation between species affecting wheat, maize, and other cereals. The range of volatile compounds produced by fungi causing grain spoilage was determined in vitro. The key groups of volatile compounds emanating from the cereal grains could form the basis for detecting fungal infection (Magan and Evans 2000).

Acidic electrolyzed water (AEW) is a product of electrolytes of a dilute solution of sodium chloride (NaCl) (0.4%, w/v) containing a low concentration of hypochlorous acid which is a very effective sanitizer at low pH. Treatment with AEW is part of a proven protocol to detect *Tilletia indica* causing Karnal bunt disease in wheat. AEW can be used to disinfect wheat seed or soil samples being tested for teliospores of *T. indica*. The AEW had a pH of 2.5–2.8 and oxidation-reduction potential of approximately 1.130 mV. In simulations of routine extractions of wheat seeds to detect teliospores of *T. indica*, the effectiveness of a 30 min AEW treatment was compared with a 2 min 0.4% sodium hypochlorite (NaOCl) treatment to eradicate bacteria and nonmut fungi. AEW treatment of wheat seed washes was standardized for eliminating contaminating microorganisms (Fig. 2.2). A significant advantage of using AEW over NaOCl was that with the former, teliospores germination was not reduced and was usually stimulated, whereas teliospores germination declined after contact with the latter (Table 2.2). The protocol facilitated detection and enumeration of viable teliospores of

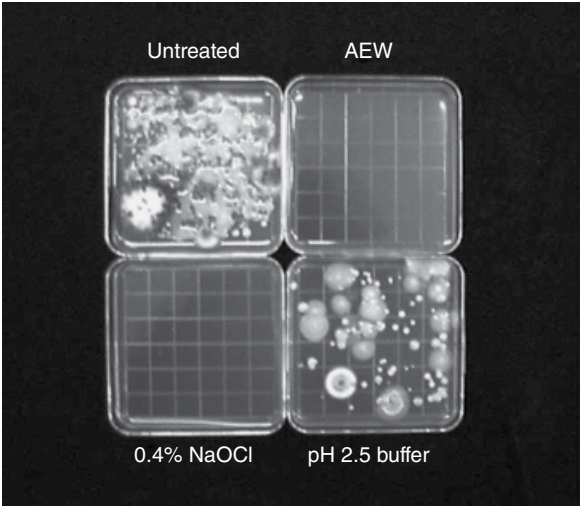


Figure 2.2 Effectiveness of acidic electrolyzed water (AEW) and sodium hypochlorite (NaOCl) in eliminating the wheat kernel bunt pathogen *Tilletia indica*. Note the inhibition of development of the pathogen in the medium amended with seed washes obtained after treatment with AEW and NaOCl. Source: Bonde et al. (2003). Reproduced with permission of the American Phytopathological Society, MN, USA.

Table 2.2 Comparative effectiveness of treatments with electrolyzed water (AEW) or sodium hypochlorite (NaOCl)^a (Bonde et al. 2003).

Treatments	Wheat lots			Mean
	Grandin HRS	Kansas #2249	Kansas #2348	
Control				
Fungi ^b	0.4	2.3	TNTC ^d	1.4
Bacteria ^b	20.0	8.5	62.7	30.4
30 min, AEW				
Fungi ^b	0.0	0.7	0.0	0.2
Bacteria ^b	0.3	0.5	0.0	0.3
Reduction (%) ^c	99.9999	99.9986	100.0	99.9995
2 min, 0.4% NaOCl				
Fungi ^b	0.3	0.0	0.3	0.2
Bacteria ^b	0.7	2.0	0.3	1.0
Reduction (%) ^c	99.9995	99.9977	>99.9999	99.9990

^a Wheat samples extracted from 18 separate 50 g sample; ^b Average numbers of CFU $\times 10^3$ of fungi and CFU $\times 10^4$ of bacteria isolated after seeding with extracts from three grain samples from a wheat lot; ^c Percent reduction based on decrease in average contaminant colony numbers compared with untreated controls; ^d Too numerous to count

T. indica in wheat seeds. Use of AEW is also useful for isolation of *T. indica* in pure cultures for identification. Assessment of the germicidal effects of AEW indicated its potential as an alternative to the seed disinfectants that were previously in use (Bonde et al. 2003; Section 2.3.2).

2.1.3 Immunoassays

Some of the fungal pathogens infecting seeds may be more easily identified up to species level based on their morphological characteristics, which exhibit greater variations compared with bacterial and viral pathogens. Morphological characteristics of bacterial and viral pathogens show limited or insufficient variations that can be used for their precise identification and differentiation. However, there are certain conditions when identification of fungal pathogens may also become difficult. Fast-growing saprophytes which may overgrow the fungal pathogens are also present in the same seed, making the isolation and examination of the fungal pathogens problematic. In addition, some fungal pathogens are known to exist in the form of strains, races, or varieties which are morphologically indistinguishable. In such cases, use of more discriminating techniques such as immunoassays and/or nucleic acid-based techniques, independent of the isolation of the pathogen concerned, become essential. In the case of rice, more than half of 56 fungal pathogens have been reported to be seedborne, necessitating application of additional tests for confirmation of the identity of the rice seedborne fungal pathogens (Mew et al. 1988). Immunology has become a multipurpose technology with numerous applications in biological sciences in general and in medicine and agriculture in particular. Immunoassays have been shown to be specific, sensitive, simple, rapid, and cost-effective. They can be automated for large-scale applications. Immunodiagnosis may be useful for confirming visible symptoms and for rapid detection of pathogens that cannot be easily isolated and identified by conventional methods. Furthermore, immunoassays have potential for early detection and precise identification of seedborne fungal pathogens that remain dormant in the seeds which do not exhibit any visible symptoms, facilitating the application of fungicides that are specific only to certain pathogens or groups of pathogens at appropriate time. Polyclonal antibodies (PABs) and monoclonal antibodies (MAbs) have been produced against fungal antigens present in whole cells, cell fractions, extracellular components, and culture filtrates. Methods of production of PABs and MAbs, principles of immunological reactions, and applications of various immunoassays have been discussed in detail in earlier publications (Narayanasamy 2001, 2005).

The discoloration of stored rice was shown to be due to *Penicillium islandicum* and *Humicola languinosa*. By employing specific monoclonal antibodies (MAbs), these fungi could be detected in discolored rice. *P. islandicum* produces a mycotoxin injurious to human beings. This mycotoxin is capable of causing liver lesions, cirrhosis, and primary liver cancer following consumption of contaminated rice grains. It is therefore necessary to rapidly detect and identify *P. islandicum* precisely in rice samples to avoid the use of contaminated rice grains as food or feed. When the effectiveness of detection of *P. islandicum* by employing specific MAb and direct-plating method was assessed, the immunoassay was found to be more efficient and rapid since direct-plating required a long time (Dewey et al. 1989, 1990). Seedborne fungi such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. are capable of producing mycotoxins that cause ailments in humans and animals. Two MAbs capable of reacting with antigens of 12

field and 27 storage fungi were generated. Further, by using PABs raised against *Penicillium aurantiogriseum* var. *melanoconidium* in direct enzyme-linked immunosorbent assay (ELISA) format, the fungal pathogens associated with barley seeds were detected efficiently. A possible linear correlation could be seen between absorbance values and population of fungal pathogens (Banks et al. 1992). The mold fungi *Aspergillus parasiticus*, *Penicillium citrinum*, and *Fusarium oxysporum* colonize rice seeds frequently. Double-antibody sandwich (DAS-) ELISA test has been applied for efficient detection of these mold fungi. Variations in absorbance values indicated the amount of mold growth present in the seeds in a reliable manner. The detection threshold of DAS-ELISA was $1 \mu\text{g mL}^{-1}$ (Chang and Yu 1997). The effectiveness and reliability of DAS-ELISA format was confirmed later for detection of nine toxigenic *Aspergillus* spp. in rice seeds and corn grains by Wang and Yu (1998). Direct competitive ELISA was applied as a post-column monitoring system after liquid chromatography for the early detection and accurate quantification of fumonisin produced by *Fusarium* spp. in maize grains. The detection limit of this procedure was 0.1 ng of FmB1 in maize samples and AAL toxin produced by *Alternaria alternata* in culture filtrates (Yu and Chiu 1998). *Sclerotinia sclerotiorum* infects a wide range of crop plants and different parts of the same plant. The infection of young petals of rapeseed by *S. sclerotiorum* plants was detected by DAS-ELISA format (Jamaux and Spire 1994). In a later investigation, mycelium and ascospores of *S. sclerotiorum* were employed as immunogens and anti-mycelium serum (Smy) and anti-ascospore serum (Ssp) were produced separately. Smy antiserum was more sensitive than Ssp antiserum in detecting the pathogen proteins in mycelial extracts. However, both antisera showed similar sensitivity to the antigen when allowed to react with ascospore. A commercial kit was used for detection of *S. sclerotiorum* on canola petals as part of the disease prediction model (Bom and Boland 2000).

Wheat Karnal bunt disease caused by *Tilletia indica* has been a cause of concern for wheat production in many countries including the US. Wheat crops suffer both quantitative and qualitative losses due to this pathogen whose entry is prevented by quarantine regulations. *T. indica* has a specific protein (64 kDa) with antigenic properties. Antibodies raised against this protein reacted specifically against the teliospores of *T. indica* in a microwell sandwich-ELISA and dipstick immunoassay (Kutilek et al. 2001). The two detection techniques – DAS-ELISA and polymerase chain reaction (PCR-) based procedure – were evaluated to assess their effectiveness in detecting the wheat common bunt pathogen *Tilletia caries* (= *T. tritici*) in young wheat plants growing from infected seeds. The DAS-ELISA test was performed using biotinylated detection antibodies. The antibodies were generated by injecting mycelial homogenates of axenic cultures of *T. caries* into rabbits. The detection limit of DAS-ELISA was 7 ng mL^{-1} of fungal protein. No cross-reactions with other fungi except for the closely related *T. controversa* were observed with DAS-ELISA test. The ELISA format did not react with spore extracts, while PCR assay was positive in detecting *T. caries*. Analysis by ELISA of shoots of individual plants grown from inoculated seeds showed that all plants tested positive. A large variability in the pathogen population was however evident in different plants, indicating quantitative differences in the degree of colonization of tissues between individual plants even in the same wheat variety. The results suggested that ELISA format allowed precise quantification of the amount of pathogen antigen present in the infected wheat plants, and was more suitable than the PCR-based assay for use in the programs for development of resistant varieties (Eibel et al. 2005a). In another investigation, the ELISA test was compared with PCR-based assay for their

efficacy in detecting *Ustilago tritici* and *U. nuda* in wheat and barley seedlings grown from infected and healthy seeds. Head infection of mature plants derived from tillers of the tested seedlings was also assessed to correlate the results of ELISA and PCR-based assay. PCR assay was more efficient than ELISA test. The results indicated that the prediction of loose smut infection by real-time PCR could be possible at the second leaf stage, and PCR assay was equally suitable for use with spring and winter wheat and barley varieties (Wunderle et al. 2012).

Ustilago nuda affecting barley causes loose smut disease which is internally seedborne. The pathogen develops internally at the growing point of infected plants. The inflorescence is transformed into smutted ears instead of grain-bearing head at maturity. Polyclonal antibodies (PABs) were raised against mycelium from the logarithmic growth phase of a shake culture of *Ustilago nuda* and biotinylated detection antibodies were used in DAS-ELISA test for detecting the pathogen. The detection limit of the test was 15 ng total protein mL⁻¹ for homologous antigen and 50 ng mL⁻¹ for extract of *U. nuda* spores, respectively. PABs reacted with other *Ustilago* spp., cross-reactivity being the highest with *U. tritici*. However, no cross-reaction occurred with the other fungal pathogens, *Tilletia*, *Rhizoctonia*, *Pythium*, and *Fusarium*. With naturally infected barley seeds, the results of the DAS-ELISA and the conventional seed embryo tests were always in good agreement. However, when seeds grown from artificially inoculated florets were tested, the DAS-ELISA indicated significantly higher infestation levels than the embryo test. Assays with halved seeds from the same lot showed that high amounts of mycelium were present in the nonembryo half. The results of DAS-ELISA on samples from barley seedlings grown from infected seeds corresponded very well with infection levels determined by staining the seed embryo, irrespective of the mode of floret inoculation (natural or artificial). DAS-ELISA format offers the possibility of assessing the efficacy of seed-treating chemicals for elimination of the pathogen mycelium from infected seeds, in addition to studying pathogen biology and characterization of resistance mechanism operating in barley plants (Eibel et al. 2005b).

A quantitative double-antibody sandwich (DAS-) ELISA format was developed using rabbit PABs directed against soluble mycelial proteins of *Leptosphaeria maculans* infecting canola (*Brassica napus*). Immunoblotting following isoelectric focusing of soluble proteins (Western blot) and DAS-ELISA tests were applied for verifying the specificity of the antiserum in the detection of *L. maculans*. No cross-reactions except for *Alternaria brassicae* were observed when the mycelial extracts of saprophytes or pathogens of *B. napus* were tested. Western blot discriminated Tox⁺ and Tox^O isolates of *L. maculans* unequivocally. However, the isolates could be distinguished using ELISA. The detection limit of the assay was less than 10 ng of fungal proteins mL⁻¹ of plant extract. The experiments to study the time-course of pathogen spread indicated that fungal growth in cotyledons was correlated with symptom scoring. Plants showing hypersensitive response were ELISA-positive only up to 34%, and they contained not more than 10 ng of fungal protein per inoculated cotyledon. On the other hand, all susceptible plants (100%) were ELISA-positive and they had more than 10 µg fungal proteins per cotyledon. Tox⁺ isolates showed significant differences in their ability to colonize cotyledonary tissues. ELISA quantification procedure was able to differentiate intermediate symptoms as late resistance responses or susceptibility (Balesdent et al. 1995).

Rhizoctonia solani capable of infecting a large number of crop plants and other plant species was detected by applying a combination of baiting and double MAb-ELISA tests. This combination of tests was more efficient and rapid in providing the results.

The pathogen could be detected in 3 days from the receipt of soil samples. The format involved receiving isolates of *R. solani* from colonized baits for determining their anastomosis group affiliation and pathogenicity. The isolates that were pathogenic belonged to AG4 group (Thornton et al. 1999). *Macrophomina phaseolina*, a seedborne and soil-borne pathogen, infects a wide range of plant species including legumes and peanut. The presence of *M. phaseolina* in cowpea seeds was detected by employing agar-plating and DAS-ELISA tests. Polyclonal antibodies (PABs) were generated against the immunogens in the mycelium and culture filtrate of the pathogen. PABs raised against the mycelium were more sensitive than the PABs raised against the culture filtrate. The detection limit of the assay was 15–30 ng protein mL⁻¹. In cowpea plants showing symptoms of infection, *M. phaseolina* was quantified in leaves, epicotyls, hypocotyls, and roots by DAS-ELISA test at 1 month after seed or soil inoculation. Both assay methods detected the pathogens in seed lots obtained from different fields in Niger and Nigeria. However, ELISA test was more sensitive since positive results were obtained with ELISA in a greater percentage of seeds than agar-plating method, which was also time-consuming (Afouda et al. 2009).

Immunofluorescence (IF) assays were performed for the detection of *Phytophthora cinnamomi* using both polyclonal and monoclonal antibodies. Specific MABs were raised against *P. cinnamomi* and they were successfully employed for the detection of the pathogen in plant tissues (Gabor et al. 1993). In another investigation, MABs were generated against the species-specific epitopes on the surface of zoospores and cysts of *Phytophthora cinnamomi*. The MABs contained a very valuable spectrum of taxonomic specificities and were tested against six isolates of *P. cinnamomi* and six species of *Pythium*. The MABs indicated the presence of spatially restricted antigens on the surface of the zoospores of *P. cinnamomi*. The MABs showed the potential for applications in the investigations on the biology and taxonomy of *P. cinnamomi* (Hardham et al. 1986). Simple procedures to visualize immunological reactions are needed for applying immunodiagnostic methods under field conditions. The 96-well microtitre plates used in the ELISA tests were replaced by dipstick formats. The dipstick immunoassay is based on the phenomena of chemotaxis and electrotaxis to attract zoospores of oomycetes to a membrane on which they encyst. The encysted zoospores are subsequently detected by immunoassay. Chemicals such as amino acids, alcohols, phenols and isovaleraldehyde, pectin, and phytohormone abscisic acid influence the chemotactic properties of zoospores. Positively charged nylon membranes strongly attract zoospores. Detection limit of the dipstick assay was as few as 40 zoospores mL⁻¹ and the results could be obtained within 45 min. The immunolabeled cysts attached to the membrane could be visualized using a light microscope after silver enhancement of the gold-labeled secondary probe. *Phytophthora cinnamomi* was rapidly detected by employing the specific monoclonal antibodies (Cahill and Hardham 1994). The relative efficacy of ELISA test and immunoblotting technique was assessed for the detection of *Phomopsis longicolla*, infecting soybean seeds. Nonspecific interference was observed in ELISA tests. Such nonspecific interference could be avoided by performing seed immunoblot assay (SIBA). Infected soybean seeds were placed on nitrocellulose paper on which the pathogen mycelium grew out. A conspicuous colored blotch was observed on the nitrocellulose paper when it was assayed. As the mycelium can only be produced from the viable spores of *P. longicolla*, SIBA test provides the distinct advantage of differentiating the living and dead spores of the pathogen. In contrast, ELISA tests do not differentiate the living and dead pathogen propagules. Such information is vital for

disease prediction in epidemiological investigations (Gleason et al. 1987). SIBA test was also applied for the detection of *Tilletia indica* infecting wheat seeds. Colored imprints were formed on nitrocellulose paper on which infected wheat seeds were placed, indicating the presence of live teliospores of the pathogen in tested seed lots (Anil Kumar et al. 1998).

2.1.4 Nucleic Acid-Based Techniques

All organisms including microorganisms contain deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA) as vital constituents that are involved in determination of genetic characteristics and the production of several enzymes and other compounds required for their metabolic activities and existence. The characteristics of nucleic acids in the fungal pathogens infecting seeds of various crops have been studied for their early detection, precise identification, and differentiation of strains, races, and biotypes within a morphologic species. Detection of fungal pathogens using nucleic acid (NA) based techniques offers certain distinct advantages over immunoassays. The fungus-like (oomycetes) and fungal pathogens are complex antigens, the nature of which varies depending on the stage of the life cycle. The antisera prepared against one type of spore or mycelium formed at a particular stage may not actively react with spores or mycelium produced at all stages in the life cycle of the target pathogen. In contrast, the presence or absence of spore-bearing structures or the slow-growing nature of some fungal pathogens will not affect the detection of the test pathogen(s) by NA-based techniques, since the nature of the genomic elements remains constant at all stages of the life cycle of the pathogen development. As the sensitivity of the NA-based techniques is generally higher, it is possible to detect, identify, and differentiate the fungal pathogens that are not amenable for detection by other methods. Obligate pathogens that have not been cultured on cell-free media so far may be detected and differentiated by employing suitable NA-based techniques. Because of the higher level of sensitivity, rapidity, reliability, and specificity, NA-based techniques are frequently preferred by researchers for the detection of microbial plant pathogens infecting various crops in different ecosystems.

2.1.4.1 Hybridization-Based Nucleic Acid Techniques

Closely related organisms generally share a greater nucleotide similarity than those that are distantly related. Techniques based on nucleic acid hybridization involve the identification of a highly specific nucleotide sequence common to the strain or isolate of the fungal pathogen to be identified, but absent from other strains or isolates or species. This selected nucleotide sequence is used to identify/detect the presence of the target strain/isolate. Likewise, a highly conserved sequence present in all strains or species in a genus may be employed to probe for the presence of any other member included in the particular genus and for establishing the relationship between the strains or species. The selection of a specific sequence as a probe may be based on one of several methods, but distinctly derived from the sequence data and screening of related organisms to determine its specificity.

Peronosclerospora sorghi, causative agent of sorghum downy mildew disease, was detected in seeds using the dot-blot hybridization procedure. The pathogen DNA was extracted by grinding sorghum seeds. The probes hybridized only with the DNA of *P. sorghi*, but not with DNA of any other fungi associated with sorghum seeds (Yao et al. 1990). The sensitivity of the test could be significantly enhanced by using

probes generated from the mtDNA rather than the chromosomal DNA. These probes were highly specific and hybridized only with the DNA of *P. sorghi* (Yao et al. 1991). Oligonucleotide probes labeled with digoxigenin (DIG) were employed in dot-blot hybridization procedure for detection of *Sporisorium reilianum*, causing head smut disease, and *Ustilago maydis*, causal agent of common smut disease of corn. Plasmid libraries of *S. reilianum* and *U. maydis* were constructed, using a shotgun cloning procedure. Clones containing strongly hybridizing species-specific DNA were selected by screening libraries with their own labeled genomic DNA, followed by cross-hybridization with genomic DNA of maize and other maize pathogenic fungi. The selected clones were used to generate subclones with short insert fragments to facilitate PCR amplification for labeling and primer design for a PCR assay. Using DIG-labeled inserts, detection of less than 0.16 ng of fungal DNA was possible by dot-blot hybridization. Primer pairs SR1 and SR3 were found to be species-specific for *S. reilianum* and UM11 was specific to *U. maydis*. It was possible to detect fungal DNA of less than 1.6 pg using SR1 and SR3 and by using UM11 even in the presence of maize DNA. *S. reilianum* could be detected in extracts of pith, node, and shank, but not in leaves from infected maize plants. Application of both dot-blot hybridization and PCR assay allowed highly sensitive detection and differentiation of smut diseases caused by *S. reilianum* and *U. maydis* (Xu et al. 1999; Section 2.3.3).

Dot-immunobinding assay (DIBA), based on similar principle as ELISA assay, involves the use of nitrocellulose or nylon membrane for immobilizing the pathogen protein (antigen) in place of polystyrene plates used in ELISA tests. The free protein sites in the membranes have to be blocked however, using bovine serum albumin (BSA) or nonfat dry milk powder or gelatin. The unconjugated pathogen-specific antibody is allowed to react with the immobilized antigen of pathogen origin in the membrane. The trapped antibody is then probed with appropriate enzyme and, in a suitable substrate, facilitating the visual detection of a colored product. DIBA test was employed for detecting the resting spores of *Plasmodiophora brassicae*, causal agent of clubroot disease of crucifers. The alkaline phosphatase conjugated goat antirabbit IgG and the substrate nitroblue tetrazolium and 5-bromo-1-chloro-3-indolyl phosphate-p-toluidine prediluted with N,N-demethylformamide were applied to detect positive reaction. Efficiency of detection of *P. brassicae* was similar to that of ELISA tests. The dilution endpoint of detection of spores in root homogenates was 1×10^2 to 1×10^3 spores mL⁻¹ (Orihara and Yamamoto 1998). The presence of *P. brassicae* in roots including hypocotyls from healthy and club-root-infected turnip plants was detected by cutting them transversely starting 1 cm below the basal parts of stems. The cut surfaces of root discs from healthy and infected plants were stamped onto nitrocellulose membrane sheets. Tissue disks produced strong positive reactions throughout the tissue-blotting marks, revealing the presence of resting spores of *P. brassicae* in all tissue samples. These techniques offer the advantage of distinguishing live fungal spores from the dead spores (Orihara and Yamamoto 1998).

2.1.4.2 PCR-Based Techniques

The polymerase chain reaction (PCR) is a simple, ingenious procedure involving amplification of target DNA sequences exponentially by in vitro DNA synthesis. Amplification proceeds rapidly with very high specificity and fidelity, using oligonucleotide primers and a DNA polymerase in an automated reaction. Nobel laureate Arthur Kornberg discovered a cellular enzyme-designated DNA polymerase which has

been used in numerous investigations, leading to the development of PCR technique (Mullis and Fallina 1987; Mullis 1990). Applications of PCR have been singularly responsible for rapid advancements in all biological sciences, including plant pathology. The effectiveness of PCR depends on its ability to amplify specific DNA or cDNA (complementary DNA) transcript in vitro from trace amounts of complex templates. Specific DNA or cDNA sequences from as short as 50 basepairs (bp) to over 1000 bp in length can be amplified to more than a million folds in a few hours in an automated DNA thermocycler. Three essential steps in PCR amplification have been recognized: (i) melting of target DNA or cDNA; (ii) annealing of two oligonucleotide primers to the denatured DNA strands; and (iii) primer extension by a thermostable DNA polymerase. Heat denaturation of the target ds-DNA is followed by hybridization of a pair of synthetic oligonucleotide primers to both strands of the target DNA, one to the 5' end of sense strand and one to the 5' end of the antisense strand by an annealing step. A thermostable *Taq* DNA polymerase from *Thermus aquaticus* (*Taq*) is used for synthesis of new DNA strands. Newly synthesized DNA strands serve as targets for subsequent DNA synthesis, as the three steps are repeated up to 50 times. The DNA sequences between the primers are produced with high fidelity and efficiency of up to 85% per cycle. This procedure is amenable for automation, if required, using heat-stable *Taq* DNA polymerase at temperatures of 60–85°C (Weier and Gray 1988).

The amplified products (amplicons) of PCR assay are commonly used for three different purposes: (i) as a target for hybridization with probe; (ii) for direct sequencing of the DNA of different strains of a pathogen; and (iii) as a specific probe. There is no need for the isolation of pathogen(s) from infected plant materials in pure cultures for performing the PCR assays for detection of single or multiple pathogens present in the seeds or planting materials. Large number of different PCR primers can be artificially synthesized at costs comparable to preparing only a few monoclonal antibodies employed in immunoassays (Henson and French 1993). A commercial product Gene Releaser is used frequently for preparing the extracts suitable for PCR amplification without the need for use of organic solvents, alcohol precipitation, or additional nucleic acid purification procedures (Levy et al. 1994).

The presence of inhibitors in the plant tissues significantly reduces the sensitivity of PCR assay. As contamination of pathogen DNA with host DNA or other DNA samples is likely to occur, resulting in false-negative results, it is essential to apply stringent conditions during all operations and to have proper negative controls. The inability of PCR assays to discriminate the viable and dead propagules of the pathogens is another major drawback of PCR assays. Development of the technique involving enrichment culturing (BIO-PCR) prior to PCR has been useful in overcoming this problem to some extent (Keer and Birceh 2003; Schaad et al. 2003; Call 2005). However, their high levels of sensitivity and specificity, in addition to rapidity and simplicity, mean that PCR assays are the preferred method of choice for routine and large-scale application in quarantine and certification programs for pathogen detection and identification.

Three DNA sequences commonly found in fungal pathogens are used for the development of diagnostic techniques based on PCR. The nuclear rDNA region, which usually consists of three genes (the large subunit gene, the small subunit gene, and the 5.8S gene) are separated by internal transcribed spacer (ITS) regions which are repeated several times in a unit. β -tubulin gene and the ascomycete mating-type gene are the other genes, the sequences of which are utilized for designing primers. The ITS region of fungal DNA is the most widely sequenced DNA region which has been most useful

for molecular systematics at the species level and even within species (McCartney et al. 2003). However, race-specific random amplified polymorphic DNA (RAPD) markers have provided more reliable for the specific detection of fungal pathogen races as in the case of *Colletotrichum lindemuthianum*, causing common bean anthracnose disease (Mesquita et al. 1998).

Smut disease symptoms are generally expressed when the earheads are formed, although infection of susceptible plants occurs much earlier. Specific primers were employed for the detection of *Sporisorium reilianum*, causal agent of head smut and *Ustilago maydis*, incitant of common smut disease in corn (maize). Primer pairs SR1 and SR3 specific for *S. reilianum* and UM11 specific for *U. maydis* (= *U. zeae*) amplified the respective sequences of pathogen DNAs. *S. reilianum* could be detected in the extracts of pith nodes and stalk, but not in leaves of infected plants. The PCR-based assays could detect fungal DNA of less than 1.7 pg using SR1 and SR3, and 8.0 pg using UM11, irrespective of the presence or absence of host plant DNA (Xu et al. 1999; Section 2.3.3).

In another investigation, *Ustilago hordei* could be detected using the primer pair designed based on the sequences of internal transcribed spacer (ITS) region of rDNA. The primers amplified a 574 bp fragment from DNA of *Ustilago* spp., but did not amplify DNA from barley or other common barley pathogens. DNA extracted from as few as four sporidia of *U. hordei* was detected by the PCR assay format. The pathogen DNA was amplified from leaf tissues of inoculated susceptible and resistant plants at different stages of plant development. The presence of *U. hordei* in the first, third, or fourth leaves was detected but not in leaves produced later in the case of resistant barley plants. The possibility of detecting *U. hordei* prior to heading would be very useful in assessing resistance of host genotypes/cultivars to barley smut disease (Willits and Sherwood 1999). A PCR assay was developed for specific amplification of DNAs of *Ustilago tritici* and *U. nuda*, infecting wheat and barley respectively, in infected plant tissues. PCR screening of *U. nuda* in seedlings from infected and healthy seed lots was compared to ELISA, microscopy, and head infections of mature plants derived from tillers of tested seedling. PCR assay was more efficient in detecting the pathogen in different samples. Prediction of loose smut infection in barley might be possible by real-time PCR format at the second leaf stage and could be used in breeding programs (Wunderle et al. 2012).

The presence of *Tilletia indica* causing Karnal bunt disease in wheat grains posed a serious threat to the US export market because of either restriction or total prohibition of wheat imports by other countries. Teliospore were concentrated by passing the seed wash of a 50 g grain sample through 53 and 20 µm pore size nylon screens. The material retained in the 20 µm screen was suspended in water, examined under the microscope, and tested by PCR assay. Two pairs of pathogen-specific primers were employed for identifying the pathogen. Both microscopic examination and PCR assay detected the pathogen in the grain samples artificially infested with at least 5 teliospores/50 g of wheat seeds. Size-selective sieving was found to be faster when compared to standard centrifuging method of concentrating the teliospores of *T. indica* (Peterson et al. 2000). The existing PCR assay was unable to differentiate *Tilletia indica* from *T. walkeri*, infecting ryegrass. An effective PCR format was developed for detection and differentiation of *Tilletia* spp. The nucleotide sequences of a 2.3 kb region of the mtDNA, earlier amplified by PCR only from three isolates of *T. indica* and three isolates of *T. walkeri*, were determined. By employing five sets of specific PCR primers, *T. indica* could be consistently detected in wheat samples. Likewise, three *T. walkeri* PCR primer sets produced single bands only with DNA extracted from *T. walkeri* isolates. No detectable

PCR amplicons from DNA of *T. indica* could be observed. Furthermore, a 212 bp amplicon was developed as a target sequence in fluorogenic 5' (prime) nuclease for PCR assay by employing the TaqMan® system to detect and differentiate *T. indica* and *T. walkeri* (Frederick et al. 2000).

Direct amplification of DNA from single ungerminated teliospores of *Tilletia indica* and *T. tritici* was effective for detection and differentiation of these fungal pathogens, infecting wheat. The need for DNA extraction step could be avoided by applying this procedure for PCR assay (McDonald et al. 1999). In the UK advisory testing for wheat seed health for *Tilletia tritici*, requiring 24 h and a lengthy agar plate testing of up to 7 days, was performed. Later PCR assay for detection of both pathogens was developed, using purified fungal DNA. The PCR Mimic technique, a form of competitive PCR was employed for the quantification of fungal infection (Mulholland and McEwan 2000). In another investigation, primer pair Tcar2A/Tcar2B was employed to detect *Tilletia caries* (= *T. tritici*) in the shoots and leaves of infected wheat plants. The PCR assay had a detection limit of 16 pg DNA/100 mg of plant tissue fresh weight. The primer pair also amplified the pathogen DNA in extracts of teliospores, while ELISA test failed to provide positive reaction for teliospores extracts (Eibel et al. 2005a).

Tilletia controversa, an important international quarantine pathogen causing wheat dwarf bunt disease, has similar teliospore morphology and genomic structure as *T. caries*, necessitating the development of a technique for differentiating these two pathogens. A random amplified polymorphic DNA (RAPD) primer-mediated asymmetric-PCR (RM-PCR) was developed to screen differential sequences between *T. controversa* and *T. caries*. A 1322 bp fragment (PR32) was selected from *T. controversa* strain 18 T and *T. caries* strain 29 T by employing RM-PCR format. The PR32 genes were specific to *T. controversa* and almost failed to exhibit any homology to *T. caries* or any other fungi in the database. When the primers designed from PR32 were employed, positive amplification from all 18 strains of *T. controversa* occurred but not with any of the 29 strains of *T. caries* by standard PCR assay. SYBR Green I and TaqMan probe real-time PCR formats were established based on PR32 for rapid and precise identification of *T. controversa*. TaqMan real-time PCR assay was able to detect *T. controversa* successfully, even in asymptomatic tissues (Yuan et al. 2009).

Rhynchosporium secalis, a barley scald disease pathogen, overwinters in plant debris and infects seeds without any visible symptoms, inducing typical scald symptoms in plants later. *R. secalis* was isolated in pure culture on lima bean agar medium and amended with Bengal rose and streptomycin. A species-specific primer set was produced based on sequence analysis of ITS region of *R. secalis*. The primer efficiently detected the pathogen in symptomless infections. The diagnostic band appeared only in the symptomless seeds of susceptible cultivar, but not in the seed extracts of resistant cultivar (Lee et al. 2001). In another investigation, a primer set RS8 and RS9 that amplified a 264 bp fragment from the DNA of all isolates of *R. secalis* was employed. This primer set did not amplify the DNA from any other fungi tested, indicating the specificity of the primer set. The detection limit of the PCR assay was 1 pg of pathogen DNA. The PCR assay could be completed within 24 h, whereas the direct-plating method required 10 days before providing results (Lee and Tewari 2001). The presence of *Rhynchosporium secalis* along with *Pyrenophora teres* in barley seeds was detected by employing pathogen species-specific primers in a PCR-based assay. The barley seeds were artificially inoculated with *R. secalis* and *P. teres*, the seed infection ranging over 70–90% in four samples tested. Adgen DNA Extraction System was used to extract the

total DNA from 1 g of barley seed powder obtained by grinding the seeds. The primers were derived from the ITS regions of rDNA of *R. secalis* and from AFLP marker in *P. teres*. PCR primers RS1-RS2 and RS8-RS9 were able to detect *R. secalis* in barley seeds and leaves efficiently. Primers PTT-F/PTT-R and PTM-F and PTM-R were detected *P. teres* in artificially inoculated barley seed. The intensity of the corresponding DNA band after amplification with primer pair RS1/RS3 was higher than that amplified with the primer pair RS8/RS9. Electrophoretic analysis of PCR amplicons were performed in 1.4% agarose gels. These pathogens were detected in all samples tested with varying disease intensities (Gubis et al. 2004).

Phytophthora sojae, causing root and stem rot in soybean, survives for several years in plants and soil. The pathogen has to be detected early to avoid carryover of the pathogen propagules to subsequent years. The ITS region of eight *P. sojae* isolates were amplified by using universal primers DC6 and ITS4 in a PCR assay. By aligning sequences of PCR products, a region specific to *P. sojae* was identified and specific primers amplified a product of c. 330 bp exclusively from the isolates of *P. sojae* among the 245 isolates representing 25 species of *Phytophthora*, revealing the specificity of the PCR assay. *P. sojae* could be detected in infected soybean tissues and residues, with a detection limit of the PCR assay being 1 fg of purified DNA of the pathogen (Wang et al. 2006). *Fusarium oxysporum* f.sp. *niveum* (FON) and *Mycosphaerella melonis* (MM) cause watermelon Fusarium wilt and gummy stem blight diseases, respectively. PCR assays were developed for rapid and accurate detection and identification of these two pathogens in infected plant tissues. Two pairs of species-specific primers Fn-1/Fn-2 and Mn-1/Mn-2 based on the differences in the ITS sequences of *Fusarium* spp. and *Mycosphaerella* spp. were synthesized. The primers specific to FON amplified a single PCR band of c. 320 bp only from FON isolates (24), whereas MM-specific primers yielded a PCR amplicon of about 420 bp only from MM isolates (22). No amplification of DNA from any of the 72 isolates from other Ascomycotina, Basidiomycotina, Deuteromycotina, and Oomycotina occurred, indicating the specificity of the primers. The sensitivity of detection was 1 fg of genomic DNA of both pathogens. The PCR assay developed in this investigation might be useful for disease diagnosis and pathogen monitoring in quarantine and certification programs and epidemiological investigations (Zhang et al. 2005).

Ramularia collo-cygni (Rcc), causing Ramularia leaf spot (RLS) in spring and winter barley in the Czech Republic, was detected and the pathogen DNA in the seeds was quantified by a real-time PCR assay. PCR primers and a TaqMan probe were designed to target Rcc-specific DNA sequence. The method was optimized using pure fungal DNA and plasmid standard dilutions. After washing, kernels were dissected into lemma, pericarp, testa, endosperm, and embryo which were individually tested by real-time PCR for quantifying Rcc, whereas the presence of other potential seedborne pathogens was checked by standard PCR format. The level of seed contamination was not the main factor influencing symptom expression. Seeds with low Rcc contamination were planted in two locations in 2009, where severe RLS disease incidence was observed. In contrast, the disease severity was much lower where seeds with higher Rcc contamination were planted in 2010. However, when RLS severity was higher, higher level of seed infection was observed. Rcc DNA was the highest in the lemma and the DNA contents in the pericarp and embryo were lower. The pathogen DNA was also detected in seed wash water, indicating that Rcc did not penetrate through the testa into endosperm of seeds (Matusinsky et al. 2011). In a later investigation, *Ramularia coll-cygni* (Rcc) DNA

was detected in barley seed samples using quantitative PCR (qPCR) assay. The seed samples were collected during 1999–2010. The proportion of seed samples tested positive for the presence of Rcc DNA increased from 2004. Quantification of pathogen DNA indicated that fungal population levels varied from season to season. The highest mean levels of pathogen DNA recorded across winter and spring barley in 2009 were 25.89 and 10.09 pg, respectively. Rcc DNA could be detected in the extracts of all emerging leaves of plants growing from infected barley seeds in controlled chamber environment. Fungicidal treatments of seeds with Raxil (tebuconazole + triazoxide) did not prevent movement of the pathogen from seeds to emerging seedlings and developing plants. A linear correlation between visual symptoms and Rcc DNA levels was observed in 2008 ($R=0.8499$, $P<0.001$). Examination of the pathogen in seed revealed its presence in both embryo and nonembryonic tissues. The pathogen was widespread in the seed samples collected at the end of the growing season (Havis et al. 2014).

A PCR assay was developed for amplifying a 496 bp fragment of internal transcribed spacer (ITS) region of *Cephalosporium gramineum* DNA at concentrations of $100 \text{ fg } \mu\text{L}^{-1}$. Winter wheat seed and seedlings were collected from field plots. Seeds were tested by PCR, using 20-seed samples bulked for DNA extraction. The PCR assay amplified a 496 bp fragment from the genomic DNA of 22 isolates of *C. gramineum*. Amplification of *C. gramineum* DNA was detected at a concentration as low as $100 \text{ fg } \mu\text{L}^{-1}$ when diluted into wheat DNA at $330 \text{ ng } \mu\text{L}^{-1}$ or into water. The addition of bovine serum albumin to the reaction mix improved repeatability of assays. PCR of DNA extracted from individual seeds of CV Stephens harvested from 2008 field plots resulted in the formation of amplicons of the expected size from 1 of 500 seeds. PCR detection was possible for *C. gramineum*, when DNA from this seed was diluted to 1:10 in DNA from healthy seed. Estimates of seed infection from plating test on semiselective medium and PCR assays were comparable at 0.18% and 0.13% of winter wheat “Stephens” ($P=0.6042$) and 0.45% and 0.58% of experimental line WA7970 ($P=0.5636$), respectively. PCR differentiated between plants with well-developed symptoms of *Cephalosporium* stripe and noninoculated plants. Positive PCR results were obtained from 22% of asymptomatic leaf blades from inoculated plants, indicating the potential of PCR assay for diagnosis of *Cephalosporium* stripe disease prior to symptom expression, facilitating application of suitable disease management strategies (Klos et al. 2012).

Polymerase chain reaction (PCR) assay can be applied for efficient pathogen detection in different plant tissues, including seeds and planting materials. However, standard PCR format cannot be used for quantification of pathogen populations. Competitive PCR format is effective for such purposes and involves the use of an internal control that can compete for the same primer set and subsequently amplifies at the same rate as target DNA. The internal control allows for quantification of initiation of contents of pathogen DNA. *Rhynchosporium secalis* biomass present in barley seeds was quantified by employing a competitive PCR format using the primer set RS1 and RS3 derived from ITS region of rDNA genes. The DNA extracted from the seeds with different intensity of infection was subjected to comp-PCR with a heterologous internal control which could compete for the same primer set in the conventional PCR, allowing the quantification of *R. secalis* biomass. The resultant PCR product ratio for each PCR (RS-amplified DNA/internal control template-amplified DNA) registered increases proportionally with increasing levels of infection in seeds. Naturally infected barley seeds collected for 4 years were tested to demonstrate the potential of comp-PCR assay as an alternative seed-health-testing technique (Lee et al. 2002).

Different *Fusarium* species – *F. culmorum*, *F. avenaceum*, *F. graminearum* (*Gibberella zeae*), *F. sporotrichoides*, and *F. sambucinum* – are more commonly associated with wheat *Fusarium* head blight (FHB) or scab disease, in addition to *Microdochium nivale* (*Monographella nivalis*). They produce a group of mycotoxins, trichothecenes among which deoxynivalenol (DON) is more harmful to humans and animals, and these toxins cause significant reduction in quality and quantity of wheat grains. A competitive PCR-based assay was developed to quantify trichothecene-producing *Fusarium* spp., based on primers derived from trichodiene synthase gene (*Tri5*). The primers specifically amplified a 260 bp product from 25 isolates of the trichothecenes-producing *Fusarium* spp. A significant correlation was observed between the amount of trichothecene-producing *Fusarium* spp. and DON concentrations in the grains (Edwards et al. 2001). *Pyrenophora teres* is predominantly seedborne, but it can infect plant debris which may also serve as sources of inoculum. On the other hand, *P. graminea* is strictly seedborne. Seed infection by *P. graminea* may therefore increase rapidly if assessment of the extent of seed infection is not carried out properly and in time. A quantitative PCR assay was developed using Scorpion Amplified Refractory Mutation System (ARMS) technology with real-time PCR detection. A single nucleotide base mismatch in primer sequences could distinguish *P. teres* from closely related *P. graminea*. The Scorpion primer set was useful to quantify *P. teres* readily after 35 cycles of amplification (Bates and Taylor 2001).

The composition and predominance of *Fusarium* species complex may vary depending on the geographical locations and environmental conditions. The components of *Fusarium* species complex could be determined by employing species-specific primers in PCR assays (Nicholson et al. 2004). The ribosomal ITS and a portion of calmodulin gene *Fusarium proliferatum*, *F. subglutinans*, and *F. verticillioides* were sequenced and analyzed to design species-specific primers. Three pairs of primers (PRO1/2, SOB1/2, and VER1/2) produced PCR products of 585, 631, and 578 bp fragments for *F. proliferatum*, *F. subglutinans*, and *F. verticillioides*, respectively. These toxigenic fungi were detected in maize kernels (Mulé et al. 2004). Fumonisin-producing and fumonisin-nonproducing strains of *F. verticillioides* could be differentiated by employing a PCR assay, based on DNA markers unrelated to fumonisin production (González-Jaén et al. 2004). In another study, two pairs of specific primers based on intergenic regions of rDNA units were employed for detection of *F. verticillioides*; the second pair could discriminate the major fumonisin-producing strains that were primarily associated with crops and a minor group of fumonisin-nonproducing strains associated with bananas. This procedure, involving the use of highly specific primer sequence, was found to be simple, rapid, and precise, providing sensitive detection, identification, and differentiation of *F. verticillioides* strains producing harmful mycotoxin (Patiño et al. 2004). *Fusarium graminearum* is the primary cause of cereal scab and maize cob rot in China. *F. graminearum* isolates (250) obtained from wheat, barley, and maize were identified as *F. graminearum* by using species-specific primers Fg16F/Fg16R. Based on sequences of *Tri13* and *Tri3* genes involved in mycotoxin biosynthetic pathway, PCR assays were effective in indicating the presence of 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and nivalenol (NIV) chemotypes. 15-ADON chemotype was predominantly observed in two provinces of China (Ji et al. 2009).

Rhynchosporium secalis, causing barley leaf blotch disease, is responsible for significant losses in the UK. In order to detect the presence of *R. secalis* in symptomless leaves, the standard PCR assay was employed. Multiplex and real-time PCR formats were

developed for detection and quantification of levels of colonization and infection of winter barley by *R. secalis*. The multiplex PCR assay was effective in detecting *R. secalis* in barley samples and for simultaneously checking the presence of PCR inhibitors. However, it was not possible to employ the multiplex PCR format for quantification of pathogen DNA. The sensitivity and specificity of PCR assays using different target genes were compared with assessment by visual examination. Three different real-time PCR assays (β -tubulin, TaqMan probe, cytochrome *b* SYBR Green I, and cytochrome *b* LNA probe assay) were highly specific for detecting *R. secalis* as there was no fluorescence signal when other fungi were tested. The cytochrome *b* LNA probe assay was the most sensitive and could detect as little as 0.1 pg of *R. secalis* DNA in the presence of 50 ng of barley leaf DNA. The detection thresholds for the cytochemical β SYBR Green I and β -tubulin TaqMan probe assays were 1.0 and 3.0 pg of pathogen DNA, respectively, in the presence of 50 ng of barley leaf DNA. The pathogen DNA contents were approximately tenfold greater in susceptible cultivar. However, resistance ratings did not correlate well with levels of disease assessed by visual examination or pathogen biomass determination by real-time PCR assays (Fountain et al. 2007).

Phoma ligulicola, causative agent of ray blight disease of pyrethrum and chrysanthemum, was considered a major threat to production. *P. ligulicola* occurred both within and outside the seed. Plate tests using surface-sterilized pyrethrum seeds showed that *P. ligulicola* could be both externally and internally seed borne. A PCR-based assay was developed for the detection of *P. ligulicola* in pyrethrum seed, using the primer pairs PL1/PL2 which amplified pathogen-specific fragment (230 bp) from the ITS region. Bovine serum albumin (BSA) was added to prevent the inhibitory effects of seed extracts. A 230 bp amplicon was generated by PCR assay from genomic DNA from infected pyrethrum seed. Sensitivity tests using serial dilutions of *P. ligulicola* DNA allowed successful detection with as little as 800 fg DNA. The PCR assay could detect the pathogen in seed lots with low levels of infection (up to 0.5%). The incidence of seedborne infection by *P. ligulicola* was quantified using PCR assay and viability assessed by plating tests. Estimation of the incidence of infection was low. Variability in seed infestation by *P. ligulicola* was affected by cultivars. Among the fungicides used for seed treatment, fludioxonil and thiabendazole/thiram significantly reduced seed infection by *P. ligulicola* (Pethybridge et al. 2006; Section 2.3.4). A PCR-based method was employed to detect and identify *Phoma valerianellae* in lamb's lettuce seed. Variations within the ITS1, 5.8S, and ITS2 region of rDNA were used to characterize the strains of *P. valerianellae* with primers within the ITS region (Pellegrino et al. 2010).

It is difficult to identify obligate fungal pathogens based only on the morphological characteristics of asexual/sexual spores/sporulating structures. Preparation of pure DNA from the obligate pathogens is problematical and the amplification of impure DNA is likely to result in wrong conclusions. Downy mildew of sunflower caused by *Plasmopara halstedii* has a worldwide distribution. Viable oospores and sporangia of *P. halstedii* may be present in a quiescent state in sunflower seeds and they may be transported with seeds in international commercial trade exchanges. To prevent unintended introduction of the pathogen, an efficient method of detection of *P. halstedii* in sunflower seeds was considered necessary. A PCR assay was developed for specific detection of *P. halstedii*, based on the ribosomal large subunit (LSU) DNA. The forward (PHAL-F) and reverse (PHAL-R) PCR primers were designed from two polymorphic regions of LSU. After screening 22 isolates of *P. halstedii* corresponding to different races

and countries and 32 other oomycetes, deuteromycete, and ascomycete isolates, the PHAL-F/R primers amplified only a single PCR band of c. 310bp from *P. halstedii* genomic DNA per 20 µL reaction volume and enabled the direct detection of *P. halstedii* in 35 g sunflower seed samples, without the need for any prior biological baiting step. An internal amplification control (IAC) was employed to help discriminate against false-negative samples due to the possible presence of inhibitory compounds in DNA extracts. Detection of *P. halstedii* was carried out on samples collected from naturally infected sunflower crops in which visual assessment of downy mildew incidence was performed. PCR assay using PHAL-F/R primers was able to detect *P. halstedii* in samples collected in plots where only 3.3% of plants showed dwarfing symptoms, which was typical of systemic infection by *P. halstedii*, resulting in seed infection (Ioos et al. 2007).

In a later investigation, a real-time PCR assay targeting *Plasmopara halstedii* in seed was developed. The reaction parameters and the DNA extraction procedure were optimized to prevent the spread or introduction of potentially new genotypes or fungicide-tolerant strains into new areas. A severely infected seed (SIS) sample originating from Clermont-Ferrand (France) was manually collected in a parcel where mixed sunflower (*Helianthus annuus*) genotypes were grown from plants showing severe systemic downy mildew symptoms such as dwarfing. The SIS powder was used to produce a series of calibrated contaminated seed samples. The real-time detection protocol targeted a region in the rDNA specific for *P. halstedii* which was strictly conserved among isolates of *P. halstedii*, regardless of their genotype, geographical origin, and fungicide tolerance pattern. The primer-probe combination did not cross-react with a high quantity of DNA from nontarget organisms, including closely related species or fungal-taxa commonly associated with sunflower seeds or soil. The qPCR assay proved to be specific and inclusive based on computer simulation and in vitro assessments, and it could detect as few as 45 copies of target DNA. A fully optimized DNA extraction method was developed starting from a sample of 1000 seeds, enabling detection of <1 infected seed per 1000 seeds. A set of controls was used systematically during the assays, including a plant-specific probe used in a duplex quantitative PCR that enabled assessment of quality of each DNA extract to ensure reliability of results. The results demonstrated that qPCR assay was robust and it could provide consistent results. Further, the procedure was well adapted to routine analyses because there was no need for subculturing a potentially harmful organism or propagating it on plant tissues (Ioos et al. 2012; Section 2.3.5).

Different *Alternaria* spp. have been reported to be seed borne. In addition to crop losses, they produce several toxic or carcinogenic compounds harmful to plants and animals. A PCR-based method was developed for the detection of *Alternaria alternata* and *A. solani* colonizing cereal grains. Primers based on the sequences of ITS1 and ITS2 regions of the 5.8S rDNA were designed. Amplification of the DNAs of both pathogens occurred, but not of the DNAs of other microorganisms or host plant species tested, indicating the specificity of the assay. The results of both PCR assay and conventional isolation method were comparable, as both methods gave positive results for eight of ten samples tested. The PCR assay required only 8 h to provide reliable results, whereas the isolation method needs several days. The time required for obtaining results is the most critical factor for accepting or rejecting shipment of grains. PCR assay, delivering results in a short time, may therefore be suitable in such cases (Zur et al. 2002). *Alternaria alternata*, *A. radicina*, and *A. dauci* are carried on carrot seeds. *A. alternata* and *A. radicina* produce highly harmful toxins, in addition to

infecting plants from seed inoculum, whereas *A. dauci* can infect plants. As the conventional plating test and deep-freezer blotter method were found to be laborious and time-consuming, a PCR-based method was developed for the detection of all three seedborne fungal pathogens. Primers were designed based on the sequences of ITS region of the rDNA repeat for the detection of *Alternaria* spp. in the seeds and roots of carrot plants. The primers were highly specific, sensitive and capable of differentiating these pathogens. As the results of PCR assay and conventional methods were similar, the PCR assay could be more suitable because of the advantage of obtaining the reliable results rapidly (Konstantinova et al. 2002).

A rapid, specific, and sensitive PCR-based assay was developed for detecting *Fusarium graminearum*, causing FHB disease in wheat and barley. The pathogen produces the mycotoxins DON, nivalenol (NIV), and zearalenone (ZEA), capable of inducing mycotoxicoses in humans and animals. A specific primer pair targeting the sequence of the *gaoA* gene, coding for the enzyme galactose oxidase, was applied for the detection of *F. graminearum* (Niessen and Vogel 1997). The Light CyclerTM technology, involving a combination of rapid in vitro amplification of DNA with real-time detection and quantification of *F. graminearum*, was applied. Based on the PCR primers specific to the *tri5* gene, a quantitative group-specific assay was developed. This system was rapid, providing the results for 32 samples in 45 min, including quantification and identification of the product. This protocol provided reproducible results (98%) in the range between 0.05 and 6.0 ng of purified *F. graminearum* DNA. The Light CyclerTM system in combination with SYBR Green I, a fluorescent dye enabling real-time detection of PCR products, was applied for the detection and quantification of *F. graminearum* both in pure culture and in contaminated wheat samples (Schnerr et al. 2001). A fast, sensitive and easy-to-handle method was developed for the detection of *F. graminearum* contamination in cereal samples. DNA Detection Test StripsTM was first tested using DNA isolated from *F. graminearum* cultures, and this procedure was compared to agarose gel electrophoresis. A minimum of 0.26 ng of purified target DNA was necessary for detection of the pathogen by the Test StripsTM and the results could be obtained within 20 min without the need for any special technical equipment or hazardous fluorescent dyes. A distinct advantage of using the Test StripsTM over conventional PCR format was the possibility of both detection and identification of target pathogen rapidly. Even in the case of nonspecific amplification, products which do not hybridize to the specific probe will not be detected by Test StripsTM method and it was comparable with gel-based DNA detection procedures. This method was found to be useful for routine and screening investigations (Knoll et al. 2002).

Wheat stalks infected by *Fusarium graminearum* and fungi associated with wheat *Fusarium* head blight (FHB) disease may become potential sources of inoculum for wheat crops. TaqMan real-time qPCR assay was employed for detection and quantification of fungal pathogens in wheat stalks exposed to natural infection. *Clonostachys rosea* (strains 016 and 1457) was applied to assess its efficacy in reducing pathogen population which was determined by real-time qPCR assay. Application of *C. rosea* caused a reduction (73%) in *F. graminearum* after 90 days, but after 180 days the pathogen population decreased to undetectable levels in the first year of treatment. During the second year, the antagonist treatment reduced the populations of *F. graminearum* and *F. avenaceum* by 68.3% and 98.9%, respectively, as reflected by genomic DNA contents of the pathogens. Similar reduction in genomic DNA contents of *F. verticillioides* (by 100%) was also observed. This investigation revealed that wheat stalks could be

important reservoirs of *Fusarium* spp. causing FHB disease in wheat, and application of the biocontrol agent (BCA) *C. rosea* could be a potential disease management strategy (Palazzini et al. 2013).

Fusarium pseudograminearum, causing wheat crown rot disease, was detected by visual discoloration of leaf sheaths and PCR assay. The pathogen colonization of tissues was quantified by quantitative PCR (qPCR), based on fungal DNA sequences. Primers specific for the translation elongation factor α sequence were employed in the PCR format. Fourteen-day-old seedlings of four hard white spring wheat genotypes differing in the level of resistance to *F. pseudograminearum* were inoculated. Assessment of extent of discoloration and fungal DNA content per unit of tissue weight was performed at weekly intervals from 7 to 35 days after inoculation (dai). Both visual and qPCR assessments indicated significant differences between the partially resistant and susceptible wheat genotypes from 14 dai. Visual discoloration of leaf sheath tissues was strongly correlated with fungal biomass estimated by qPCR in all four genotypes. However, this correlation became weaker with increasing time after inoculation. Evaluation of plants at 14 dai provided a rapid test which provided clear discrimination between lines for both parameters and was the time point of closest correlation between extent of fungal colonization and intensity of disease symptoms. The results indicated the usefulness of qPCR format for correlating the amount of pathogen DNA with the level of resistance to wheat crown rot disease caused by *F. pseudograminearum* (Knight et al. 2012).

Colletotrichum lindemuthianum, a seedborne pathogen, is responsible for anthracnose disease causing significant losses in common bean in many countries. Seed infection by *C. lindemuthianum* reduces seed quality and may lead to introduction of disease or new races of the pathogen into new geographic locations. Planting pathogen-free seed is a key recommendation for effective management of common bean anthracnose disease. A PCR-based assay was developed to specifically detect *C. lindemuthianum* from pure fungal DNA and mixed DNA from infected bean tissues such as seed and leaves. Based on sequence data of rDNA region consisting of the 5.8S gene and ITS1 and ITS2 of four *C. lindemuthianum* races and 17 *Colletotrichum* species downloaded from GenBank, five forward primers were designed. Among them one forward primer C1F4 was selected for combining with the universal reverse primer ITS4 to detect *C. lindemuthianum* DNA. A 461 bp specific band was amplified from the genomic DNA template of 16 representative isolates of *C. lindemuthianum*, but not from 58 representative isolates of 17 other *Colletotrichum* spp. or 10 bean pathogens. Using single-step PCR, the results were unstable and the sensitivity was low for genomic DNA from infected leaves and different mixtures of infected and healthy bean seed powder. The nested PCR format was therefore applied. A 461 bp DNA fragment was produced from genomic DNA of individual infected leaves and seed powder with nested PCR assay. Visible PCR products were detectable following electrophoresis in ethidium bromide-stained gels. The nested PCR assay could detect as little as 10 fg of *C. lindemuthianum* genomic DNA and in samples with 1% of infected seed powder mixed with 99% healthy seed powder (Fig. 2.3). The nested PCR format provided the results within 24 h, making it suitable for personnel with no taxonomic expertise (Chen et al. 2007; Section 2.3.6). A real-time PCR assay was developed for detection and quantification of *C. lindemuthianum* in infected common bean seeds of the commercial bean class navy bean. The ribosomal DNA (rDNA) region consisting of part of the 18S rDNA, 5.8S rDNA, ITS1, 2 and part of the 28S rDNA of seven races of *C. lindemuthianum*, 21 isolates of *Colletotrichum* spp., and 9 other bean pathogens were sequenced with the universal

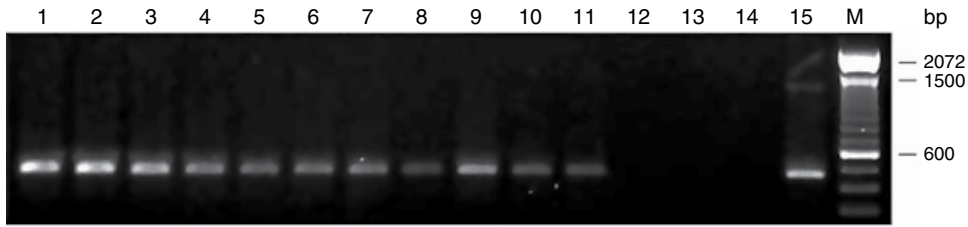


Figure 2.3 Detection of *Colletotrichum lindemuthianum* in infected bean seed powder containing different concentrations of the pathogen, using nested PCR assay. Lanes: M-100-bp DNA ladder; 1, 100%; 2, 80%; 3, 60%; 4, 40%; 5, 20%; 6, 10%; 7, 8%; 8, 6%; 9, 4%; 10, 2%; 11, 1%; 12, 0%; 13, anthracnose-resistant genotype G2333; 14, negative control (water); and 15, positive control (pathogen DNA). Source: Chen et al. (2007). Reproduced with permission of the American Phytopathological Society, MN, USA.

primer set ITS5/ITS4. Based on the aligned sequence matrix, one primer set and a probe were designed for a SYBR Green dye assay and a TaqMan MGB (minor groove binder) assay. The primer set was specific and sensitive for detection of *C. lindemuthianum*, with a detection limit of 5 fg of pathogen genomic DNA (Chen et al. 2013).

A duplex PCR was developed to detect *Colletotrichum lindemuthianum* infection in common bean. Based on the differences in the sequences of the ITS region of 22 *Colletotrichum* spp. retrieved from GenBank database, one pair of specific primers CY1/CY2 was designed. A single product of 442 bp was amplified by the primer CY1/CY2 only from *C. lindemuthianum* and *C. orbiculare*, but not from any other *Colletotrichum* spp. tested. This product was cloned, sequenced, and used for designing a species-specific primer pair of CD1/CD2. The CD1/CD2 primer pair could distinguish *C. lindemuthianum* from *C. orbiculare* by a 638 bp PCR band. A duplex PCR method employing both pairs of primers, CY1/CY2 and CD1/CD2, was used to detect *C. lindemuthianum* infection in common bean. The sensitivity of detection with duplex PCR format was 1.0 pg of pure genomic DNA from the pathogen DNA, indicating the effectiveness of the assay for detection of *C. lindemuthianum* in common bean (Wang et al. 2008).

Colletotrichum capsici, incitant of anthracnose and fruit rot of pepper (*Capsicum annuum*), is both internally and externally seedborne. A PCR assay based on sequence-characterized amplified region (SCAR) marker was developed for specific detection of *C. capsici* in fruits and seeds of pepper. The SCAR primers specifically amplified a 250 bp fragment from the genomic DNA of seven isolates of *C. capsici*. Amplification of the pathogen-specific fragment also occurred from DNA extracted from infected fruits. The PCR assay had a detection limit of 1 pg of purified DNA of *C. capsici*. A real-time PCR format using SYBR Green was employed for the detection of *C. capsici* in seeds and fruits. The PCR format developed in this investigation might be useful for rapid and sensitive detection of the pathogen in seeds in quarantine programs (Srinivasan et al. 2014).

Infection of small-grain cereals by *Fusarium* spp. results in accumulation of different mycotoxins in food and feed in addition to significant yield losses. *F. poae* and *F. sporotrichoides* are the principal pathogens with serious toxigenic profile. A highly sensitive duplex PCR assay based on multicopy rDNA sequences was developed for the differential detection of *F. poae* and *F. sporotrichoides*. The specificity of the PCR assay was evaluated, using genomic DNA extracted from 27 *Fusarium* species comprising 105 isolates. The primer FspITS2SK detected only *F. sporotrichoides* belonging to the

FstITS genotype and it was not specific to isolates with FslI genotype present in *F. sporotrichoides*/*F. langsethiae* group of species. The sensitivity of the duplex PCR assay was determined on 24 symptomless wheat seed samples naturally contaminated with *Fusarium* spp. originating from northern Poland. The assay was compared with PCR assay developed earlier based on single-copy primers, Fp82F/R and FsporF1/LansporR, specific for *F. poae* and *F. sporotrichoides*, respectively. Results of duplex PCR format showed that all 24 samples were positive for the presence of *F. poae*, whereas PCR assay employing single-copy primer could detect *F. poae* in 21 samples only. A 288-nucleotide product characteristic of *F. sporotrichoides* was generated in 19 samples when duplex PCR format was employed. In contrast, only seven samples showed a positive reaction for *F. sporotrichoides* with single-copy PCR assay. Morphological analysis showed the presence of *F. sporotrichoides* in 16 samples, indicating the lesser efficiency of single-copy PCR format (Kulik 2008). A multiplex PCR assay was developed for simultaneous identification of the species and trichothecene chemotypes of *Fusarium graminearum* and *F. asiaticum* sensu stricto, based on the genes related to trichothecene biosynthesis. PCR assay was performed in a single reaction where three pairs of primers designed for the *tri6* region and one pair of primers designed for *tri3* were employed. Multiplex PCR assay was able to identify species and chemotypes for all tested strains of *F. asiaticum* and *F. graminearum* isolated in Japan. The multiplex PCR protocol might be useful for investigations for disease diagnosis, epidemiology, and population structures of *F. graminearum* complex (Suzuki et al. 2010).

Aspergillus flavus group encloses *A. flavus* and *A. parasiticus* as aflatoxin producers and *A. oryzae* and *A. sojae* as koji molds. A PCR-based assay was employed to identify the four aflatoxin-synthesizing genes encoding norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1*), and a regulatory protein (*apa-2*) involved in aflatoxin biosynthesis. Fourteen strains of *A. flavus* were found to possess the four target DNA fragments. When peanut kernels were artificially contaminated with *A. parasiticus* and *A. niger* for 7 days, the contaminant DNA was extracted from a piece of cotyledons (c. 100 mg) and subjected to multiplex PCR assay using four pairs of primers coding for the genes mentioned above. They were successfully detected by this assay. The target DNA fragments were detected in kernels infected by *A. parasiticus*, while none was detected in uninoculated kernels or other kernels infected with *A. niger* (Ruey Shyang et al. 2002). *Fusarium oxysporum* f.sp. *vasinfectum*, causal agent of the important wilt disease of cotton, occurs in the form of several races that differ in the virulence on cotton genotypes. Although the seed transmission of *F. oxysporum* f.sp. *vasinfectum* has been reported in many countries, attempts to detect the pathogen in cotton seed from California were unsuccessful. Incidence of wilt disease in isolated spots in previously uninfested fields indicated the possibility of transmission of the virulent race 4 of *F. oxysporum* f.sp. *vasinfectum* through cotton seeds. The pathogen was isolated from individual as well as a group of seeds using Komada's medium. Race 4 was frequently detected in Pima cultivar DP744. Race-specific primers were employed in the PCR assay for the detection of race 4. The DNA was extracted from the mycelium of single-spore cultures, using the FastDNA Kit and FastPrep Instrument (QBiogene, Irvine, CA) or the PowerSoil DNA Isolation Kit (MOBIO Laboratories, CA) as per the manufacturer's recommendations. Amplicons specific to race 4 were obtained only from infected seeds of Pima cultivar DP744, but not from the seeds of Maxxa seed (negative control). The results suggested that acid-delinting of cotton seed might reduce the seed transmission of *F. oxysporum* f.sp. *vasinfectum* to some extent and that dispersal of race 4 by infected seed might occur to new areas (Bennett et al. 2008).

Rice crops are infected by clavicipitaceous pathogens *Ustilaginoidea virens*, causative agent of false smut disease, and *Ephelis oryzae*, incitant of udbatta disease. A PCR-based technique for detection of these pathogens was developed. A comparison was made for the genetic diversity in the rDNA internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene of *U. virens*, *Ephelis japonica*, as well as some other clavicipitaceous fungi. Each fungal pathogen was successfully detected by using a specific primer set with high sensitivity. Species-specific primers designed in this investigation were able to detect the target pathogens in plant tissues. PCR detection results correlated well with conventional histological observations. The nested PCR protocol was sensitive and reliable for the detection of *U. virens* and *E. japonica*. The procedure could be used to study disease cycles and early prediction of incidence of false smut and udbatta diseases under field conditions (Zhou et al. 2003).

Rice plants infected by *Ustilaginoidea virens* remain asymptomatic until heading stage and it is difficult to locate the presence of pathogen spores on contaminated seeds. Although infection by *U. virens* may not reduce the yield, the quality of seeds may be affected because of the presence of the toxin ustiloxin, a microtubule inhibitor, toxic to animals (Koiso et al. 1994). An efficient method of detecting the pathogen in rice seeds was therefore developed. The nested PCR technique, using primers designed from rDNA internal transcribed spacers and the 5.8S rRNA gene of *U. virens*, was found to be sensitive and specific in detecting the pathogen in rice seeds. The DNA fragment of *U. virens* was amplified producing a specific band of 380 bp that could be detected in agarose gel after electrophoresis. The nested PCR assay could rapidly and reliably identify the pathogen in and on the rice seeds, facilitating the elimination of infected seed lots. The primer specificity was confirmed by the amplification of the target DNA of *U. virens* pathogenic to rice, while no amplification occurred in samples of DNA extracted from *Pyricularia grisea*, the causal agent of rice blast disease (Ditmore and TeBeest 2005). Detection of *Ustilaginoidea virens* in rice seeds was achieved by employing a simple and rapid PCR assay. The primer pair specific to *U. virens* was designed from 5.8S rDNA intra- and interspecific variations in the nucleotide sequences of the pathogen. A 346 bp fragment could be amplified using specific primers uvr-F and uvr-R in *U. virens*. This primer set did not amplify any DNA fragment of *Fusarium verticillioides*, *Sclerotinia sclerotiorum*, *Tilletia barclayana*, *Fusarium graminearum*, and *Magnaporthe grisea*, indicating the specificity of the primers used in PCR assay. *U. virens* could be detected in 9 of the 24 (37.5%) rice seed samples tested. The PCR protocol developed may be useful for screening large number of rice samples and for assessing the rice seed infection by *U. virens* (Chen et al. 2014).

Verticillium dahliae has been demonstrated to be seedborne in lettuce (Vallad et al. 2005) and spinach (Toit et al. 2005). Verticillium wilt disease caused by *V. dahliae* is one of the most destructive diseases affecting olive (*Olea europaea*) trees. Transmission of the pathogen through seeds has been suggested as a possible mode of dissemination to new locations. Seeds and seedlings from symptomatic and asymptomatic mother plants of olive were tested by direct-plating and nested PCR methods. Primers were designed, using the sequences of ITS regions of nuclear rRNA genes of *V. dahliae*. The sensitivity of the nested PCR assay was evaluated by amplifying crude DNA of conidia. A significantly higher level of infection in seeds and seedlings by *V. dahliae* was detected by nested PCR assay than direct-plating method, when both symptomatic and asymptomatic trees were tested. Seed transmission of *V. dahliae* to the progeny seedlings of two olive cultivars varied considerably. The results clearly showed that infected olive seeds could be a potential source of inoculum carrying the pathogen to new geographical

locations (Karajeh 2006). Transmission of *Verticillium dahliae* from infested spinach seeds to lettuce was investigated in microplots. Verticillium wilt developed following two or three plantings of *Verticillium*-infested spinach in independent experiments. The pathogen recovered from infected lettuce plants from microplots was confirmed as *V. dahliae* by PCR assays. In greenhouse experiments, transmission of a green fluorescent protein (GFP) -tagged mutant strain of *V. dahliae* from spinach to lettuce roots was demonstrated after two cycles of incorporation of infected spinach residue into the soil. The results confirmed that *V. dahliae* introduced through spinach seed could cause Verticillium wilt disease in lettuce (Short et al. 2015).

In order to enhance the sensitivity of detection of *Phytophthora nicotianae*, causing tobacco black shank disease, single-closed tube nested PCR and conventional PCR formats were employed. Two new specific primers designed from the pathogen DNA ITS1 and ITS2 regions, internal to the nucleotide sequence flanked by universal primers ITS4 and ITS6, were used. Morphologically characterized isolates of *P. nicotianae* (36) were compared in this investigation. Positive reaction was inferred by the amplification of 737 bp product from DNA of all isolates of *P. nicotianae* and two *P. nicotianae/cactorum* hybrids. No amplification occurred when other *Phytophthora* spp. and genera were tested, indicating the specificity of the assay. Nested PCR format was at least 1000-fold more sensitive than conventional PCR format. In addition, the presence of *P. nicotianae* in different infection sites, origins, crops, samples from nutrient solutions, water, and rockwool used in hydroponic cultures could be detected, validating the specificity and reliability of the nested PCR procedure developed in this investigation (Grote et al. 2002). As *Phytophthora nicotianae* can also spread through irrigation water, it is necessary to detect the pathogen in water samples to restrict the spread of the disease. A species-specific PCR assay was developed involving the use of a pair of species-specific primers (PN) and customization of a commercial soil DNA extraction kit for purification of DNA propagules present in the irrigation water. PN primers were designed, based on the sequences of the elicitor gene *parA*. PN primers allowed more efficient detection of *P. nicotianae* isolates, while PP primers were specific to *P. parasitica*. PN primers specifically amplified the DNA sequences of 31 isolates of *P. nicotianae* only, but none of the 102 isolates of 15 species of *Phytophthora* and also 64 isolates of other oomycetes, true fungi, and bacteria were tested. The detection limit of the PCR assay was between 80 and 800 fg of DNA/ μ L of *P. nicotianae*. This PCR protocol could detect the pathogen in naturally infested water samples from tobacco nurseries more rapidly and precisely than the standard isolation-based methods. The procedure had the potential for wider field application to enable the growers to take appropriate disease management decisions in time (Kong et al. 2003).

Another investigation demonstrated that *Phytophthora nicotianae* could be efficiently detected by using primers designed based on the sequences of a Ras-related protein gene (*Ypt1*) in PCR assay. Fungal isolates (115) representing 26 species of *Phytophthora* and 29 other pathogenic fungi were tested to evaluate the specificity of the selected primers. A specific 389 bp amplicon was produced only from the isolates of *P. nicotianae*, but not from other *Phytophthora* spp. and fungal pathogens. The detection limit of the PCR assay using the species-specific Pn primers was 1 ng of genomic DNA. In the nested PCR format, the primer pair YPt-1F/YPt1R was used for the first round amplification. The sensitivity of the nested PCR method was enhanced by 100 folds. The standard PCR format was successful in detecting *P. nicotianae* in naturally infected tobacco tissues, as well as in the soil samples. This PCR format might form a simple method for diagnosis

of infection of tobacco by *P. nicotianae* and it would be effective for monitoring pathogen population, facilitating application of suitable disease management strategies (Meng and Wang 2010). As the distribution and concentration of pathogen in soil and irrigation water may not be uniform, the sensitivity of detection has to be enhanced. A pair of *Phytophthora* universal primer pair Ypt-1F/Ypt-1R was employed for the first round amplification in the nested PCR format. The amplicons were then amplified with species-specific primer pair Pn1/Pn2. The detection limit of the nested PCR was 10 pg DNA of *P. nicotianae*. This protocol was very sensitive for the detection of *P. nicotianae* in the soil samples and it has the potential for tracking the pathogen movement in soil and irrigation water (Meng and Wang 2010). In another study, primers targeting ITS regions of rDNA genes of *Phytophthora* spp. were identified. Based on the difference in the nucleotide sequences of ITS2 of 15 different *Phytophthora* spp., primers specific for amplification of DNA fragment from *P. nicotianae* were selected for use in the real-time PCR assay. The detection limit of standard PCR was 10 pg μL^{-1} of pathogen DNA. SYBR Green I PCR format could detect *P. nicotianae* at concentrations as low as 1.2 fg μL^{-1} , while TaqMan PCR could detect 1.2 pg μL^{-1} of *P. nicotianae* DNA. The real-time PCR assays were 10^4 to 10^5 times more sensitive than the standard PCR assay. This protocol maximized the yield and quality of the DNA recovered from soils samples rapidly. The real-time PCR protocol detected *P. nicotianae* in the soil with a detection limit of 10 pg μL^{-1} (Huang et al. 2010).

Group-specific detection of trichothecene- and fumonisin-producing *Fusarium* spp. and specific identification of *F. graminearum* and *F. verticillioides* in field-collected barley and corn samples were accomplished by employing a fluorogenic (TaqMan®) real-time PCR assay. Primers and probes were designed from genes involved in mycotoxin biosynthesis (*TR1b* and *FUM1*). In addition, primers and probes were prepared based on the rDNA sequences of *Fusarium* to provide a genus-specific internal positive control. Barley and corn samples infected by *F. graminearum* and *F. verticillioides* tested positive for the presence of trichothecene and fumonisin (Bluhm et al. 2004). A real-time PCR assay useful for monitoring and quantifying the major *Fusarium* spp. involved in FHB complex was developed. TaqMan® primers and probes showing high specificity to *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, and *Microdochium nivale* var. *majus* were designed. By maintaining an internal PCR control and using proper dilutions of pure genomic DNAs of the pathogens, the DNA contents of each pathogen in leaves, ears, and in harvested grains of winter wheat could be determined. TaqMan® technology was shown to be useful to quantify and monitor the dynamics of individual species of the *Fusarium* species complex causing FHB disease in cereals (Waalwijk et al. 2004). A real-time PCR assay was developed for specific detection of *Fusarium culmorum* in infected wheat and barley seeds. Primers and a TaqMan minor groove binder probe were derived from the sequences of a *Fusarium*-specific PCR product. The specificity of the assay was confirmed by testing *Fusarium* species and 21 other fungal species, with serial dilutions of purified genomic DNA per reaction. A significant correlation (r^2 average = 0.982) and colinearity was found between DNA concentration and Ct (cycle threshold) values of real-time PCR assay with serially diluted DNAs extracted from seed samples with different deoxynivalenol (DON) content. Eight barley and nine wheat varieties infected by *F. culmorum* isolate B were evaluated in 1 year (barley sample) and in 4 years (wheat samples). The results of real-time PCR analysis and ELISA testing for DON content were compared and a significant correlation was found for barley samples (r^2 = 0.935). The relationship between Ct values and DON contents was

rather a complicated one, because of the environmental variations under field conditions. The real-time PCR assay was found to be highly specific and sensitive (Leiřová et al. 2006).

Fusarium verticillioides (teleomorph, *Gibberella fujikuroi*), a component of corn ear rot complex, produces fumonisins capable of contaminating corn and sorghum grains that become unfit for human and animal consumption. Fumonisin levels were high in corn grains, while sorghum grain had a low level of this mycotoxin. The genetic diversity of *F. verticillioides* strains isolated from corn (22) and sorghum (21) was investigated. Differences in the genetic profiles were observed among the strains isolated from two substrates, using a single primer amplification reaction by polymerase chain reaction (SPAR-PCR). Fumonisin levels were higher in strains isolated from corn than those in the isolated from sorghum. The *MAT-1* and *MAT-2* alleles were identified by PCR and the isolates were subsequently crossed with *F. thapsinum* (*G. fujikuroi* mating population F) reference strains, because this species is morphologically similar to *F. verticillioides* and produced low levels of fumonisins. The SPAR haplotypes of some strains isolated from sorghum were similar to the *F. thapsinum* reference strain haplotypes (da Silva et al. 2006). Many genes potentially involved in the virulence of *Fusarium graminearum*, the predominant causal agent of Fusarium head blight (FHB) disease complex, have been identified. It is essential to detect and quantify the wild-type and mutant strains occurring in the fields to understand the role of genes in disease development. A SYBR-based real-time PCR assay was developed to quantify the total genomic DNA in a plant sample as well as the total genome DNA of *F. graminearum* that contributed from a strain containing a common selectable marker used to create deletion mutants. This procedure allows correlation of the amount of disease observed in wheat field trials with the mutants of *F. graminearum* under investigation (Dyer et al. 2006). In order to identify *Fusarium* spp. present in grains and feed samples, the DNA extraction methods such as DNeasy® Plant Mini Spin Column (Qiagen), the Biorobot EZ1 (Qiagen) with the DNeasy® Blood and Tissue Kit (Qiagen), and the Fast-DNA® Spin Kit for Soil (Qbiogene) were tested. DNeasy® Plant Mini Spin Columns in combination with sonication gave the best results with respect to *Fusarium* DNA yield. The modified DNeasy® Plant Mini Spin protocol was used to analyze 31 wheat samples for the presence of *F. graminearum* and *F. culmorum*. The DNA level of *F. graminearum* and *F. culmorum* could be correlated to the level of DON ($r^2 = 0.9$) and ZEN ($r^2 = 0.5$), whereas there was no correlation between *F. culmorum* and DON/ZEA. The results indicated that *F. graminearum* and not *F. culmorum* was the main producer of DON in Swedish wheat during 2006 (Fredlund et al. 2008).

Fusarium graminearum, primarily a pathogen of cereals, can also infect noncereal crops such as potato and sugar beet in the USA. Isolates of *F. graminearum* collected from potato, sugar beet, and wheat produced white fluffy mycelium and distinct macroconidia on potato dextrose agar (PDA) and carnation leaf agar (CLA) media. These isolates induced typical FHB symptoms in susceptible wheat cultivar and were capable of producing a wide range of trichothecenes. Significant variation among isolates in relation to virulence, FgDNA content and mycotoxin production was observed. Most of the potato and sugar beet isolates produced equal or even greater FHB severity, FgDNA content, and higher mycotoxin accumulation compared with wheat isolates. Potato isolates showed greater variability in producing different mycotoxins than sugar beet and wheat isolates. Most potato isolates produced high levels of deoxynivalenol (DON). A real-time PCR assay was developed based on intergenic sequences specific to the

trichodiene synthase gene (*Tri5*) from *F. graminearum*. TaqMan probe and primers were designed and used to estimate DNA content of the pathogen (FgDNA) in the heads of susceptible wheat variety after inoculation with 21 isolates of *F. graminearum* collected from potato, sugar beet, and wheat under artificial conditions. The presence of nine mycotoxins was analyzed in the inoculated wheat heads by gas chromatography and mass spectrometry. All isolates contained *Tri5* gene and were virulent to susceptible variety. Isolates of *F. graminearum* differed significantly in virulence, as reflected by disease severity, FgDNA content, and mycotoxin accumulation. Potato isolates showed greater variability in producing different mycotoxins than sugar beet and wheat isolates. Correlation analysis showed a significant positive relationship ($P < 0.001$) between FgDNA and FHB severity or deoxynivalenol (DON) production. Further, a significant ($P < 0.001$) positive correlation between FHB severity and DON content was observed. The results revealed that *F. graminearum*, causing potato dry rot and sugar beet decay, could be potential sources of inoculum for wheat FHB epidemics. Real-time PCR assay could offer sensitive and accurate quantification of *F. graminearum* in wheat and could be useful for monitoring the colonization of wheat grains by *F. graminearum* in controlled environments and evaluating wheat germplasm for resistance to FHB disease (Burlakoti et al. 2007).

A simple and rapid quantification technique such as real-time PCR was considered essential to assess the mycotoxin risk in cereals applied in cereal-based industry. The TMTR1 and TMFg12, two variants of TaqMan technology, were applied to quantify trichothecene-producing *Fusarium* DNA and *Fusarium graminearum* DNA present in barley grains and malt samples. The contents of deoxynivalenol (DON) in barley grains were represented by the *Fusarium* DNA contents determined by TMTR1-trichothecene assay. On the other hand, the TMFg12 format for *F. graminearum* provided relative DON contents in the North American barley and malt samples (Sarlin et al. 2006). Species-specific PCR primers were used to detect *Fusarium* spp. and *Microdochium* spp. associated with FHB disease complex. The DNA from the fungal pathogens was extracted from seeds (milled flour) and chaff in diagnostic PCR (dPCR) and competitive PCR formats. The incidence of FHB pathogen in quantifiable amounts of DNA was significantly less ($P < 0.05$) in the grain than in the chaff for all FHB pathogens, except for *F. culmorum* for which there was no significant difference. The amount of quantifiable DNA in chaff was as much as ten times greater than in the grain. Accumulation of DON was most strongly associated with the presence of *F. graminearum*, while NIV accumulation was associated only with *F. culmorum*. On the other hand, production of zearalenone (ZON) was associated with all three *Fusarium* spp. However, the amount of the pathogen DNAs and mycotoxins varied greatly at different locations (Xu et al. 2008).

Microdochium nivale (= *M. nivale* var. *nivale*) and *M. majus* (= *M. nivale* var. *majus*) are associated with *Fusarium* head blight (FHB) disease and seedling blight disease complex of small grain cereals. In contrast to *Fusarium* species of FHB complex, *Microdochium* spp. do not produce mycotoxins but they cause poor germination and crop establishment. Quantitative real-time PCR assays were designed to differentiate the two *Microdochium* spp. based on the translation elongation factor-1 α gene (TEF-1 α) and used to analyze total of 374 seed samples of wheat, barley, triticale rye, and oat sampled from farmers' field across Denmark from 2003 to 2007. Both *Microdochium* spp. were detected in all cereal crops. *M. majus* was found more predominantly compared with *M. nivale* in most years in all cereals except rye, in which *M. nivale* represented a larger proportion of the biomass. Historical samples of wheat and barley

Table 2.3 DNA contents (pg/μg plant DNA) of *Microdochium majus* and *M. nivale* in pooled historical grain samples of wheat and barley during (1957–2000) (Nielsen et al. 2013). From each period, the sample was pooled using one sample per year which was again pooled from four samples in the individual year.

Year	<i>M. majus</i>		<i>M. nivale</i>	
	Wheat	Barley	Wheat	Barley
1957–1960	559	0	4	9
1965–1968	666	704	3	12
1977–1980	67	102	7	31
1981–1984	465	479	5	36
1985–1988	249	1044	59	234
1989–1992	158	6372	36	33
1993–1996	174	939	18	31
1997–2000	1104	20,058	12	479

form 1957 to 2000 similarly showed a strong prevalence of *M. majus* over *M. nivale*. PCR analysis of the two quantified *Microdochium* species in wheat, barley and triticale samples generally showed co-existence of *M. majus* and *M. nivale* in all three cereal species (Table 2.3). Strobilurin resistance in *M. nivale/majus* was analyzed in selected wheat (2003–2007), barley (2007), and historical (1957–2000) samples, since detection of strobilurin resistance is of importance to plan future fungicide control strategies (Nielsen et al. 2013).

Septoria tritici (*Mycosphaerella tritici*) and *Stagonospora nodorum*, causing wheat leaf blotch and glume blotch diseases, constitute the Septoria complex. The ITS regions of these pathogens were cloned and sequenced. Conserved sequences of the rDNA were used for designing specific primers that successfully amplified similar-sized fragments from infected wheat plant tissues (Beck and Ligon 1995). In combination with ELISA test, the PCR-based assay was adapted to a microplate format for quantification of pathogen population, reflecting disease pressure (Beck et al. 1996). A robust and quantitative PCR assay was developed using the β -tubulin gene as target for detection and quantification of *S. tritici* infection levels on wheat tissues. Specific primers were designed by aligning and comparing β -tubulin sequences from other fungi. The final primer set was selected after testing against several fungi and also against *S. tritici*-infected and healthy wheat samples from different locations. A single DNA fragment (496 bp) was amplified from DNA of *S. tritici*, but not from DNA of wheat plant or other fungi tested. The detection limit of the assay was c. 2 pg of pathogen DNA. The amounts of PCR-amplified products were directly quantified in microtitre plate wells with a fluorimeter using the dye PicoGreen which fluoresced specifically upon binding with the ds-DNA. The PicoGreen assay required c. 10 pg of *S. tritici* DNA/reaction, showing that this assay was less sensitive compared to conventional PCR assay. However, PicoGreen was shown to be quantitative, rapid, and easy to perform in a microtitre format, allowing high-throughput screening. In addition, the PCR/PicoGreen assays

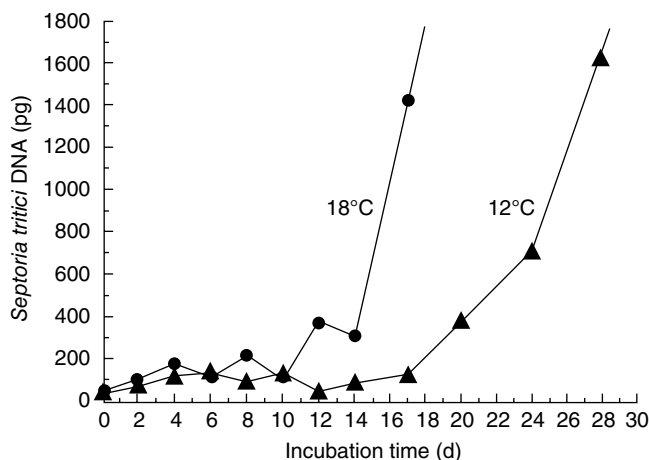


Figure 2.4 Estimation of biomass of *Septoria tritici* in inoculated wheat leaves, after different periods of incubation at 12°C and 18°C employing PCR/PicoGreen assay. Source: Fraaije et al. (1999). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

were found to be useful to study the colonization, infection, and subsequent disease development of *S. tritici* on wheat under in vitro, glasshouse, and field conditions (Fig. 2.4; Fraaije et al. 1999).

Fusarium crown rot (FCR) disease is caused by primarily by *Fusarium culmorum* and *F. pseudograminearum*. A real-time quantitative PCR (QPCR) assay was developed for the detection of these fungal pathogens, using primers and probes specific for a segment of the trichodiene synthase (*tri5*) gene. The utility of the QPCR assay was tested under field conditions by inoculating spring and durum wheat cultivars to produce different levels of disease severity. At 2 weeks after harvest, plants were analyzed by QPCR assay. Disease severity scores (DSS), based on visual assessment and *Fusarium* DNA contents determined by QPCR, were positively correlated with each other for all three cultivars in 2004 but only for durum cultivar in 2005 ($P < 0.005$). Grain yields for both spring and durum wheat and cultivars were negatively correlated with *Fusarium* DNA contents (Hogg et al. 2007). Quantification of biomass of different *Fusarium* spp. in wheat and maize is necessary to understand the role of individual species in disease development. Quantitative real-time PCR assays were developed, based on the primers and probes designed on the sequences of the elongation factor 1 α (EF1 α) gene for 11 *Fusarium* spp.: *F. graminearum*, *F. culmorum*, *F. poae*, *F. langsethiae*, *F. sporotrichoides*, *F. equiseti*, *F. trinctum*, *F. avenaceum*, *F. verticillioides*, *F. subglutinans*, and *F. proliferatum*. The biomass assessment for different *Fusarium* spp. was performed in 24 wheat and 24 maize samples. The assays were found to be specific and sensitive. The results of quantitative real-time PCR assays were correlated with the mycotoxin data of the field samples (Nicolaisen et al. 2009). Sorghum ergot disease is caused by *Claviceps africana* in several countries, including Zimbabwe and the USA, and *C. sorghicola* and *C. sorghi* are involved in this disease in Japan and India, respectively. These three *Claviceps* spp. show close morphological similarities, so it was necessary to develop a technique for rapid identification and differentiation of these pathogens. PCR primers and probes were designed from the sequences of the intron 3 region of β -tubulin gene (for *C. africana* and *C. sorghi*) and sequences of the intron 4 region of EF-1 α

(for *C. sorghicola*). They were tested for their efficiency and specificity by real-time PCR with purified DNA from respective *Claviceps* spp., and the results indicated the sensitivity and specificity of the real-time PCR protocol developed in this investigation. The assay could be employed for detecting sorghum ergot pathogens in seed and grain shipments and for differentiating them to specifically determine the identity of *Claviceps* sp. present in the samples tested, thereby providing required information for regulatory decision-making process (Tooley et al. 2010).

Fusarium circinatum, causal agent of pitch canker disease affecting many *Pinus* spp., is a serious threat to pine forests due to reduction in growth and timber quality and extensive tree mortality. *F. circinatum* has been listed as a quarantine pathogen in many countries. Development of a specific and sensitive tool for detection of *F. circinatum* in conifer seed was therefore found to be necessary. A protocol based on a biological enrichment step followed by a real-time PCR assay was developed. Several enrichment protocols were compared and a 72 h incubation of the seed with potato dextrose broth was the most efficient technique to increase *F. circinatum* biomass before DNA extraction. The relative accuracy, specificity, and sensitivity of the real-time PCR assay was evaluated in comparison with conventional PCR format on 420 seed DNA extracts. The real-time PCR was found to be highly specific and significantly more sensitive than the conventional PCR assay. The real-time PCR assay enabled the detection of *F. circinatum* in samples artificially contaminated with less than 1/1000 infected seeds, as well as in naturally infected samples. In order to routinely check the quality of the seed DNA extracts, a primer probe combination that targeted a highly conserved region within the 18S rDNA in plants or fungi was successfully developed. The real-time PCR protocol could rapidly and reliably detect *F. circinatum* in pine seed and prevent long-distance spread of the pathogen via contaminated seed lots (Ioos et al. 2009).

Banana Fusarium (Panama) wilt pathogen *Fusarium oxysporum* f.sp. *cubense* race 1 (FOC1) and race 4 (FOC4) infect banana crops in tropical and subtropical zones worldwide. A real-time fluorescent PCR assay was developed based on specific conserved primers from the markers of sequence-characterized amplified region (SCAR) and TaqMan probes for detecting FOC1 and FOC4. Both races could be detected specifically and efficiently within 90 min after extraction of DNA from samples. The protocol could be employed to differentiate FOC races from other *Fusarium* spp. and *Penicillium* spp. The real-time fluorescent PCR procedure was highly sensitive, yielding a total copy number of 91,528 which was a factor of 100 higher than that of end-point PCR format. Race 1 was found to be more predominant (approximately 36 times) than race 4 in banana cultivar Gungfen #1 pseudostem at 14 days after infection. The single-tube duplex real-time PCR format might be employed by quarantine programs because of the high specificity, sensitivity, and rapidity in detecting *F. oxysporum* f.sp. *cubense* race 1 and 4 (Yang et al. 2015). The development of diagnostic techniques capable of reliably distinguishing races of *F. oxysporum* f.sp. *cubense* (FOC) is of prime importance for effective management of the Fusarium wilt disease of banana. The putative effector genes known as *SIX* genes have been efficiently used to differentiate the three races of *F. oxysporum* f.sp. *lycopersici*, incitant of tomato wilt disease. In line with this approach, an international collection of FOC isolates was screened for the presence of the putative effector *SIX8*. Using the PCR assay and sequencing approach, variation in *FOC-SIX8* was identified, facilitating the differentiation of race 4 from race 1 isolates and tropical and subtropical race 4 isolates from one another precisely (Fraser-Smith et al. 2014).

The proposal of “seed treatment according to need” was aimed at improvement of both seed health and seed-production efficiency. Development of a high-throughput, quantifiable test was considered to be necessary. A quantification real-time PCR assay using TaqMan chemistry was developed to quantify the level of contamination with *Tilletia* spp. in wheat seed lots. In the UK, wheat seed was predominantly contaminated with *T. caries* (= *T. tritici*). DNA standards prepared from spores were calibrated, using a set of 26 seed samples with *T. caries* contamination levels ranging from 0 to 1000 spores/seed. The linear calibration model obtained by the regression $\log_{10}$ (number of spores/seed + 1) on mean $\log_{10}$ DNA (μg) produced a coefficient of determination (R^2) of 0.904. The calibration model was tested using 226 seed samples; of these, 21 samples were outside the limits, 16 were over-predictions, and 5 were under-predictions. The five under-predictions were all from seed samples where contamination was less than one spore/seed. The model predicted that samples with 44 pg of DNA would be below one spore/seed with 95% probability. Of the 226 test samples compared with this threshold, 99 contained less than 44 pg DNA and these had less than one spore/seed by microscopic assessment. The real-time assay allowed an increase in test throughput and provided the sensitivity required for an advisory threshold of one spore/seed (McNeil et al. 2004). A molecular assay with enhanced sensitivity and specificity was developed for rapid detection and differentiation of *Tilletia indica*, the quarantineable Karnal bunt pathogen which had significant impact on international wheat trade. The technique to multiplex PCR by probe color in real-time PCR analysis was employed to design a single-tube, five-plex fluorescent assay to detect and identify different pathogen species simultaneously. Different probes were labeled with dyes that had nonoverlapping specific emission spectra for simultaneous detection. The procedure involved the release of DNA from bunt spores, PCR amplification to enrich *Tilletia*-specific templates from released DNA, and five-plex real-time PCR to detect, identify, and differentiate *T. indica*, *T. walkeri*, *T. ehrahartae*, *T. horrida* and a group consisting of *T. caries*, *T. laevis*, *T. controversa*, *T. bromi*, and *T. fusca* in wheat grains. This fluorescent technique could detect even one spore and thus bypass the germination step which is mandatory for testing seeds/grains for the presence of fungal pathogens. This assay utilizes five dual-labeled, species-specific primer pairs in a PCR mix in a single tube. The amplicons are detected simultaneously by five different fluorescence spectra. This protocol is effective and economically sustainable as a result of the high specificity and sensitivity of the assay, reducing labor and reagents requirements. In addition, this technique could be also used for the identification of other contaminant *Tilletia* spp. present in wheat grains (Tan et al. 2009).

Pyrenophora graminea and *P. teres* present in the barley seeds were detected by using DNA primer sequences in PCR assay. The PCR assay was performed in a LightCycler with a real-time measurement of the amplicons using a fluorescent dye. *P. teres* infection was quantified, while *P. graminea* infection was quantitatively determined as present or absent. Test results could be obtained within 48 h. *P. graminea* infection in the UK was detected infrequently by PCR assay, while incidence of *P. teres* was more variable. This rapid seed-health-testing method offered the advantage of applying seed treatment with chemicals, if required (Thomas et al. 2000). In a later investigation, a real-time PCR assay was developed to detect and quantify seedborne infection of *Pyrenophora graminea* and closely related *P. teres*. The seed infection threshold for *P. graminea* is lower than that of *P. teres*. The conventional freezing blotter procedure could not distinguish *P. graminea* and *P. teres*. PCR primers and a TaqMan probe were

designed to target a *P. graminea*-specific DNA sequence. DNA contents of *P. graminea* determined in barley seeds were positively correlated with seed infection incidence, as assessed by blotter method and also with the incidence of disease on plants in the greenhouse, correlation coefficient being $R^2 = 0.59$ ($P < 0.001$). Combining real-time PCR with a fast DNA extraction procedure provided the opportunity for rapid and automated detection and quantification of *P. graminea* DNA in seed samples. The time required for detection of *P. graminea* in barley seed was reduced from 30 days needed for conventional method (greenhouse) to 1 day by adopting the protocol developed in this investigation. The major advantages of the real-time technique are the precision, specificity, and speed of providing reliable results to prevent rejection of seed lots (Justesen et al. 2008).

Alternaria brassicae causes an important seedborne black spot disease of cruciferous crops with which *A. brassicola* and *A. japonica* (= *A. raphani*) are also associated. These pathogens are transmitted by seeds of many plant species belonging to the genera *Brassica* and *Raphanus*. The disease could be destructive for seed producers, as infection often leads to premature pod-shattering and shriveling of seeds. Detection and identification of *A. brassicae* in seed is a critical step in the process of production of disease-free seeds which constitutes an important component of disease management system. A PCR-based detection technique was formulated, using specific primers designed from the sequences of ITS regions of nuclear rDNA of *A. brassicae*, *A. brassicola*, and *A. japonica*. These pathogens could be detected in the seed macerates (Iacomi-Vasilescu et al. 2002).

Fungal pathogen genes involved in toxin production have been successfully employed to develop molecular diagnostic tools. For the detection of *Alternaria brassicicola* present in seeds of crucifers, a PCR-based diagnostic assay was developed. The assay involved a combination of DNA extraction from seed using Nucleospin Food Kit and real-time PCR in the presence of SYBR Green. Primer pairs were designed based on the nucleotide sequence of a potential pathogenic cluster of two *A. brassicae* genes encoding a nonribosomal peptide synthase (NRPS) gene and an ATP-binding cassette (ABC) transporter. ABC sens/rev primers were employed first in a PCR reaction with genomic DNA extracted from mycelial cultures of different fungal isolates to test the specificity of the primers. No increasing fluorescent signal exceeding the background fluorescence (baseline threshold) was observed, except when *A. brassicae* DNA was used as template. No cross-hybridization with other *A. brassicae*-related sequences occurred. A standard curve was plotted using known amounts of *A. brassicae* DNA against the Ct values. The mean Ct values obtained with 100% and 10% artificially contaminated radish seed batches were 25.56 and 32.55 respectively, corresponding to approximately 250 pg and 3.5 pg of *A. brassicae* DNA, respectively. Using the real-time PCR format developed in this investigation, *A. brassicae* could be detected in naturally contaminated radish seeds within 50 h. *A. brassicola* was specifically detected in the DNA extracted from seed. Real-time PCR format provided more sensitive and specific detection and differentiation of *A. brassicola* and *A. japonica* in radish, *Alternaria alternata* in radish and cabbage, *Stemphylium botryosum*, *Penicillium* sp., and *Aspergillus* in cabbage, and *Verticillium* sp. in tomato seeds. Real-time PCR assay could be used for quantification of natural infection levels in cabbage and radish seeds (Guillemete et al. 2004; Section 2.3.7).

Cruciferous crops are infected by *Alternaria brassicae* and *A. brassicola*, causing black leaf spot diseases. *Alternaria* spp. were isolated from infected cabbage, cauliflower, broccoli, and mustard plants on which the symptoms were indistinct and

overlapping, making it difficult for the identification of the pathogens in mixed infections. A simple polymerase chain reaction (PCR) assay was developed using specific primers from conserved ITS rDNA regions to detect *A. brassicicola* in infected plant and seed samples from cruciferous crops. About 85–100% of seed infection was observed in samples collected from infected fields under natural and artificially inoculated conditions. DNA extracted from healthy and infected seeds of all cruciferous plants were employed as template in the PCR assay with Acola-sens/reverse primer set. A fragment of c. 600 bp was amplified from extracts of seeds of all crops infected by *A. brassicicola* only. No amplification occurred from extracts of seeds infected by *A. brassicae*, indicating the specificity of the primer set employed. The detection limit of the PCR assay was 100 pg of *A. brassicicola* DNA. The PCR format was found to be simple, sensitive, and capable of providing the results rapidly. The primers did not amplify any fragment from the DNA extracts of *A. alternata* (infecting crucifers) and *A. porri* (infecting onions) (Sharma et al. 2013a).

Phytophthora capsici, causing Phytophthora blight disease of pepper (chilli), was detected by using specific primers based on ITS region of rDNA in PCR assay in artificially inoculated and naturally infected plants. In order to estimate pathogen DNA quantitatively, a real-time PCR assay was developed. The pathogen DNA was quantified by employing specific primers and SYBR Green dye (ds-DNA-binding dye). The DNA content is reflected by the dye which fluoresces. The minimal amount of pathogen DNA quantified by this assay was 10 pg which could be detected as early as 8 h post inoculation in susceptible pepper cultivar. Pathogen biomass accumulated in the tissues to the maximum extent (Silvar et al. 2005). Oospores of *Phytophthora capsici* present in the soil might form an important source of inoculum for infecting pepper plants. Combination of a sieving–centrifugation and real-time quantitative PCR (QPCR) assay was applied for detection of *P. capsici*. Five samples representing three different soil textures infested with oospores of *P. capsici* were suspended in water and then passed through 100, 63, and 38 µm metal sieves and finally through a 20 µm filter. After resuspending the material retained in the filter in water and centrifugation, the pellet was subjected to sucrose extraction process. The oospores concentration was assessed, using a hemocytometer. The QPCR procedure using primers based on the sequence of rDNA was employed for quantification of the pathogen DNA in the oospores in soil samples. A positive relationship was observed between the quantities of DNA of *P. capsici* oospores recovered from the soil samples and the number of oospores incorporated into the soil (Pavon et al. 2008).

Single-zoospore cultures of *Phytophthora capsici* from pepper, cucurbits, eggplant, and other vegetable crops were obtained by cultivating them on 5% V8 juice agar containing 1.0 g CaCO₃, 16 g agar and 950 mL distilled water. The isolates from root, stem, and fruit of bell pepper, watermelon, squash, cantaloupe, cucumber, and zucchini from commercial production fields in Georgia, USA were identified tentatively based on morphologic characteristics. The identity of the isolates was confirmed by performing PCR assay. *P. capsici*-specific primers PC-1 and PC-2 amplified a fragment of 560 bp from the genomic DNA of the pathogen. All the isolates induced disease symptoms in squash plants, when inoculated with zoospore suspensions under greenhouse conditions (Jackson et al. 2012). In a later investigation, a species-specific PCR format was developed for rapid and accurate detection and identification of *Phytophthora capsici* in infected plant tissues, soil, and water. The PCR primers were designed based on Ras-related protein (*Ypt1*) gene. To verify the specificity of the primers, 236 isolates

representing 12 species of *Phytophthora* and 26 fungal plant pathogens were utilized. A PCR product of 364 bp was amplified by the species-specific primer from all isolates of *P. capsici* only, but not from any other isolates of *Phytophthora* spp. or other fungal pathogens tested. Sensitivity of detection by the PCR assay using the primer pair Pc1F/Pc1R was 10 pg of genomic DNA of *P. capsici*. Application of a nested PCR format enhanced the sensitivity of detection by 1000 fold, as the assay was capable of detecting 10 fg of pathogen DNA. Nested PCR format used Pc1F/Pc1R primer pair as the first-round primers along with Pc1F/Pc2R as second-round primers. The PCR format showed potential for detection of *P. capsici* in infected plant tissues, as well as in infested soil and water. This format could be used for disease diagnosis and pathogen monitoring (Lan et al. 2013).

A rapid and sensitive PCR-based assay was developed for detecting *Phytophthora sojae* from soil carried with transported soybeans or in currently cultivated soil. Real-time PCR combined with fluorescent SYBR Green I dye was applied for detection and quantification of *P. sojae* in the soil. The universal primers of DC6 and ITS4 amplified the sequences of ITS regions of eight *P. sojae* isolates. After alignment of the amplified sequences, a region specific to *P. sojae* was used to design the specific primers PS1 and PS2. A product of 330 bp of PCR amplification was detected exclusively from the isolates of *P. sojae*, but not in any of the isolates of 25 species of *Phytophthora* spp. tested. Combination of PCR assay and a simple soil screening method resulted in the early detection of *P. sojae* in soil samples within 6 h with a detection limit of two oospores in 20 g soil samples. A real-time fluorescent quantitative PCR using primers PS1 and PS2 was used to determine populations of *P. sojae* precisely in two field soil samples and one soil sample collected from soybeans imported from the USA (Wang et al. 2006). *Phytophthora cinnamomi* is capable of infecting a wide range of plant species, including avocado and eucalypt. Primers LPC2 and RPC3 were synthesized as per the instructions of the manufacturer (Pacific Oligos). The primers were species-specific in detecting *P. cinnamomi*. An internal standard DNA fragment amplified by the same primers, but producing an amplicon of different sizes, was added to reaction mixture to detect false-negative reactions caused by inhibition of amplification. The use of PCR for detecting *P. cinnamomi* effectively eliminated the need to use the traditional baiting method. Further, the possibility of processing a large number of samples in a short time by applying the PCR protocol was found to be a distinct advantage over baiting method (O'Brien 2008).

Visual examination of infection of rice by *Rhizoctonia solani*, causing sheath blight disease, was not found to be reliable for determination of disease severity. A real-time, quantitative (Q) PCR assay was developed to detect and quantify *R. solani* AG-1-1A DNA in infected rice plant tissues. A specific primer pair based on ITS region of rDNA of the pathogen was designed. The assay procedure could detect and quantify pathogen DNA content as low as 1 pg, enabling specific and reliable detection of *R. solani* and quantification of fungal DNA. Further, the assay could be employed for the evaluation of levels of resistance of rice cultivars to sheath blight disease, based on the pathogen DNA contents of rice cultivars tested (Sayler and Yang 2007). *Rhizoctonia* root rot caused by *R. solani* and *R. oryzae* is a serious limiting factor in cereal production in Pacific Northwest in the USA. A SYBR green I-based real-time quantitative (q) PCR assay was developed for detection, identification, and quantification of the pathogens. Primers specific to ITS1 and ITS2 of nuclear DNA of *R. solani* and *R. oryzae* were designed. The assays specifically detected *R. solani* AG2-1, AG-8, and AG-10, three genotypes of *R. oryzae*, and an AG-1-like binucleate *Rhizoctonia* spp. The results could

be reproduced quantitatively at or below a cycle threshold (Ct) of 33 or 2–10 fg of mycelial DNA from cultured pathogens, 200–500 fg of pathogen DNA from root extracts, and 20–50 fg of pathogen DNA from soil extracts. Detection of the pathogen was possible at concentrations well below that required for quantification of pathogen populations (Okubara et al. 2008). *Colletotrichum lagenarium* infects cucurbits such as watermelon (*Citrullus lunatus*), cucumber (*Cucumis sativus*), squash (*Cucurbita moschata*), and muskmelon (*Cucumis melo*). The pathogen is transmitted through seed of watermelon and cucumber. Rapid and precise methods of detection of pathogen, using conventional PCR and real-time PCR formats were developed. PCR assays amplified different ITS sequences from *C. lagenarium* effectively in infected tissues. PCR analysis with the primer co-m-337F1/R was able to differentiate *C. lagenarium* from other fungal pathogens, including *Colletotrichum* spp., *Fusarium* spp., *Alternaria* spp., and *Didymella* spp. An optimized real-time PCR assay was developed to detect and monitor *C. lagenarium* in plant materials. The technique had a detection limit of 1 pg of pathogen DNA and demonstrated the potential for rapid detection and diagnosis of the disease caused by *C. lagenarium* (Kuan et al. 2011).

Magnaporthe grisea, causal agent of rice blast disease, was detected using SYBR Green I real-time quantitative PCR procedure in pathogen culture, rice plants, and rice seeds. The absolute qPCR format showed that *M. grisea* could be detected at as low as 6.9×10^{-5} ng of genomic DNA. Seeds of all 10 rice cultivars contained *M. grisea* DNA, indicating that the blast pathogen was generally widespread in rice seed. The qPCR protocol was successfully applied to quantify DNA of *M. grisea* in rice leaves collected from the field-grown plants using rice actin gene as a housekeeping gene. The results indicated the practical significance of assessing potential fungal infection in rice seedlings before planting (Sun et al. 2015). Bakanae disease caused by *Gibberella fujikuroi* (*Fusarium fujikuroi*) infects rice plants at seedling and tillering stages. Knowledge of pathogen population structure and aggressiveness (virulence) of isolates will be useful to formulate effective disease management strategies. Twenty isolates of *F. fujikuroi* from different locations in the Philippines were characterized by universally primed (UP-) PCR assay using AA2M2 and L45 primers. Sixty-six unique banding patterns were generated and a dendrogram was prepared. A high degree of variation was observed among the isolates. At 90% similarity, 15 clusters were formed, suggesting that sexual reproduction played a significant role in generating variation in isolates of *F. fujikuroi* populations in the field (Cumagun et al. 2011; Section 2.3.8). *Colletotrichum* spp., causing anthracnose diseases in a wide range of crop plant species, accounts for significant losses in agricultural and horticultural crops. *C. gloeosporioides* is carried in soybean seeds. As this pathogen could infect plants in the field and also harvested produce, rapid and reliable detection and identification of the pathogen was considered to be necessary. Morphological examination of pathogen, arbitrarily-primed PCR (ap-PCR), and DNA sequencing studies were applied for the precise identification of *C. gloeosporioides* infecting lychee plants and fruits. The ap-PCR assay identified 21 genotypes of *C. gloeosporioides*, three of which were predominant. A specific genotype identified using ap-PCR assay was associated with the production of the teleomorph in culture. Analysis of sequence data of ITS and β -tubulin regions of representative isolates did not group the lychee isolates into a monophyletic clade. As the majority of the isolates were from one of the three genotypes as determined by using ap-PCR assay, it might be possible that the isolates from lychee could form a specific group within *C. gloeosporioides* (Anderson et al. 2013).

Onion neck rot disease is caused by three fungal pathogens – *Botrytis aclada*, *B. alli*, and *B. byssoidea* – carried on seeds. The ribosomal intergenic spacer (IGS) regions of the three *Botrytis* spp. and nontarget *Botrytis* sp. were sequenced and aligned for designing primer pairs specific to *B. aclada*, *B. alli*, and *B. byssoidea*. These primers were employed in a real-time fluorescent PCR assay using SYBR chemistry for detection and quantification of these seedborne pathogens. The primers reliably detected 10 fg/reaction of the pathogen genomic DNA extracted from pathogen cultures. The pathogens present in the seeds of 23 commercial seed lots were quantified by applying real-time PCR format, which was shown to be more sensitive than the conventional plating method. A total of 5 of 23 seed lots tested negative to the agar plating method. In contrast, the presence of the pathogens in these 5 seed lots was detected by the PCR-based protocol developed in this investigation. However, there was no relationship between the extent of seed infection by *Botrytis* spp. detected by real-time PCR assay and incidence of neck rot disease (Chilvers et al. 2007).

A DNA-based method for quantification of airborne inoculum of *Botrytis squamosa*, causing leaf blight disease in onion, was developed. The DNA from the conidia trapped in the rotating-arm samples was purified and analyzed using quantitative TaqMan real-time PCR (qPCR) technique. The sensitivity of the qPCR procedure was high with a detection limit of two conidia per rod. A positive correlation was observed between the numbers of conidia counted, using a light microscope and amount of DNA estimated by qPCR assay. Field samples (142) from commercial onion fields were analyzed to assess the possibility of predicting the disease risk being low or above damage threshold (Dth) level. At the damage thresholds of five or ten lesions/leaf, conidia quantification with qPCR assay was more reliable at predicting disease risk than by counting conidia using the microscope. The results indicated that the qPCR protocol could be used for reliably quantifying the airborne pathogen population in commercial onion field and for assessment of disease risk (Carrise et al. 2009).

Verticillium dahliae, a soilborne fungal pathogen capable of infecting a wide range of crops worldwide, is seedborne in spinach seeds. Spinach seeds produced in the USA and Europe are commonly infected by *V. dahliae*. If infected seeds are planted soil inoculum density is increased, contributing to the *Verticillium* wilt disease epidemics on lettuce and other crops grown in rotation with spinach. A rapid, sensitive, and reliable method of detecting *V. dahliae* in spinach seed is therefore essential. The threat posed by seedborne *V. dahliae* was established by planting seed lots with infection levels above designated threshold. As the conventional plating method and blotter test do not discriminate between *V. dahliae* and other closely related *V. tricorpus*, in addition to being time-consuming, a sensitive, rapid, and reliable method of detection and quantification of *V. dahliae* in spinach seed was developed. A quantitative real-time PCR assay was optimized and applied for detection and quantification of *V. dahliae* in germplasm and 15 commercial spinach seed lots. *V. dahliae*-specific primer pair VertBt-F/VertBt-R was employed. The assay was able to quantify *V. dahliae* in spinach seed reliably with a sensitivity limit of c. 1 seed per 100 (1.3% infection in a seed lot). The relationship between pathogen DNA copy number and percentage seed infection was linear and highly significant (Table 2.4; $R^2 = 0.99$; $P = 0.0001$). By employing the VertBt primer pair, 115 bp β -tubulin amplicon was detectable by PCR from both *V. dahliae* and *V. longisporum*. Extracts from the 15 commercial seed lots were screened with a *V. longisporum*-specific primer pair. The amplicon of the expected size 340 bp unique to *V. longisporum* was not amplified from any of the spinach seed lots tested,

Table 2.4 Quantification of *V. dahliae* using quantitative polymerase chain reaction (qPCR, mean $\pm$ standard deviation) in commercial spinach seed lots (Duressa et al. 2012).

Seed lot	Pathogen copy number	Cq value
1	544.68 $\pm$ 94.97	26.20 $\pm$ 0.26
2	2149.19 $\pm$ 728.40	25.38 $\pm$ 0.20
3	292.55 $\pm$ 135.17	27.01 $\pm$ 0.05
4	638.01 $\pm$ 186.33	26.32 $\pm$ 0.53
5	301.17 $\pm$ 120.47	27.78 $\pm$ 0.68
6	666.32 $\pm$ 185.07	25.94 $\pm$ 0.48
7	514.10 $\pm$ 88.69	26.45 $\pm$ 0.05
8	146.64 $\pm$ 10.10	28.77 $\pm$ 0.11
9	96.87 $\pm$ 6.99	29.02 $\pm$ 0.15
10	844.28 $\pm$ 165.10	26.09 $\pm$ 0.31
11	255.98 $\pm$ 47.16	27.20 $\pm$ 0.28
12	56.94 $\pm$ 8.02	29.60 $\pm$ 0.23
13	29.72 $\pm$ 4.87	31.11 $\pm$ 0.50
14	1.46 $\pm$ 0.45	35.81 $\pm$ 0.81
15	4.72 $\pm$ 1.32	34.44 $\pm$ 0.54

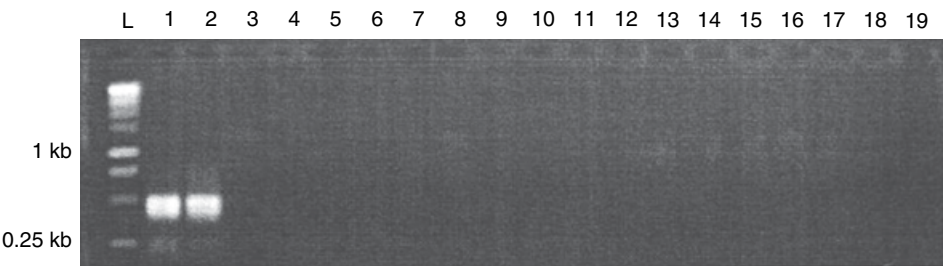


Figure 2.5 Detection of *Verticillium longisporum* in spinach seed predominantly infected by *V. dahliae*. Note the presence of 340bp amplicon unique to *V. longisporum* detected by PCR assay. Lane 1: DNA template extracted from a pure culture of *V. longisporum* strain Bob 70; lane 2: DNA template of *V. longisporum* strain Bob 70 in seed DNA background; lane 3: DNA templates extracted from *V. dahliae* strain VdLs 16; lanes 4–18: templates expressed in seed lots; lane 19: negative control (water); lane L: DNA standards. Source: Duressa et al. (2012). Reproduced with permission of the American Phytopathological Society, MN, USA.

indicating the absence of *V. longisporum* in the commercial seed lots (Fig. 2.5). The assay could be effectively used to quantitatively assess the seed infection levels and the results may be helpful to apply required disease management tactics to reduce disease incidence (Duressa et al. 2012; Section 2.3.9).

Anisogramma anomola, incitant of eastern filbert blight (EFB) disease of European hazelnut (*Corylus avellana*) causes significant economic losses in Oregon, USA. As the symptoms of infection could be visually recognized only after 12–16 months, detection,

and identification of the pathogen by conventional isolation-dependent methods was not useful. A TaqMan real-time PCR assay was developed, based on rDNA internal transcribed spacer (ITS) for detection and identification of *A. anomola*. The assay was validated with multiple isolates of *A. anomola*, closely related species, common environmental microorganisms, and over 100 samples of *C. avellana*. Using the real-time PCR assay, it was possible to detect as low as 0.12 pg of the genomic DNA of *A. anomola*. The assay positively diagnosed EFB on 82% of asymptomatic plants as early as 15 weeks after infection. The real-time PCR assay proved to be more sensitive and rapid than the conventional diagnostic methods. The real-time PCR has potential for facilitating hazelnut breeding and disease management by early and precise diagnosis of eastern filbert blight of hazelnut (Molnar et al. 2013).

Sclerotinia sclerotiorum, causative agent of stem rot of oilseed rape (SROR) with a wide host range of about 400 plant species, could be detected by employing a real-time PCR technique. A pair of primers (SsF/SsR) was designed based on the sequences of a DNA region amplified by a microsatellite primer M13. The primer generated a 252 bp DNA fragment from each of the 65 isolates of *S. sclerotiorum* from oilseed rape collected at different locations and in different years, but not from any other fungal species tested, indicating the specificity of the primer pair. For a rapid detection of early infection on petals of oilseed rape a real-time PCR format was developed, employing this pair of primers used in the standard PCR format. Sensitivity tests revealed that the target fragment from 5 pg of pathogen DNA spiked with 2 ng of oilseed rape DNA could be detected. The real-time PCR could detect *S. sclerotiorum* in petals containing 0.0252–0.1111 ng mg⁻¹ of petals. In order to eliminate PCR inhibitors, 2% polyvinylpyrrolidone (PVPP) was added to the extraction buffer and the extracts were further purified using a commercial UNIQ gel extraction kit. The cost of purification was only US \$0.18. This procedure provided a high-quality template DNA for PCR amplification of the target DNA fragment (Yin et al. 2009).

With a wide host range including many crop plants, *Sclerotinia sclerotiorum* initiates infection through airborne conidia. A specific diagnostic technique was developed to reliably detect and precisely quantify the airborne inoculum levels. The airborne ascospores were trapped using Buckard (Hirst-type) spore traps and rotating-arm traps. The DNA was extracted from the spores trapped in wax-coated plastic traps. A SYBR Green quantitative PCR (qPCR) technique was applied to determine the pathogen population. A linear relationship between the number of ascospores and DNA of *S. sclerotiorum* (mean 0.35 pg of DNA/spore) was observed. It was possible to detect as few as two ascospores present in the spore trap. The specificity of the assay was revealed by the absence of amplification of host plant (*Brassica napus*) DNA and other fungal pathogens, including *Sclerotinia minor*. No relationship between rainfall and airborne ascospores of *S. Sclerotiorum* was observed under Rothamsted conditions. The qPCR procedure developed in this investigation might be useful for detection and quantification of population of *S. sclerotiorum* in seeds and other plant tissues such as stem or petals, as well as in soil samples (Fig. 2.6; Rogers et al. 2009). The population structure of *Sclerotinia sclerotiorum* from different crop plants was investigated using eight microsatellite markers and sequenced sections of the intergenic spacer (IGS) region of the rRNA gene and the elongation factor (EF) 1- α gene. A total of 228 microsatellite haplotypes were identified within 384 isolates from 12 *S. sclerotiorum* population samples from England and Wales. One microsatellite haplotype was generally found at high frequency in each population and was widely distributed across different hosts,

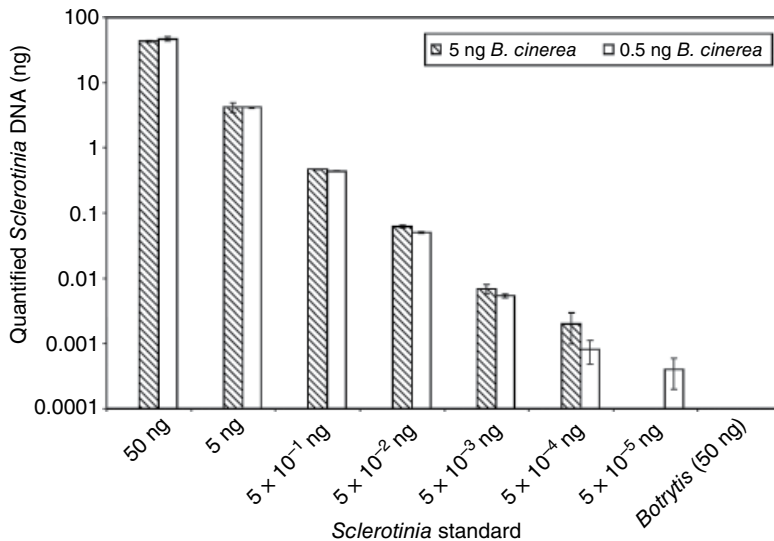


Figure 2.6 Assessment of DNA contents of *Sclerotinia sclerotiorum* present along with *Botrytis cinerea* in mixed populations using quantitative PCR assay. Bars indicate the standard errors of means. Source: Rogers et al. (2009). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

location, and years. Fourteen IGS and five EF haplotypes were detected in twelve populations studied, with six IGS haplotypes and one EF haplotype exclusive to buttercup (*Ranunculus acris*). Analysis of published sequences of *S. sclerotiorum* populations from the USA, Canada, New Zealand, and Norway showed that three of the IGS haplotypes and one EF haplotype were widely distributed, while eight IGS haplotypes were present only in the UK. Overall, there was no consistent differentiation of *S. sclerotiorum* populations from different crop host plant species. *S. sclerotiorum* has a multiclonal population structure in the UK, as indicated by this investigation (Clarkson et al. 2013).

Isolates of *Sclerotinia sclerotiorum* infecting canola from China and the USA were investigated to assess their genotypic and phenotypic diversity and population differentiation. Genetic diversity was determined using eight microsatellite markers and mycelial compatibility groups (MCGs). Phenotypic diversity was assessed based on their sensitivity to three fungicides, production of oxalate and sclerotia, growth rate, and virulence on two canola cultivars. No shared MCGs or multilocus haplotypes were detected between the two populations which differed significantly ($P < 0.001$). Recombination occurred in both populations, while the Chinese population exhibited a greater frequency of recombination. A PCR-based assay was useful for detection of the isolates of both populations. About 60% of the isolates were inversion-plus at mating type locus. Both *S. sclerotiorum* populations differed significantly for all phenotypic traits (< 0.05), except for the sensitivity to fungicide fluazinam and virulence. The phenotypic characteristics were inherited in the same way in both populations. Significant correlations were obtained in five phenotypic traits. Cross-resistance to benomyl and iprodione was detected, while virulence was not significantly correlated with another phenotypic trait and had the least heritability. Regarding virulence, both populations showed similar levels of aggressiveness on canola cultivars, irrespective of their level of susceptibility/resistance to *S. sclerotiorum* (Attanayake et al. 2013).

Analysis of race structure and genotypic diversity of four populations of *Leptosphaeria maculans* isolated from *Brassica* spp. (broccoli, cauliflower, and cabbage) in Mexico was carried out. Race structure of *L. maculans* showed unusually low diversity at three out of four locations. However, by employing 14 minisatellite markers, high proportions of repeated multilocus genotypes could be recognized in these populations in addition to a significant linkage disequilibrium and strong clonal fraction (65–87%). The occurrence of the mating-type idiomorphs always significantly departed from the 1:1 proportion expected in the case of random mating. Each population therefore consisted of a few (4–9) multilocus genotypes which were specific to each location. The results suggested the presence of exclusive or a high rate of clonal multiplication. A shift in reproductive behavior of the pathogen appeared to be primarily man-mediated when cropping practices for *B. oleracea* and *B. napus* were compared (Dilmaghani et al. 2013).

Common bean (*Phaseolus vulgaris*) seeds infested by *Fusarium oxysporum* f.sp. *phaseoli* (*Fop*) could introduce the pathogen into the fields, resulting in significant economic losses. In order to prevent short- and long-distance dissemination of *Fop* through infested seeds, a rapid, precise, and sensitive real-time PCR (qPCR) assay was developed for detection of the pathogen in common bean seeds. Seed lots of seven cultivars with infection levels ranging over 0.25–20% were prepared by mixing known quantities of *Fop*-infected seeds with *Fop*-free seeds. The efficiencies of SYBR Green and TaqMan PCR methods were compared, using primers based on the *Fop* virulence factor *ftf1*. The primers amplified a 63 bp product for highly virulent strains of *Fop*, but did not amplify the 63 bp amplicon from the nonpathogenic or weakly pathogenic isolates of *F. oxysporum* from *P. vulgaris* or other hosts. Both qPCR assays detected *Fop* infection at low levels of seed infection (0.25%). However, the results suggested that TaqMan assay was more reliable for quantification of pathogen population than SYBR Green assay. TaqMan assay developed in this investigation has the potential for application as an effective tool for the detection and quantification of *F. oxysporum* f.sp. *phaseoli* in common bean seeds (de Sousa et al. 2015).

2.1.4.3 Combination of PCR-Based Assays with Other Techniques

Polymerase chain reaction (PCR) -based assays are combined with other techniques to overcome the problem of inhibition of PCR by compounds coextracted with DNA from the substrates or to improve the efficiency of PCR assays. *Didymella bryoniae*, incitant of cucurbit gummy stem blight disease, was frequently isolated from infected plants along with another pathogen *Phoma* sp. A combination of PCR with enzyme-linked immunosorbent assay (ELISA) by employing primers specific to *D. bryoniae* and *Phoma* sp. was applied in microplates. Primers were modified by addition of a fluorescein and a biotin label to the 5' ends of the forward and reverse primers, respectively. PCR amplifications were detected in an ELISA test, using horseradish peroxidase-conjugated anti-fluorescent antibody and three substrates that yielded three colored products. PCR-ELISA format successfully detected *D. bryoniae* in all samples except one (45 of 46 samples) and *Phoma* sp. isolates (13) tested, providing results comparable with those of gel electrophoresis. One isolate of *D. bryoniae* that could not be detected by the PCR-ELISA format had a large DNA fragment, as visualized in agar gels. When “blind” fungal samples were tested, all of the seven isolates of *D. bryoniae* and *Phoma* were detected and differentiated by PCR-ELISA protocol which was found to be simple, specific, rapid, and convenient. However, the sensitivity of PCR-ELISA format was less when compared with that of gel electrophoresis procedure (Somai et al. 2002).

When combined with real-time PCR assay, magnetic capture hybridization (MCH) technique enhances the sensitivity and specificity of detection of plant pathogens significantly. MCH involves the use of single-stranded biotinylated probe, conjugated to streptavidin-coated magnetic beads (SCBs) to hybridize with single-stranded target DNA from crude samples. Target nucleic acids that hybridize to capture probes may be concentrated by magnetic force, eliminating nontarget DNA and PCR inhibitors by rinsing. After elution, the enriched target DNA may be amplified and detected by real-time PCR assay. In order to overcome the adverse effects of PCR inhibitors, a MCH-PCR assay was developed for the detection of *Botrytis aclada*, causal agent of onion neck rot disease, in onion seed lots. MCH-PCR assay detected the pathogen DNA from aqueous solutions containing 100 fg DNA and reduced the time required to test onion seeds to less than 24 h compared to the 10–14 days needed for conventional plating test. MCH-PCR assay in combination with a Mini-Beadbeater DNA extraction procedure allowed detection of *B. aclada* in suspensions with 1×10^4 and 1×10^3 conidia mL^{-1} in 100% and 60% of tests, respectively. In suspensions with 10 conidia mL^{-1} , the detection frequency was reduced to 10%, indicating the need for maintaining the DNA content at optimal concentration for obtaining positive reaction. MCH-PCR format was more sensitive and efficient than conventional PCR in detecting the pathogen in seed lots with 4.8 and 9.9% (as determined by agar-plating method) natural *B. aclada* infestation (80 and 70% tests, respectively). MCH-PCR assay successfully detected *B. aclada* DNA in seed lots with natural infestation levels ranging from 0.25 to 90.0% (Walcott et al. 2004).

MCH multiplex real-time PCR procedure was developed for the simultaneous detection of a fungal pathogen, *Didymella bryoniae* (anamorph *Phoma cucurbitacearum*), causal agent of gummy blight disease and the bacterial pathogen *Acidovorax avenae* pv. *citrulli* (*Aac*), the incitant of bacterial fruit blotch (BFB) disease affecting melon and watermelon seeds. Single-stranded DNA hybridization capture probes target DNA of *D. bryoniae* and *Aac* that were covalently attached to magnetic beads and employed for selective concentration of template DNA from cucurbit seed samples. Sequestered template DNAs were subsequently amplified by multiplex real-time PCR, using pathogen-specific TaqMan PCR assays. The *D. bryoniae*-specific TaqMan PCR assay produced the predicted 86 bp fragment and Texas-Red-based fluorescence with *D. bryoniae* template DNA, but did not amplify DNA from *D. lycopersici* or other fungal genera tested. Genomic DNA from a majority (93%) of *D. bryoniae* RG1 isolates was amplified with TaqMan PCR assay. The comparative effectiveness of direct real-time PCR and MCH real-time PCR formats for the detection of *D. bryoniae* was assessed. The fungal pathogen was not detected in the negative control samples by both assay formats. A detection threshold for *D. bryoniae* of 10^5 conidia mL^{-1} was observed for both direct real-time PCR and MCH real-time PCR assays. Additionally, the Ct values associated with positive reactions were similar for both techniques, suggesting that MCH had no additive effect on real-time PCR sensitivity. However, MCH multiplex real-time PCR was consistently effective for simultaneous detection of *D. bryoniae* and *Acidovorax avenae* subsp. *citrulli* in naturally infested melon seed lots. The MCH multiplex real-time PCR assay detected *Aac* in 100% of seed lots without *D. bryoniae* infestation. In samples to which 1, 5, or 10 *D. bryoniae*-infested seeds were added, both pathogens were consistently detected with mean Ct values of 34.0, 32.8, and 31.8 respectively. In contrast, crude DNA extraction and direct multiplex real-time PCR failed to detect either pathogen in naturally infested melon seed lots. Nevertheless, *D. bryoniae* and *A. avenae* subsp. *citrulli* were consistently detected in melon seed samples ($n=5000$ seeds) with 0.02%

natural infestation by both pathogens within 48 h. The MCH real-time PCR protocol developed in this investigation might be useful for evaluating seed infestation by either of the two pathogens and may be more amenable to high-throughput testing of seed samples than other standard assays (Ha et al. 2009).

2.1.4.4 Reverse-Transcription (RT) Polymerase Chain Reaction

Reverse-transcription (RT-) PCR is an elegant technique which detects target mRNA. It is possible to quantify viable populations of pathogens, which is otherwise not possible by employing other PCR-based methods. In contrast to DNA which has quite a stable structure, mRNA species have half-lives of only a few minutes (Ingle and Kushner 1996). Detection of mRNA by RT-PCR assay as compared to DNA detection methods is considered a better indicator of cell viability (Sheridan et al. 1998). RT-PCR has been applied to detect and quantify the expression of fungal pathogen genes during pathogenesis (process of disease development) or host plant genes, leading to induction of resistance to diseases or other stresses (McMaugh and Lyon 2003; Schenk et al. 2003). The RNA is transcribed into cDNA that can be detected by employing specific primers or probes in PCR assay format. For the detection of viable populations of *Mycosphaerella graminicola* in wheat plants which were asymptomatic a RT-PCR technique was developed using a specific primer set E1/STSP2R, based on the sequences of β -tubulin genes. The primer set amplified one single fragment only from the total RNA of the pathogen and infected wheat leaves. RT-PCR assay could detect *M. graminicola* at least 4 days before symptom expression in inoculated plants. After symptoms of infection became visible, the increase in band intensity of the amplified RT-PCR products in the gel was in general agreement with visual disease severity assessment. Presymptomatic detection is valuable, because *M. graminicola* has a latent period of at least 4 weeks in northern Germany (Verreet et al. 2000). The β -tubulin genes are among the most conserved genes with high copy numbers, making them suitable targets for reliable detection. The efficacy of two formats of RT-PCR was found to be less (100 pg total RNA) than that of one-step RT-PCR format (5 ng total RNA) (Guo et al. 2005).

2.1.4.5 Random Amplified Polymorphic DNA Technique

Random amplified polymorphic DNA (RAPD) technique is a PCR format that uses arbitrary primers applicable to differentiate races, strains, pathotypes, and pathogenic or nonpathogenic isolates of fungi. Primers used in this procedure are very short pieces (ten or fewer bases) of DNA from a known source. It is highly probable that these primers may be able to find some complementary sequences in the target DNA, producing a mixture of DNA fragments of various sizes. When the products from such a reaction are analyzed by the gel electrophoresis technique, distinct banding patterns are produced; some of these patterns may prove to be specific to certain species or varieties or strains of the fungal pathogens. The patterns themselves may be useful for detection and diagnosis of some pathogenic fungi and the diseases caused by them, but some of these patterns may prove to be specific to certain species or varieties or strains. In certain cases, some of the bands may be cut out of gels and sequenced to produce specific primers for more precise PCR analyses or probes for dot hybridization and other detection procedures.

Fusarium moniliforme (= *F. verticillioides*) and *F. subglutinans*, two anamorphs (asexual stage) of the so-called "*Gibberella fujikuroi*" species complex, are destructive maize pathogens which cause heavy quantitative and qualitative losses. A PCR format was developed for early and sensitive detection and identification of *G. fujikuroi*

anamorphs in maize kernels. The primer pairs based on sequences of random amplified polymorphic DNA (RAPD) fragments were specific to *F. moniliforme* and *F. subglutinans*. The PCR assays were independent of the high phenotypic variability of traits which might complicate classification by morphological characters. The primers could detect the 100–200 fungal genomes, even in the presence of excess of maize DNA. A rapid and easy method of extracting DNA from maize kernels without introducing inhibitory substance was developed in this investigation. The procedure was successfully applied to identify field isolates from Poland and to detect the pathogens in corn ears of various hybrids in Germany. The protocol showed the potential for application in breeding programs for early detection and identification of *Fusarium* spp., infecting corn kernels (Möller et al. 1999). *Fusarium verticillioides* (= *F. moniliforme*), frequently present in corn seeds, was found to be associated with symptomatic and also asymptomatic plants, possibly as a secondary invader or an endophyte. *Fusarium* was detected at a frequency of 0–100% relative to the total number of fungi isolated from two populations of *Zea mays* (BR105 and BR106) and their respective inbred lines. The frequency of occurrence of this genus was practically the same in the field sites (around 60%). *F. verticillioides* strains (21) were analyzed using the RAPD technique using 10 random primers. Variability analysis of endophytic isolates via RAPD showed genomic polymorphism taxa of species around 60%. Endophytic isolates were clustered by their sites of origin. On the other hand, RAPD analysis clustered the endophytic isolates by their corn inbred line hosts (Mil-01 to Mil-06), whereas at site A they clustered into two major groups related to the corn gene pool (BR105 or BR106 population). All strains from seeds collected at site A (except strains L9 and L10) were subgrouped according to maize inbred lines. Further, a discrete subgrouping of isolates was discernible at site B. A coevolution process involving endophytic isolates of *F. verticillioides* might be the reason for genetic variability of strains observed (Pamphile and Azevedo 2002). In another study, RAPD analysis was used to assess genetic variation among 28 isolates of *F. moniliforme* isolated from rice, sorghum, maize, cotton, hibiscus, and parkia collected from Burkino Faso. Scorable markers (128) were obtained from 20 random decamer primers. Cluster analysis with UPGMA using genetic distances showed that the isolates were of five main groups. In general, isolates from the same host species were found to group together in the same cluster. The isolates from cotton and hibiscus were more closely related to each other than to the isolates from sorghum, rice, and maize. Host preference seemed to be a factor in the differentiation of isolates (Kini et al. 2002).

Toxigenic isolates of cereal pathogens *Fusarium culmorum*, *F. graminearum*, *F. crookwellense*, and *F. avenaceum* from various countries such as Poland, England, New Zealand, Italy, and Canada were investigated using RAPD-PCR assay, sequence-characterized polymorphic regions (SCARs), morphological characteristics, and mycotoxin production under laboratory conditions. The DNA products were compared by RAPD-PCR assay which showed species-specific bands and the greatest diversity among isolates of *F. avenaceum*. PCR assay using three 20-mer primer pairs reported earlier to be useful for identification of *F. culmorum* and *F. graminearum* group 2 confirmed their species specificity. The same species-specific PCR product was observed in isolates of both nivalenol and deoxynivalenol chemotypes of *F. culmorum* or *F. graminearum*. A clear relationship was found between morphological and species-specific PCR identification of *F. culmorum* and *F. graminearum* isolates. However, appearance of the same band (272 bp) in the gel for *F. avenaceum* and *F. tricinatum* when the primers FA-ITS F/R (SCAR2-14) were used might lead to wrong identification of the isolates of *Fusarium* spp. (Chelkowski et al. 1999).

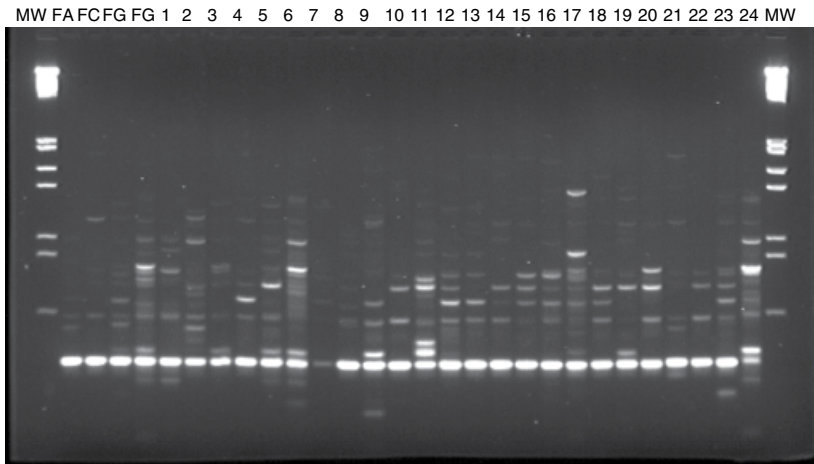


Figure 2.7 Differentiation of isolates of *Fusarium graminearum*, *F. avenaceum* and *F. culmorum* based on random amplified polymorphic DNA (RAPD) profiles formed by Operon primer OPB15. Lane 1: Molecular standards; lane 2: *F. avenaceum*; lane 3: *F. culmorum* (R-6565); Lanes 4 and 5: *F. graminearum* (R-6914 and R-6925) and lanes 6 to 29: isolates 1 to 24 of *F. graminearum* from North Carolina, USA. Source: Walker et al. (2001). Reproduced with permission of the American Phytopathological Society, MN, USA.

Understanding the genetic diversity of *Fusarium* spp. associated with wheat Fusarium head blight (FHB) disease may help improve the effectiveness of disease management strategies. Sixty-six isolates of *F. graminearum* collected in North Carolina were tested for in vitro growth rate, production of deoxynivalenol (DON) and zearalenone, and pathogenicity on three cultivars of soft red winter wheat. Significant variations for all three characteristics were recorded. Randomly amplified polymorphic DNA (RAPD) analysis revealed high levels of genotypic diversity among isolates. In vivo levels of DON were determined for the five isolates inducing highest disease severity scores and the five isolates causing lowest disease severity scores, and no significant differences were observed among these isolates. However, all ten isolates did produce detectable concentrations of DON in vivo. RAPD analysis revealed a large amount of genotypic variability among isolates. Ninety-six polymorphisms were repeatable in two tests of each primer (Fig. 2.7). A multiple regression model using the abovementioned pathogen isolate characteristics and cultural practices showed that genotypic and phenotypic diversity were significant under conventional and reduced tillage conditions and diversity was high, regardless of whether the previous crop was a host or nonhost for *F. graminearum*. The results indicated that variable pathogen populations of *F. graminearum* existed in the test location (North Carolina) and the pathogen population could be highly pathogenic on wheat, capable of producing high concentrations of mycotoxins (Walker et al. 2001; Section 2.3.10).

The mycotoxin-producing potential and molecular variability of *Fusarium culmorum* isolates (37) collected from the Pan-Northern Hemisphere, together with isolates representing other *Fusarium* spp., were investigated. Mycotoxin-producing potential of the isolates was tested by thin-layer chromatography (TLC) and by PCR assay, using primer pairs specific to the *Tri7* and *Tri13* genes. Thirty isolates belonged to chemotype I (capable of producing DON and 3-acetyl-deoxynivalenol), while seven isolates

constituted chemotype II (capable of producing nivalenol and/or fusarenone X). The presence of a functional *Tri7* gene correlated with nivalenol production. Isolates of chemotype I were generally more virulent than those included in chemotype II. Phylogenetic analysis of RAPD profiles showed clustering of isolates into different groups. The similarity of RAPD profiles of isolates from one geographical location (Hungary) was recognized. A similar clustering was observed on the tree based on restriction analysis of the intergenic spacer region data. The isolates contained either *MAT-1* or *MAT-2* gene (mating-type gene) homologues. No correlation could be established between clustering of isolates based on RAPD, restriction analysis of intergenic spacer region or sequence data, and distribution of MAT idiomorphs. Likewise, mycotoxin-producing potential, virulence, or molecular characteristics of the isolates showed no relationship between them. The results seemed to suggest that *Fusarium culmorum* had a recombining population structure (Tóth et al. 2004). *Fusarium verticillioides* is the principal source of fumonisins contaminating cereal grains which are produced during colonization and infection of host plants, even when disease symptoms are not visible. It is essential to have efficient methods of detection and identification of *F. verticillioides* to prevent entry of fumonisins in the food chains. DNA-based strategies based either on DNA markers unrelated to fumonisin production or information about the genes involved in fumonisin production have been applied for early detection and precise identification of *F. verticillioides* (González-Jaén et al. 2004).

Claviceps purpurea infects rye and other grasses, producing ergot sclerotia which contain alkaloids with medicinal value. Three groups within this species (G1, G2, and G3) were formed based on habitat association, sclerotia, conidial morphology, and alkaloid production. *C. purpurea* isolates (21) collected from a wide range of host plant species and various geographical locations were analyzed by RAPD and amplified fragment length polymorphism (AFLP) techniques. High genetic variability was noted between these groups within less than 2% of polymorphic markers that shared across all isolates. The results supported the recognition of three discrete groups within *C. purpurea*. In order to determine whether *C. purpurea* complex was made up of one or multiple species, phylogenetic and population genetic analyses were performed. The isolates of G1 type were found to be more integrated with one another. The results suggested that little or no gene flow occurred between the different ecotypes and that this process might be driven by adaptations to ecological habitats (Douhan et al. 2008).

Comparative potential of repetitive-sequence-based polymerase chain reaction (rep-PCR) fingerprinting of fungal genomic DNA and RAPD analysis was investigated for assessing phylogenetic relationship and use as a diagnostic tool for *Tilletia* spp. DNA primers (BOX, ERIC, and REP) corresponding to conserved repetitive elements motif, originally described for prokaryotes, were employed to generate fingerprints of *T. indica*, *T. walkeri*, *T. controversa*, *T. laevis*, *T. tritici*, *T. goloskokovii*, *T. barclayana*, and members of *T. fusca* complex. Computer-assisted analysis of the database of combined fingerprints clearly distinguished each taxon and indicated phylogenetic relationships consistent with previously reported RAPD analyses. Three main cluster groups based on extent of similarity (35–40%) were recognized. Group I included *T. indica* and *T. walkeri*, while group II enclosed members of *T. fusca* complex, *T. controversa*, *T. laevis*, *T. tritici*, and *T. goloskokovii*. Isolates of *T. barclayana* formed group III. The results indicated that rep-PCR format could be used as a simple taxonomic tool and also as a diagnostic technique (McDonald et al. 2000).

Tilletia controversa, an important international quarantine pathogen causing wheat dwarf bunt disease, has similar teliospores morphology and genomic structure as *T. caries*. An RAPD primer-mediated asymmetric-PCR (RM-PCR) was developed to differentiate *T. controversa* and *T. caries* and to screen differential sequences between these two pathogens of wheat. Using RM-PCR assay, a 1332 bp DNA format (PR32) was selected from strains of *T. controversa* (18) and *T. caries* (29). The PR32 genes were specific to *T. controversa* without any homology to *T. caries* or other fungi in the database. Primers designed from PR32 amplified the DNA fragment of *T. controversa* from all strains of *T. controversa* only. On the other hand, no amplification occurred in any of the strains of *T. caries*, indicating the specificity of the primers. By employing SYBR Green I and TaqMan probe real-time assay based on PR32, *T. controversa* was identified rapidly and precisely. TaqMan real-time PCR assay was successfully applied to detect *T. controversa* in asymptomatic wheat tissues (Yuan et al. 2009). PCR-based RAPD procedure was applied to differentiate isolates of *Tilletia indica* (20) India. The isolates were classified into two major clusters; one enclosed 5 isolates from hilly areas, and the other included 15 isolates collected from plains. The groups identified based on pathogenicity levels did not match well with clusters differentiated by PCR-based RAPD technique (Thirumalaisamy and Singh 2012).

Genetic variability of isolates of *Plasmopara halstedii*, causal agent of downy mildew of sunflower, was studied. *P. halstedii* isolates (77) collected from 12 countries from four continents were investigated for RAPD polymorphism, employing 21 primers. A binary matrix derived from the results was subjected to various complementary analyses. Similarity indices ranged over 89–100%. No clustering of the isolates could be observed and the groups did not fit race or geographical origins. Phylogenies were probably observed by limited diversity. Most of the genetic diversity was attributable to individual differences. Even the most differentiated races had the lowest within-diversity indices. The results suggested that *P. halstedii* is probably homothallic or has an asexual mode of reproduction, and the gene flow among countries could occur through commercial exchanges. This investigation aimed to investigate the international genetic diversity of the sunflower downy mildew pathogen which may provide knowledge of the pathogen population structure at an international level, enabling the formulation of strategies for managing the sunflower downy mildew disease (Roeckel-Drevet et al. 2003).

Isolates of *Peronsclerospora sorghi*, causal agent of downy mildews of sorghum and corn, show pathogenic and genetic variability. In order to investigate genetic variability of *P. sorghi* isolates, a DNA sequence-characterized amplified region (SCAR) marker was identified, using PCR for the identification of isolates of the pathogen. The RAPD primer OPB15 was able to consistently amplify a 1000 bp product in PCR only from DNA of *P. sorghi* isolates from corn and not from those of sorghum. A PCR-amplified 1000 bp product was cloned and sequenced. Specific primers were designed based on the sequence of SCAR marker to identify the maize isolates of *P. sorghi*. The SCAR primers amplified a 800 bp fragment from the DNA of isolates of the pathogen from corn only. The results indicated the usefulness of SCAR primers for specific detection and identification of isolates of *P. sorghi* from two important cereal crops (Ladhalakshmi et al. 2009).

Colletotrichum lindemuthianum, incitant of common bean anthracnose disease, exhibits wide variations in its pathogenicity, resulting in identification of several races based on the type of infection on different bean cultivars. A total of 10 races were identified among 48 isolates. Random amplified polymorphic DNA (RAPD) analysis was performed in the same 48 isolates of *C. lindemuthianum*. The analysis revealed that

a wide genetic diversity was in existence among the isolates which formed five groups at a maximum similarity value of 89.6%. No clear relationship was observed between the loci sampled by RAPD markers and pathogenic potential of isolates. Analysis of molecular variance showed that 96.06% of the variability was observed within regions and only 3.94% among regions, indicating a high exchange of genetic material among the regions of a state in Brazil. Most of the variability was detected within races (75.24%). The results of pathogenicity assessment and RAPD analysis indicated similar broad genetic diversity of the pathogen, and were found to be useful for breeding for resistance to anthracnose disease of common bean (Silva et al. 2007). *Aspergillus flavus*, causal agent of peanut aflaroot disease and discoloration of rice grains, produces aflatoxin B₁ (AFB₁), a mycotoxin harmful to humans and animals consuming contaminated grains and feed. *A. flavus* strains (22) were isolated from rice grain samples from 20 states in India. The potential to produce AFB₁ in different media by the strains was determined. The isolates were investigated for their genetic variability using RAPD assay. The isolates showed variable genetic similarity, varying over 17–80% of the standard *A. flvus* (MTCC2799). One strain of *A. flavus* each from Tamil Nadu (DRAF 009) and Maharashtra (DRAF 015) produced a high amount of AFB₁ in all media tested. Aflatoxin production potential and genetic similarity did not seem to be related (Reddy et al. 2009).

Cucurbit crops are seriously affected by gummy stem blight disease caused by *Didymella bryoniae* (= *Phoma cucurbitacearum*). In order to differentiate isolates of *D. bryoniae* collected from various countries across the world, RAPD profiles and specific PCR primers were employed. Two major genotype groups, RAPD Group (RG) I and RG II, were recognized based on the results. A real-time PCR assay developed previously could detect and differentiate only the isolates belonging to RG I genotype. However, a new set of primers and TaqMan probe in conjunction with real-time PCR have been employed for successful detection and differentiation of both genotypes of *D. bryoniae*. The new protocol generated strong Ct values from a worldwide collection of *D. bryoniae*, including 105 RG I, 13 RG II, and 7 unassigned isolates. This broad spectrum and specific real-time PCR system might prove to be useful for routine seed and plant health diagnosis for *D. bryoniae* (Ling et al. 2010). *Alternaria brassicae*, causing dark leaf spot disease of cauliflower and mustard, is responsible for significant qualitative and quantitative losses. RAPD analysis and intersimple sequence repeat primers revealed that genetic variation among 32 pathogenic isolates of *A. brassicae* had the mean similarity coefficient of 0.73 and 0.84, respectively. In addition, internal transcribed spacer analysis showed that all isolates exhibited 90–100% similarity, although their pathogenic potential varied considerably (Sharma et al. 2013b).

Rhizoctonia solani, capable of being seed borne in some plant species of its wide host range, exists in the form at least 13 distinct anastomosis groups (AGs). *R. solani* AG-3 was found to be highly virulent, necessitating development of a method for rapid and reliable identification of the pathogen. RAPD technique involving the use of specific genetic markers was applied for the identification of AG-3 isolates. RAPD amplification revealed the presence of a specific fragment (26 kb) in all AG-3 isolates. A PCR-based restriction mapping method was developed, using restriction enzyme *Xho*I for the identification of AG-3 isolates. These virulent isolates could be rapidly detected in infected plant tissues (Bounou et al. 1999). *R. solani* AG-8 was subdivided on the basis of zymogram patterns into zymogram groups (ZGs). RAPD-PCR technique was applied to determine relationships between four ZGs within RG-8. *R. solani* AG-8 isolates (79),

representing four pectic isozyme groups (ZG 1-1, 1-2, 1-4, and 1-5) from different locations in southern Australia, were analyzed by RAPD-PCR assay. Six primers were employed to determine the relatedness of the isolates. The results indicated that AG-8 populations included four distinct groups that matched four ZGs, providing support to the concept that ZGs are distinct intraspecific groups (MacNish and O'Brien 2005).

2.1.4.6 Amplified Fragment Length Polymorphism

Investigations of population dynamics require more information over and above rapid detection and precise identification of target pathogens. *Pyrenophora teres*, causing net blotch disease in barley, was identified by amplified fragment length polymorphism (AFLP) fingerprinting technique. Specific primers amplified DNA from *P. teres* f. *teres* (net form), but not from closely related *P. teres* f. *maculata* (spot form), indicating the specificity of detection and the potential of this procedure for differentiating closely related pathogen species/strains. Form-specific PCR products were generated due to high specificity of the primers (Leišova et al. 2005a). The Czech isolates of *Pyrenophora teres*, *P. graminea*, *P. tritici-repentis*, and *Helminthosporium sativum* were analyzed by AFLP technique. All the four pathogens had distinct AFLP profiles. Detection of 948 polymorphic bands was accomplished by employing 19 primer combinations. All main clusters in the dendrogram corresponded to the individual pathogen species investigated. The two forms of *P. teres* f. *teres* (PTT) and *P. teres* f. *maculata* (PTM), inducing net and spot symptoms respectively, could be differentiated as they formed different clusters. Genetic diversity with regard to the location and year of sampling was analyzed separately within the AFLP-based dendrogram cluster of PTT and PTM. Unweighted pair group method analysis (UPGMA) of the 37 isolates of PTT and 30 isolates of PTM, using 469 polymorphic bands, showed that the variability seemed to have been influenced more by the year of sampling than by the geographic origin of the isolate. The presence of intermediate haplotypes with a relatively high number of shared markers between the two groups indicated that hybridization between the forms of *P. teres* could occur, but it was probably often overlapped by selection pressure or genetic drift (Leišova et al. 2005b).

It is essential to gather genetically diverse collection of isolates for assessment of genetic variability within a natural population of target pathogen. Different *Ustilago* species, causing smut diseases of wheat, barley, and oat, have been classified based on taxonomic characteristics such as spore size, shape, morphology, germination type, and host range. However, these characteristics are not useful to determine relationships within smut fungi and other groups of fungi. Pathogen populations depending on host range, geographical locations, and environmental conditions may vary with different sampling periods. The genetic relationships among different species of *Tilletia* spp. causing bunt disease have been studied using RAPD and RFLP analysis. Intersimple sequence repeats (ISSRs) have been used to characterize genetic variations within fungi. This technique using primers based on microsatellite sequences and degenerate anchors at the 5' end is highly reproducible, allows the detection of interspecific and intraspecific DNA-polymorphisms, and is applicable to a diverse range of fungal pathogens. The phylogenetic relationships among seven species of *Ustilago* – *U. avenae*, *U. bullata*, *U. hordei*, *U. kolleri*, *U. nigra*, *U. nuda*, and *U. tritici* – were investigated using ISSRs and amplified fragment length polymorphisms (AFLPs) to compare their DNA profiles. Fifty-four isolates of different *Ustilago* spp. were analyzed using ISSR primers and 16 isolates of *Ustilago* spp. were studied using AFLP primers. The variability among

isolates within species was low for all species except *U. bullata*. The isolates of *U. bullata*, *U. nuda*, and *U. tritici* were well separated. *U. avenae* and *U. kolleri* isolates did not separate from each other and no significant variability could be seen between these species. Likewise, *U. hordei* and *U. nigra* isolates did not show significant variability between the species. However, the isolates from each species grouped together. Use of molecular data for classification of fungi such as smut fungi, which have limited variability in morphologic characteristics, may be highly valuable. The ISSR and AFLP analysis of *Ustilago* spp. was useful in determining genetic variability among them. The AFLP technique appeared to be more sensitive to detect differences in DNA sequences than ISSR technique (Menzies et al. 2003).

The genetic relationships of molecular variability and virulence patterns of races of *Ustilago tritici*, causing loose smut disease of wheat existing in western Canada, were investigated. Isolates (85) of 19 races of *U. tritici* collected from durum and bread wheat were studied using molecular and virulence data. Eleven *EcoRI* and *MseI* primer pair combinations were employed for AFLP analysis. A total of 52 polymorphic fragments were generated. Virulence data from the wheat differential set for loose smut were used to perform cluster analysis, using unweighted pair group method with arithmetic averages method. Cluster analysis from molecular and virulence data indicated that races collected from durum wheat cultivars were grouped separately from those of bread wheat cultivars. Most races were pathogenic to either bread wheat or durum wheat. Race T39 had a complex virulence pattern, enabling it to infect both durum and bread wheat cultivars. This investigation demonstrated that molecular diversity of *U. tritici* could be associated with virulence patterns and suggested that emergence of new races would be possible by recombination of virulence of different races of *U. tritici* (Randhawa et al. 2009).

In another investigation, the genetic diversity of *Ustilago tritici* was studied using isolates obtained from intensive sampling (80 isolates from four field) and extensive sampling (81 isolates from 81 fields). Genetic diversity of the isolates was assessed using virulence data and amplified fragment length polymorphism (AFLP) profiles. The virulence potential of the isolates of *U. tritici* was assessed by inoculating five differential hosts. The AFLP data were gathered, using 10 selective primers that yielded 23 polymorphic bands. Genetic diversity of the isolates was similar within extensive- and intensive-sampling collections based on virulence assessments. On the other hand, genetic diversity was variable within the intensive-sample collection than within the extensive-sampling collection when the AFLP data were considered. Generally, AFLP analysis indicated a greater degree of genetic variability than virulence data revealed. Host genotypes might strongly influence the genetic variability within populations of an obligate pathogen such as *U. tritici*. When the variability was determined using virulence data, host genotype had a weaker influence on pathogen variability as reflected by AFLP profiles (Popovic and Menzies 2006).

Sporisorium reilianum, a soilborne, facultative biotrophic fungal pathogen, causes the sorghum head smut disease. The population structure and genetic diversity of *S. reilianum* isolates were studied, using AFLP genotyping analysis. An efficient and reliable method of inoculation for assessing the levels of resistance of sorghum genotypes to head smut disease was also developed. The syringe injection method was the most effective among the four methods tested. Highest disease incidence was observed on three differentials, namely, BT x 643, SC170-6-17, and T x 7078 in the range 83–98%.

Two isolates were classified as race 1, but race 2 or 3 isolates were not detected among the 32 isolates from Texas, USA. Using 16 AFLP primer combinations, genetic diversity was assessed in DNA samples from 49 isolates of *S. reilianum* from Texas, Uganda, Mali, and Mexico. Single-base extensions with *EcoRI* and *MseI* primers in the selective amplification increased the number of informative polymorphic bands. High genetic dissimilarity (50%) was detected between isolates originating from maize and those originating from sorghum. Isolates of *S. reilianum* from Texas were grouped into four clusters with $\geq 82\%$ similarity. The AFLP technique permitted the uncovering of cryptic genetic variations of strains or closely related species, even at the isolate level, which may not be possible using morphological or other molecular systematic characteristics (Prom et al. 2011).

Genetic variability of *Pyricularia oryzae* (*Magnaporthe grisea*) was assessed by AFLP technique. Isolates of *P. oryzae* (114) collected from rice-growing areas in the Red River Delta during 2001 and other isolates (9) in Vietnam were analyzed based on 160 polymorphic AFLP markers. Based on DNA similarity and cluster analysis, the isolates were classified into 12 different AFLP genetic groups. Two separate groups were formed to accommodate isolates of *P. oryzae* from *japonica* and *indica* cultivars which exhibited at least 60% dissimilarity with little evidence for gene flow between these two clusters. On the other hand, isolates from *indica*, forming the predominant group, showed significant gene flow between these populations found south and north of Red River. Pathogenicity tests indicated the presence of two avirulent isolates and 23 pathotypes which were differentiated into 12 AFLP groups genetically (Thuan et al. 2006). In order to assess the levels of genetic diversity and population structure of *Microdochium* spp., 172 isolates were collected from five populations of infected wheat seeds. These isolates were classified into *M. majus* and *M. nivale* using PCR assay with specific primers. *M. majus* was the predominant one with 165 isolates assigned, and 7 isolates belonged to *M. nivale* involved in the Fusarium head blight (FHB) disease. Intersimple sequence repeat (ISSR) analysis showed that the total genetic diversity was 0.023 when estimated by Nei's gene diversity index within populations dominated by *M. majus*. The results indicated the existence of little genetic diversity among the five populations of *M. majus* examined. Based on the unweighted pair group method of cluster analysis using the ISSR data, all isolates were identified as one of eight haplotypes in *M. majus* or six haplotypes in *M. nivale*, allowing the construction of a dendrogram with two clades corresponding to each species. The results showed that migration of the pathogen may play an important role in the population biology of *M. majus* (Hayashi et al. 2014).

Fusarium graminearum and *F. asiaticum* isolates (437) collected from wheat spikes from all regions in China were analyzed to determine the extent of their genetic diversity and geographic distribution. Sequence-characterized amplified region (SCAR) analyses of 437 isolates resolved both species, with 21% being *F. graminearum* (SCAR type 1) and 79% being *F. asiaticum* (SCAR type 5). SCAR and AFLP analyses were completely congruent, revealing two distinctly different patterns that could resolve the Chinese Fg complex. They were differentiated by combining SCAR typing with SSCP analysis. No variants were detected within either SSCP-SCAR class, suggesting that the primer set Fg16F/FgR amplified highly conserved sequences that may be fixed in these two species. AFLP profiles clearly resolved two groups A and B that were completely congruent with both species. However, more diversity was detected by AFLP, revealing several groups within each group. In several cases, even for isolates from the same

district, AFLP analysis revealed the presence of several groups within each group. AFLP haplotypes differed markedly. Phylogenetic analyses of multilocus DNA sequence data indicated that all isolates of SCAR type 1, AFLP group A were *F. graminearum*, whereas isolates of SCAR type 5, AFLP group B were *P. asiaticum*. The results suggested that *F. asiaticum* was the predominant FHB species occurring on wheat in China, although a significant population of *F. graminearum* was also present. The geographic distribution of *F. asiaticum* and *F. graminearum* across China appeared to be related to the annual average temperature of different locations. Of this 92 isolates of *F. graminearum*, 80 isolates were found in cooler areas where the annual average temperature was 15°C or lower. AFLP analysis identified considerable diversity within *F. asiaticum* and *F. graminearum*. Only five pairs of isolates displayed identical AFLP patterns. No identical AFLP patterns were observed for any two isolates originating from geographically distant districts in China (Qu et al. 2008).

Alternaria tenuissima populations isolated from wheat kernels were investigated for their genetic diversity. Amplified fragment length polymorphism (AFLP) analysis was applied on 101 isolates of *A. tenuissima* to determine genetic differences between isolates from wheat kernels and other hosts. Using four primer combinations, AFLP banding patterns indicated significant genetic distance and at the same time a low level of gene flow between the Far East and the two other groups of isolates originating from the European part of the country. The degree of similarity between the northwest and Caucasus populations was very high and the migration rate was also greater. Isolates analyzed by UPGMA-based cluster analysis were grouped according to the location of origin, but irrespective of plant host. The group of isolates from wheat and barley did not differ significantly from each other (Gannibal et al. 2007). The genetic variability within vegetative compatibility groups (VCGs) of *Verticillium dahliae* was investigated by employing AFLP technique. Isolates of *V. dahliae* from cotton (96) and olive trees (45) were analyzed by AFLP and PCR techniques. Clustering of isolates within VCGs correlated with respect to VCGs, irrespective of host plant species and geographical locations. However, VCGs exhibited molecular diversity, the variability being highest in VCG2A. In addition, VCG2B isolates from artichoke formed two distinct clusters based on PCR markers of 334 bp or 824 bp. Moreover, correlations between molecular differences and their virulences of VCG2B isolates pathogenic to cotton cultivars were observed (Collado-Romero et al. 2006).

2.1.4.7 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) analysis has been shown to be useful for the identification and taxonomy of fungal pathogens. The RFLP technique is based on the natural variations in the genomes of different groups or strains of organisms. Variations (polymorphisms) in fragment sizes may be generated by loss or gain or by other events that influence fragment size such as deletions or insertions in the DNA sequences. The restriction enzymes cleave the DNA at specific sites, resulting in a different number of fragments depending on the test fungal species or strains. The DNA fragments are separated by electrophoresis in agarose or polyacrylamide gel to detect the differences in the size/number of DNA fragments. The number and size of the fragments vary due to the distribution of restriction sites in the DNA. A specific set of fragments constituting the “fingerprint” for a test strain/isolate can be recognized, depending on the combination of each restriction enzyme and target DNA. Southern blot analysis facilitates the identification of the restriction sites of DNA fragments

(Hamer et al. 1989; Leach and White 1991). By staining gels with ethidium bromide, the restriction sites may be directly observed under ultraviolet light (Klich and Mullaney 1987). The DNA fragments are then transferred to nitrocellulose or nylon membrane and hybridized with an appropriate probe.

Probe-specific RFLP for a dispersed, repeated sequence (known as MGR) was employed for constructing genotype-specific *Eco*RI restriction fragment length profiles (MGR-DNA fingerprints) from field isolates of rice blast pathogen *Magnaporthe grisea* in the USA. The major pathotypes of *M. grisea* could be identified precisely based on the MGR-DNA fingerprints. Organization of clonal lineage within and among pathotype groups was also defined by examining the fingerprints (Levy et al. 1991). The genetic relationships among isolates of *Pyricularia grisea* from rice and other grass hosts were investigated by RFLP analysis by using the repetitive probe MGR 586. Rice blast isolates representing four distinct races differentiated by inoculating Korean differential rice varieties showed multiple bands hybridizing to the probe MGR586. The results indicated the possibility of *Pyricularia grisea* populations from nonrice hosts, providing inoculum for the rice crops (Han et al. 1995). *Tilletia indica*, wheat kernel bunt pathogen, is a target of international quarantines by several wheat-growing countries and its presence in wheat grains poses serious risks of rejection by importing nations. *T. indica* and *T. walkeri* (causing bunt disease of annual rye grass) were found as contaminants of wheat seeds. The PCR assay using the *T. indica*-specific primer pairs Ti17M1/Ti57M1/M2 and Ti57M1/M2 amplified products of similar size from both *T. indica* and *T. walkeri*, but did not amplify products from *T. horrida* DNA (Fig. 2.8). Sequence analysis of the entire ITS region identified a *Sca*I restriction enzyme site in the ITS1 region of *T. walkeri* that was not present in *T. indica* or other *Tilletia* spp. examined. The ITS *Sca*I RFLP-PCR test (*Sca*I test) was applied to screen 36 isolates of *T. indica*, 15 isolates of *T. walkeri*, and 34 isolates of taxa in the genus *Tilletia* from wheat and other grasses (Fig. 2.9). DNA sequencing and restriction digest results showed that *T. walkeri* could be easily distinguished from *T. indica* and other morphologically similar bunt fungi with a *Sca*I restriction digest of either the ITS1 region alone or the entire ITA1, 5.8S, and ITA2 region of nuclear rDNA repeat unit. Phylogenetic analysis of taxa based on ITS sequence data indicated a close relationship between the two species and other morphologically similar taxa (Levy et al. 2001; Section 2.3.11).

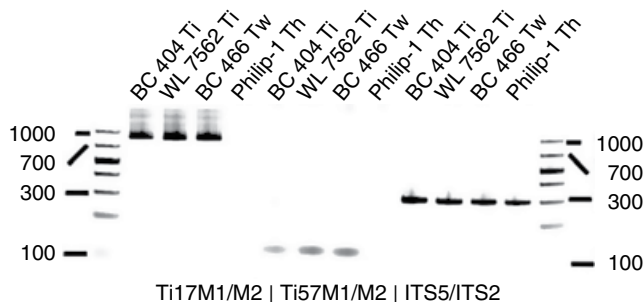


Figure 2.8 Detection of *Tilletia indica*, *T. walkeri* and *T. horrida* by PCR assay using *T. indica*-specific primer pairs Ti17M/M2 and Ti57M1/M2. Note products of similar size produced from DNA of *T. indica* and *T. walkeri*, but not from *T. horrida*. Lanes 1 and 14: Molecular ladder; lanes 2, 3, 6, 7, 10 and 11: *T. indica*; lanes 4, 8 and 12: *T. walkeri* and lanes 5, 9 and 13: *T. horrida*. Source: Levy et al. (2001). Reproduced with permission of the American Phytopathological Society, MN, USA.

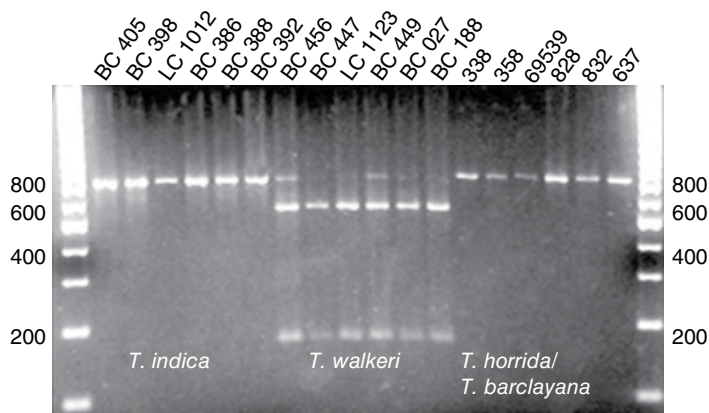


Figure 2.9 Differentiation of isolates of *T. indica*, *T. walkeri*, *T. horrida* and *T. barclayana* based on the sequence analysis of ITS region *ScaI* restriction enzyme digest of ITS, 5.8S and ITS ribosomal DNA PCR products differentiated by agarose gel electrophoresis. Lanes 1 and 20: DNA ladder; lanes 2–7: *T. indica*; lanes 8–13: *T. walkeri*; lanes 17–19: *T. barclayana*. Source: Levy et al. (2001). Reproduced with permission of the American Phytopathological Society, MN, USA.

Phomopsis longicolla is the primary pathogen causing soybean seed decay, but *Diaporthe phaseolorum* is also found frequently associated with this disease. Conventional isolation-based methods were not useful to reliably detect and differentiate these two fungal pathogens present in latent and systemic infections. RFLP analysis of PCR amplification products was demonstrated to be effective in differentiating these pathogens present in soybean seeds. Specific primers Phom I and Phom II were designed from polymorphic regions of *P. longicolla* and *D. phaseolorum* isolates from soybean. It was possible to detect specific bands in PCR amplifications from ten pooled seed samples and also from individually infected seeds. Likewise, the presence of *P. longicolla* and *D. phaseolorum* could be detected in asymptomatic soybean plants inoculated with these pathogens based on the recognition of specific band by RFLP analysis (Zhang et al. 1997). Classification of *formae speciales* and races is based on the variations in pathogenic potential of the isolates of *Fusarium* spp. RFLP analysis of the total pathogen DNA was performed after digestion with restriction enzymes *Pst*I, *Hind*II, and *Eco*RI followed by Southern blotting and hybridization with mtDNA polypore from *Fusarium oxysporum* f.sp. *niveum* (Fon). Unique RFLP patterns were observed for each *formae specialis*. Genetic divergence in *F. oxysporum* f.sp. *cucumerinum* (Foc) was seen to the maximum extent, whereas genetic divergence was only minimal in Fon (Kim et al. 1993). Several species of *Fusarium* have been reported to be associated with infection of cereal grains which are contaminated with different kinds of mycotoxins capable of causing ailments in humans and animals. By employing specific genetic markers, the toxigenic isolates producing fumonisins could be differentiated from nontoxigenic isolates. Two sets of primers VERT F-1 and VERT F-2 were employed in PCR-RFLP technique for specific detection of toxigenic isolates of *F. verticillioides* which could produce fumonisins (Patiño et al. 2004).

Pseudocercospora herpotrichoides infecting rye has been shown to exist in the form of four different pathotypes, R, N, C, or S, which exhibit varying virulence levels. A pathotype-specific DNA probe was effective for the identification of R-type isolates

of *P. herpotrichoides*. A 6.7 kb DNA fragment from R-type isolates hybridized with R-type isolates only, but not with N, C, or S pathotypes of *P. herpotrichoides* or *P. anguioides*. Rye seedling infected by R-type isolates could be identified by hybridization of the probe to DNA extracted from infected rye plants (Nicholson et al. 1994). Genetic diversity in the clones of *P. herpotrichoides* was assessed by applying RFLP analysis. Polymorphic pathogen-specific probes were efficient in differentiating the clones directly in DNA preparations obtained from infected plants without the need for isolation of the pathogen (Frei and Wenzel 1993). RFLP analysis was useful in establishing a correlation between molecular and biological characteristics of the biotypes of *Stagonospora nodorum* from barley and wheat. The genetic similarity was very low (0.12) between the two biotypes of *S. nodorum* (Ueng et al. 1995). In order to assess the genetic relationships among different isolates of *Verticillium* spp., the sequences of intergenic spacer (IGS) region and β -tubulin gene formed the basis of differentiation. Four distinct groups were formed to include isolates of *V. albo-atrum*, *V. tricorpus*, and *V. dahliae* from cruciferous and noncruciferous crops. Furthermore, four subgroups were formed to differentiate isolates of *V. dahliae* from noncruciferous hosts based on the sequence similarity within the IGS region (Qin et al. 2006).

Fusarium graminearum (teleomorph *Gibberella zeae*), incitant of Fusarium head blight (FHB) disease, is responsible for heavy losses in several countries in Europe, the USA, and China. *F. graminearum* developed resistance to carbendazim fungicides under high disease pressure and excessive use of these fungicides. Primer-induced restriction analysis (PIRA) -PCR assay was developed to detect isolates of *F. graminearum* with moderate resistance to carbendazim. Two primers were designed and synthesized based on the nucleotide sequence of the β 2-tubulin gene from *F. graminearum*. Fragments of 163 bp were amplified by nested PCR from isolates differing in their carbendazim sensitivities. A *Hind*III restriction enzyme recognition site was introduced artificially by inner primers to detect a mutation at codon 167, and the *Taa*I restriction enzyme was used to detect a mutation at codon 200. The final PCR product amplified by the primer pair *Hind* 3F + *Hind* 3R was digested by restriction enzyme *Hind*III and *Taa*I. The mismatched primer *Hind* 3F introduced a *Hind*III recognition site into PIRA-PCR product of isolates nonmutated at codon 167. The sensitivity of isolates to carbendazim was determined by analyzing electrophoresis patterns of the resulting PCR products after simultaneous digestion with both *Hind*III and *Taa*I. PIRA-PCR assay was faster in providing the results (within 7–8 h), whereas conventional methods require 5–7 days to yield results. However, both assays gave identical results. PIRA-PCR assay has the potential to monitor the appearance of isolates with moderate resistance to carbendazim rapidly and consistently (Luo et al. 2009).

Aspergillus flavus may infect seed of several crops such as corn, cotton, and peanut and contaminate them with harmful aflatoxins which are hepatotoxic and carcinogenic in nature. Vegetative compatibility groups (VCGs) were identified based on complementation tests between nitrate-nonutilizing (*nit*) mutants and have been used to examine the genetic diversity of *A. flavus* populations in cotton and peanut fields. Using a species-specific DNA probe pAF28 that hybridized strongly only to strains of *A. flavus*, developed and based on the DNA fingerprints, it was possible to precisely distinguish the 22 VCGs among 29 strains of *A. flavus* (Papa 1986). The pAF28 hybridized to multiple and distinct bands for DNA from different subspecies of *A. flavus* (Wicklowsky et al. 1998). The ability of species-specific DNA probe pAF28 to correctly match 75 strains of *A. flavus* isolated from a peanut field in Georgia with 1 of 44 distinct VCGs was assessed.

Multiple strains belonging to the same VCG typically produced identical DNA fingerprints, with the exception of VCG 17 and VCG 24 which contained strains that showed 83 and 87% similarity, respectively. *A. flavus* isolates sharing more than 80% of fragments were included in the same DNA fingerprint group, each represented by a single isolate which produced unique DNA fingerprints. The results confirmed that pAF28 probe was able to distinguish *A. flavus* VCGs based on DNA fingerprints, and that it could be the basis for predicting the approximate number of VCGs in a sample population of *A. flavus*. Furthermore, the DNA probe also hybridized strongly and produce multiple and distinct bands with other species of *Aspergillus* section *Flavi*. However, the DNA fingerprints with multiple bands produced by representative strains could not be used to classify these strains according to species in section *Flavi*. The VCG analysis was found to be cumbersome in genetic analysis studies on large pathogen population. On the other hand, DNA fingerprinting of *A. flavus* allowed ready comparison of new DNA fingerprints with those in stored electronic database. It is therefore possible to more accurately predict that *A. flavus* isolates belonging to the same pAF28 DNA fingerprint group were also vegetatively compatible (McAlpin et al. 2002).

Verticillium dahliae isolates (27) from cotton were investigated by RAPD and specific PCR assays. By using nitrite nonutilizing (nit) mutants, the isolates were assigned to VCG1 (13 isolates), VCG2B (8 isolates), and VCG4B (6 isolates). DNA extracted from the isolates was used for RAPD and specific PCR assays. RAPD assays of VCG2B and VCG4B isolates consistently produced the DNA bands diagnostic of the nondefoliating (ND) pathotype. Similarly, assays of VCG1 isolates (except one) yielded RAPD markers diagnostic for the defoliating (D) pathotype. As there was lack of consistency, all isolates were examined as a next step by specific PCR assays using D- and ND-specific primers. The results of the PCR assays confirmed that all VCG2B and VCG4B isolates belonged to ND-pathotype, as well as that of all VCG1 isolates except three isolates that belonged to ND-pathotype. The latter three VCG1 isolates did not yield any amplification products with either D- or ND-specific primers. The isolates of VCG2B and VCG4B from Israel appeared to be molecularly homogenous, since all of those tested yielded the DNA bands characteristic of the ND-pathotype in RAPD and the specific PCR assays. VCG1 isolates appeared to be molecularly homogenous however, because three of them failed to amplify the D-pathotype-associated marker (0.55 kb) although they induced defoliation symptoms in cotton as other D-pathotype isolates (Korolev et al. 2008). Two pathogenic races of *Verticillium dahliae* infecting tomato and lettuce were recognized based on the host resistance specific to race 1 governed by plant immune receptors that recognize the race-1-specific fungal effector *Ave 1*. Further, race 1 could be identified by employing PCR assay. In order to identify and differentiate races 1 and 2 of *Verticillium dahliae*, two isolates from each race were examined for the presence of potential markers in the genome sequences, and PCR primers capable of distinguishing them were identified. One primer pair VdR1F/VdR2R consistently yielded a 256 bp amplicon in all race 2 isolates exclusively. DNA from 677 isolates of *V. dahliae*, including 340 from spinach seed lots was screened using the selected primer pair and race-1-specific primer pair published earlier. DNA from isolates that did not amplify with race-1-specific PCRs amplified with race-2-specific primers. For validation, two differential lines of lettuce were inoculated with 53 arbitrarily selected isolates from spinach seed and their pathogenicity and virulence were assessed by greenhouse tests. The results strongly supported that of PCR assays. Isolates from lettuce, pepper, and strawberry from California as well as isolates from spinach seed from two of four countries contained both races,

whereas only race 2 was detected in cotton, olive, mint, and potato. The results indicated the need to differentiate the races of *V. dahliae* for epidemiological and breeding for resistance investigations (Short et al. 2014).

2.1.4.8 DNA Sequence Analysis

Microsatellites or simple sequence repeats (SSRs) have been used as molecular markers as they are multiallelic, codominant, and highly polymorphic. Microsatellites have been employed to investigate the genetic structure and reproductive biology of some pathogens. The cost-effectiveness of the diagnostic method is a vital factor in determining its wider application. In order to replace the microsatellite-based method, which is both expensive and laborious, a novel strategy was developed to substantially reduce the cost of developing SSRs (by 50%). This procedure is based on anchored PCR format in which microsatellite amplification is achieved with one primer complementary to the flanking sequence and one that is specific to the repeat motif (Hayden et al. 2004). Markers amplified by anchored PCR were designated for sequence-amplified microsatellite (SAM) analysis and sequence-tagged microsatellite (STM) profiling (Hayden et al. 2006).

Sequence-tagged microsatellites (STMs) (168) were generated from DNA of *Rhynchosporium secalis*, causing barley scald disease, by employing a rapid and cost-effective technique. Anchored PCR assay directly generated a DNA library highly enriched for microsatellite clones, which was screened by colony PCR to identify uniquely sized simple sequence repeat (SSR) fragments. By adopting this procedure, DNA sequencing attempts to select SSR clones suitable for primer design could be reduced. In addition, the need to design specific flanking on only one side of the microsatellite repeat halved the amount of primer synthesis compared to conventional SSRs, which require a pair of primers flanking the repeat. STMs (66) that amplified 66 loci indicated a high level of polymorphism among a diverse set of 16 Australian isolates. The cost of performing fluorescence-based microsatellite assay could be considerably reduced by the method followed in this study to generate STMs (Keipfer et al. 2006). The two forms of lesions produced in barley infected by *Pyrenophora teres* (*Drechslera teres*) were differentiated as spot form induced by *P. teres* f. *maculata* and net form caused by *P. teres* f. *teres*. These two forms could not be reliably differentiated based on symptoms and conidial morphology. The genetic distinctiveness of these two forms could be determined by generating microsatellite markers (SSRs, 80) for the two barley *P. teres* forms. A modified sequence-tagged microsatellite profiling technique was developed for fungi with small genomes like *Rhynchosporium secalis* (Keipfer et al. 2006, 2007). In a further investigation, nine SSRs were found to amplify loci in one *P. teres* form only when tested on the same set of six field-sampled isolates derived from single spores of each *P. teres* form. Loci with different allele size ranges in each form or different loci in each form were identified. The use of SSRs has the potential for application as diagnostic markers to detect and differentiate *P. teres* spot and net forms (Keipfer et al. 2008).

Analysis of race structure and genotypic diversity of four populations of *Leptosphaeria maculans* isolated from *Brassica oleracea* (broccoli, cauliflower, and cabbage) in central Mexico was performed. Race structure was characterized by an unusually low diversity at three out of four locations. Fourteen minisatellite markers were employed in the assay. A high proportion of repeated multilocus genotypes was detected in these populations, combined with a significant linkage disequilibrium and

strong clonal fraction (65–87%). Assessment of mating-type idiomorphs showed significant departure from the 1:1 proportion expected in the case of random mating. Each population consisted of a few (4–9) multilocus genotypes which were specific to each location. The data suggested that an exclusive or high rate of clonal multiplication might occur in these test locations in central Mexico (Dilmaghani et al. 2013).

The coexistence of genetically divergent populations of *F. graminearum* sensu stricto has been reported. The molecular diversity within 12 individual field populations of *F. graminearum* in Germany was assessed using 19 simple sequence repeat (SSR) markers. The variation in aggressiveness within individual field populations as well as the deoxynivalenol (DON) concentration in wheat under natural conditions was measured. Three populations of *F. graminearum*, each consisting of 30 single-spore isolates, were compared with 11 isolates collected from other countries. All isolates were characterized using 19 SSR markers. The genotypic proportion of phenotypic variance within population was significant (<0.001) for Fusarium head blight (FHB) infection and DON concentrations in grains. Ranges in mean FHB severity and DON concentration in the host were only slightly smaller for the field populations in Germany than for the *F. graminearum* collections from other countries. Both traits were significantly correlated ($P < 0.05$) within and across populations. The ability of fungal pathogens to adapt to environmental factors, including resistant cultivars, largely depends on genetic variation within natural populations governed by the evolutionary forces of mutation, recombination system, selection, genetic drift, and gene flow (Talas et al. 2012).

Genetic diversity and relatedness of isolates of *Pyrenophora tritici-repentis*, causing tan spot disease of wheat, were investigated using simple sequence repeat (SSR) markers derived from genomic DNA of the pathogen. SSR markers amplified 31 different loci in the pathogen isolates. A significant genetic differentiation of isolates could be detected. Nei's analysis of genetic diversity and genetic distance indicated that isolates of *P. tritici-repentis* could be grouped into four distinct populations based on their region of origin. Pathogenic isolates were also compared by race, genetic distance, and cluster analysis based on pooled allele frequency. PtrToxA (toxin produced by the pathogen) -producing and nonproducing isolates clustered together but they were distantly related to PtrToxA-producing isolates, suggesting that host specificity imposed by the different Ptr toxins could form the basis of differentiation of *P. tritici-repentis* isolates (Aboukhaddour et al. 2011).

Total DNA of *Plasmopara halstedii*, causal agent of sunflower downy mildew disease, was extracted for analysis of polymorphisms that could be useful to differentiate field isolates with respect to epidemiological investigations or pathotype characterization. The DNA was obtained from mitotically produced zoosporangia, which were the only cellular structure of the biotrophic pathogen accessible and from its host. DNA fingerprinting with minisatellite and simple sequence repeat (SSR) primers were performed using total DNA of the pathogen. Polymorphisms that allowed single field isolates could be identified, but they were not correlated with either physiologic races or geographic origin of isolates. Using specific differentiating primers, single-spore strains of *P. halstedii* isolates were analyzed with respect to genetic homogeneity. Minor variation was visible in the mitotically derived offspring, but the overall appearance of these patterns was mostly uniform with those of the respective parental isolate (Intelmann and Spring 2002). In a later investigation, 15 novel microsatellite markers were isolated from *Fusarium graminearum*, causing Fusarium head blight (FHB) disease in cereals. The level of polymorphism at these novel and 13 previously published

markers was analyzed in 33 strains of *F. graminearum* from Europe, North America, and Nepal. The number of alleles for each of the novel markers ranged from 4 to 20 and gene diversity from 0.417 to 0.962. The resolution for distinguishing different strains was slightly better than could be obtained from earlier investigations. Twenty-seven markers were also detectable in three *F. culmorum* strains and one *F. crookwellense* strain. None of the markers could be detected in three of *F. avenaceum* and four of *F. poae* strains, indicating the potential of these microsatellite markers for wider application for species differentiation (Vogelgsang et al. 2009).

Tilletia controversa, causative agent of wheat dwarf bunt disease, is an important international quarantine pathogen. A diagnostic molecular marker generated from intersimple sequence repeat (ISSR) for rapid identification of *T. controversa* was identified. A total of 60 primers were tested by ISSR to detect DNA polymorphisms between *T. controversa* and related species. The primer ISSR 818 generated a polymorphic pattern displaying a 952bp DNA fragment specific for *T. controversa*. The marker was converted into a sequence-characterized amplified region (SCAR) and specific primers (TCKSF2/TCSR2) were designed for use in a PCR detection assay. The detection limit of the assay was 1 ng of DNA which could be liberated from 1.1 µg of teliospores in a 25 µL PCR assay. The procedure, using a SCAR marker developed in this study, showed potential for reliably distinguishing *T. controversa* from related pathogenic fungi (Gao et al. 2011).

The extent of genetic diversity and population structure of 72 rice-infecting isolates of *Rhizoctonia solani* AG-1 IA, causal agent of rice sheath blight disease, collected from 12 counties in south China, were investigated using nine intersimple sequence repeat (ISSR) markers. A total of 116 bands were amplified with a majority of amplified fragments ranging from 500bp to 2500bp in size, of which 110 (94.8%) were polymorphic. Six major clusters with 73% genetic similarity coefficient were recognized among 72 isolates of *R. solani*. The mean coefficient of gene differentiation (*Gst*) was 0.165, indicating that 83.5% of the genetic diversity resided within the population. The gene flow among the sampled populations analysis of molecular variance (AMOVA) showed that there was high level (81.93%) genetic variation within subpopulations. No significant correlation was revealed between genetic differentiation and geographical (distance) isolation (Shu et al. 2014).

Bipolaris sorokiniana causes wheat blotch disease which is responsible for serious losses, especially in slightly warm and humid environmental conditions. The molecular variability of isolates of *B. sorokiniana* (40) collected from different regions of India was assessed by employing the universal rice primer (URP) -PCR technique. The URPs were derived from DNA repeat sequences in the rice genome. Of the 12 URP markers selected, 10 markers were effective in producing polymorphic fingerprint patterns from DNA of isolates of *B. sorokiniana*. Analysis of the entire fingerprint profile using the unweighted pair group method with arithmetic averages (UPGMA) differentiated isolates of the pathogen gathered from different regions of India. An isolate BS-53 from a northern hilly region differed significantly from other isolates, showing less than 50% similarity. Based on the genetic similarity, the isolates of *B. sorokiniana* formed three major clusters. The isolates from the northwestern and northeastern plains and the peninsular zone clustered separately as three groups, as the isolates within each cluster exhibited more than 75% similarity among them. One of the markers (URP-2F) amplified three monomorphic bands of 0.60, 0.80, and 0.90kb size, and could be employed as a specific marker for the identification of *B. sorokiniana* and differentiation of its isolates (Aggarwal et al. 2010a, b).

In a further study, a pathogen-specific marker derived from its genomic DNA was used to develop a quick and reliable PCR-based diagnostic assay. A PCR-amplified band of 650bp obtained from *B. sorokiniana* isolates using universal rice primer (URP) was cloned in pGEMT easy vector and sequenced. Based on their sequences, six primers were designed from which a primer pair RBSF1 and RBSF2 amplified a sequence of 600 bp in *B. sorokiniana*. The specificity of the marker was tested against 40 isolates of *B. sorokiniana*, 7 isolates of *Bipolaris* spp., and 27 isolates of other wheat pathogens and other crops. The results showed specific amplification of 600 bp only from DNA of *B. sorokiniana* isolates. The detection limit of the assay was 50 pg of genomic DNA. *B. sorokiniana* could be detected in presymptomatic leaves of wheat plants inoculated with the pathogen. The SCAR marker SCARBS₆₀₀ could precisely detect and differentiate *B. sorokiniana* from other wheat pathogens, including other *Bipolaris* spp. The protocol developed in this investigation showed potential for use in diagnosis of infection by blotch disease in wheat even before visible symptoms appear (Aggarwal et al. 2011).

Tilletia indica, causing Karnal bunt disease of wheat, occurs in several countries including the USA. The pathogen exhibits high genetic variability due to its heterothallic nature. The pathogenic potential of different isolates of *T. indica* from different locations in India was assessed. Three pathotypes were recognized based on their virulence on a set of differential wheat genotypes. Monosporidial culture lines (5 each) from KB1, KB2, KB4, and KB5 and the three KB3 lines were established. These lines were genetically analyzed, employing 12 universal rice primers (URPs). Amplification of DNAs from the selected monosporidial culture lines revealed 98.44% polymorphism. The primer UR13R produced 100% polymorphic bands. Similarity in maximum extent (83%) was observed between KB1MsB and KB1MsD as calculated by Jaccard's similarity coefficient, while minimum similarity was seen between KB1MsC and KB4MsB, and KB1MsE and KB3MsA (46%). Differentiation of the monosporidial culture lines of *T. indica* could be achieved efficiently by using URPs (Aggarwal et al. 2011).

Characterization of isolates of *Colletotrichum lindemuthianum*, causing anthracnose disease of bean, was carried out. Based on the temperature effect on its biological characteristics, molecular diversity was revealed by RAM and ERIC-BOX PCR analysis. Isolates originating from certain locations showed better adaptation at the highest temperature (24°C) tested while some isolates showed similar better adaptation at lower temperatures (12 and 18°C), depending on their geographical origin in Greece. On the other hand, molecular diversity was detected using RAM and ERIC-BOX PCR primer. Both approaches revealed two main clusters of isolates which were in agreement with the results of RAPD and RFLP analyses. The majority of the tested isolates were grouped in the same main cluster, underlying high levels of genotype similarities between Greek populations of *C. lindemuthianum* (Bardas et al. 2009).

Fungal plant pathogens have been detected and identified based on their morphological characteristics and/or sequences of specific DNA segments, as in the case of *Phytophthora cinnamomi*. Amplification of pathogen DNA segments containing ITS1, 5.8S RNA gene, and ITS2 was performed by PCR, employing universal ITS primers 1 and 4. Amplified fragments were separated by agarose electrophoresis and purified using spin columns. After ligating to appropriate plasmid vector, the selected amplified fragments were sequenced. All isolates of *P. cinnamomi* (6) tested exhibited identical ITS region sequences. The isolates identified as *P. cinnamomi* showed all morphological characteristics attributed to this species (Polashock et al. 2005).

2.1.4.9 Phylogenetic Analysis

Different *Fusarium* spp. cause Fusarium head blight (FHB) disease in wheat and other small grains. Differences in virulence and production of chemotoxins and other characteristics of pathogen isolates/species used in breeding programs may influence the reactions of host genotypes, affecting the reliability of the results obtained. The genetic structure of 15-acetyldeoxynivalenol (15-ADON) chemotypes of *Fusarium graminearum* (teleomorph *Gibberella zeae*) populations collected from Canada was investigated using restriction fragment length polymorphism (RFLP) and intersimple sequence repeat (ISSR) molecular markers. The genetic analysis revealed that 15-ADON producing *F. graminearum* populations from different provinces of Canada are genetically homogeneous. Mismatch distribution analysis was used to examine the evolutionary factors, leading to genetic homogeneity among populations. The results of mismatch distributions showed evidence of a recent burst in population growth and demographic expansion across Canada, and suggested the need for vigilant monitoring of trends of population growth of *F. graminearum* for effective management of this devastating pathogen (Mishra et al. 2009).

In order to assess the differences among *Fusarium* isolates from different geographical regions, the phylogenetic and chemotypic characteristics and virulence of 58 isolates collected from wheat-producing countries such as Canada, Mexico, and Iran were investigated. Phylogenetic relationships among the isolates were characterized using the *Tri101* gene sequence. All Canadian and Iranian isolates clustered in a single group and were identified as *F. graminearum* lineage 7 (= *F. graminearum sensu stricto*) within the *F. graminearum* (Fg) clade. The isolates from Mexico were identified as either *F. graminearum* lineage 3 (= *F. boothi*) within Fg clade or *F. crookwellense*. A PCR assay based on the *Tri12* gene identified three trichothecene chemotypes of deoxynivalenol (15-ADON) type, 3-acetyldeoxynivalenol (3-ADON) and nivalenol (NIV), with 15-ADON being the most common chemotype. All *F. boothi* isolates from Mexico were of 15-ADON chemotype, while all *F. crookwellense* isolates were found to be NIV producers. None of the Canadian isolates produced NIV, while 25.6% of Iranian isolates were NIV producers. A high level of variation in virulence was observed among isolates of *F. graminearum*. They were more virulent than the other species of *Fusarium*, indicating that the variability in host genotype reactions could be due to the differences in the aggressiveness of isolates of FHB pathogens (Malihipour et al. 2012).

Fusarium head blight (FHB) disease is primarily caused by *F. graminearum* (Fg) species complex in China. At least 14 phylogenetically distinct species constitute the *Fusarium* species complex. *Fusarium* isolates (55) collected from wheat with FHB symptoms along the north of the Yangtze River were investigated for their phylogenetic relationships. An additional phylogenetic species of *F. graminearum* complex was identified for the first time in China as *F. meridionale*. In addition, *F. acuminatum*, distantly related to Fg complex, was also identified in the isolates from Northern China. Mycotoxin genotype analysis indicated that *F. asiaticum* isolates were potential producers of 3-ADON and NIV mycotoxin (Wang et al. 2010). In order to determine the population structure of pathogens involved in FHB disease in a naturally infected disease nursery in China, 160 isolates of *F. graminearum* complex were recovered from symptomatic wheat spike samples collected in 2008 and 2009. Isolates were characterized using species- and chemotype-specific PCR reaction and also variable number tandem repeat (VNTR) markers. All isolates were identified as *F. asiaticum*, except for one isolate which was identified as *F. avenaceum*. Among the 159 *F. asiaticum* isolates,

120 (79%) isolates were of the nivalenol (NIV) type, 29 (28%) isolates were of 15-ADON type, and only 4 (3%) isolates were of the 3-ADON type. By using VNTR markers, 124 distinct haplotypes and 76 polymorphic alleles across the whole population were identified. The results suggested that FHB in the nursery was caused by a genetically homogeneous and nonrandom mating population of *F. asiaticum* which consisted of all three chemotypes with different levels of virulence (Puri et al. 2012).

2.1.4.10 DNA Array Technology

DNA array technology was first developed to detect human genetic disorders. DNA arrays and chips are powerful tools for gene expression profiling, and it has the potential for application in the precise identification and differentiation of microorganisms including microbial plant pathogens. Common assay systems such as microplates or standard blotting membranes have been used in arrays which can be created by hand or by employing robotics to dispense samples. Two types of arrays have been commonly used. Macroarrays contain sample spot sizes of about 300 μm diameter or larger and can be easily imaged by available gel or blot scanners. The membrane-based arrays or filter arrays belong to this category. On the other hand, microarrays have spot sizes less than 200 μm in diameter and are able to accommodate thousands of spots. Specialized robotics and imaging equipment are necessary for successful operation for microarray system (Shi et al. 2003). When a single nucleotide is substituted, mismatches occur at a specific position of nucleotide sequence. Selectivity of a specific single nucleotide polymorphism (SNP) oligonucleotide depending on its sequence could be observed. High discriminatory potential associated with immobilized detector oligonucleotide has been shown to be crucial for diagnostic applications, since closely related pathogen species may differ in only a single base pair for a target gene. The SNP profiles of fungal pathogens have been used as the basis of detection of large number of pathogens simultaneously (Lievens and Thomma 2005).

The target pathogens *Verticillium dahliae* and *Fusarium* spp. were isolated in pure cultures. The perfect match oligonucleotides were selected from either ITS1 or ITS2 sequences from which the detector sequences with a melting temperature of $55 \pm 5^\circ\text{C}$ were obtained by adjusting the length of these oligonucleotides. Selectivity of a single SNP oligonucleotide, depending on its sequence, was observed in *V. dahliae*. The results indicated that high specificity could be achieved with DNA arrays that allow discrimination of single nucleotide sequences; closely related fungi could therefore be differentiated. It was suggested that multiple nucleotides harboring unique polymorphism at different positions may be employed to differentiate SNPs (Lievens et al. 2006). Robust SCAR markers were developed based on RAPD markers which could be used for specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* (FOC) and *F. oxysporum* f.sp. *radicis-cucumerinum* (FORC) in infected plant tissues. By employing these markers in a DNA macroarray, multiple pathogens belonging to the genus *Fusarium* and *Fusarium oxysporum* could be detected simultaneously with a high level of sensitivity. Three and two samples out of five samples were diagnosed with FORC and FOC, respectively. The identity of the pathogens established using DNA microarray was confirmed by isolating the pathogens on selective medium and examining their morphological characteristics (Lievens et al. 2007). *Fusarium solani* species complex (FSSC) contains morphologically similar, but more than 45 distinct, lineages. They were chosen as targets for the development of a macroarray detection system that could be adapted broadly. Oligonucleotides (17-27-mers) were designed from the ITS

of the rDNA genes of 17 FSSC isolates that belong to 12 phylogenetically closely related species. Of the 33 oligonucleotides on the array 21 were able to discriminate all 12 species, some of which differed by only a single nucleotide. High specificity of the array system was found to be due to optimization of the hybridization temperature and oligo probe length which had a more substantial effect on the array performance. The array was validated by testing samples from inoculated and field-infected plants (Zhang et al. 2007).

2.1.4.11 Matrix-Assisted Laser Desorption Ionization

Time-of-Flight Mass Spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) technique involves separation of small DNA molecules on the basis of their charge to mass ratio. The separation time is on the order of microseconds although several analyses are typically performed for each sample, extending the overall time to a couple of seconds. Small DNA molecules of less than 100bp and differing in size by a single base can be easily differentiated. The high-throughput capability of the technique, as up to 800 samples can be processed simultaneously and automatically, makes it attractive as an alternative diagnostic tool for detection and differentiation of microorganisms. The location of soilborne pathogens such as *Phytophthora cinnamomi* has to be mapped to prevent their spread to new areas. Precise mapping of pathogen presence involves the testing of a large number of soil samples, for which the application of MALDI-TOF MS procedure was shown to be effective. Appropriate sites for the design of genotyping primers for *P. cinnamomi* were located by visually scanning an alignment of the sequences of the ITS1 and ITS2 regions of *Phytophthora*. Two prominent peaks, one for the unextended primer and one for the extended primer, were seen. The extension product was seen only when *P. cinnamomi* was present. The peaks were clearly differentiated. A similar profile was obtained for DNA extracted from the mycelium–soil mixture, demonstrating that the profile was not altered by contaminants coextracted with DNA from soil. The results of MALDI-TOF-MS technique were shown to be reproducible and rapid and to have potential for wider application. The presence of PCR inhibitors in the soil samples tested did not affect the reactions (Siricord and O'Brien 2008).

2.1.4.12 Biochemical Methods

2.1.4.12.1 Detection of GUS Activity

Constitutive expression of β -glucuronidase (GUS) in the fungal pathogen transformed with GUS reporter gene has been successfully applied to establish the identity of the pathogen causing the disease under investigation. *Verticillium dahliae* infects a wide range of plant species, including oilseed rape (*Brassica napus* subsp. *oleifera*). *V. longisporum* was also associated with the wilt disease assumed to be caused by *V. dahliae*. In order to determine the true cause of the disease, the infection patterns of *V. dahliae* and *V. longisporum* on the roots of *B. napus* were studied. These two fungi were labeled with green fluorescent protein (GFP) from the jelly fish (*Aequorea victoria*). Colonization of the root tissues by *Verticillium* spp. was visualized by confocal laser scanning microscopy (CLSM). GFP was stably expressed in the fungi, following *Agrobacterium tumefaciens* (At-) mediated transformation. *V. longisporum* was able to readily spread into the vascular system. In contrast, the systemic growth of *V. dahliae* was strongly inhibited and the hyphae were loosely attached to the root surface only. The results clearly

revealed the pathogenic nature of *V. longisporum* to oilseed rape and the inability of *V. dahliae* to infect this host, establishing that the earlier belief of *V. dahliae* being pathogenic to oilseed rape was incorrect (Eynck et al. 2007).

2.1.4.12.2 Isozyme Analysis

By separating the proteins extracted from the target fungal pathogen, protein profiles may be obtained by using electrophoresis techniques. Electrophoretic techniques are employed to separate amino acids and sometimes to purify macromolecules such as proteins or nucleic acids that differ in size, charge, or conformation. Agarose or polyacrylamide gels are commonly used. Isozymes may be defined as multiple molecular forms of a single enzyme and these forms have similar enzymatic properties, but have slightly different amino acid sequences. The usefulness of isozyme electrophoresis for detection and identification of fungal pathogens has been demonstrated. Isozyme analysis is a powerful biochemical technique that is useful for detection, identification, and differentiation of morphologically similar or closely related species, varieties of *formae speciales*.

Downy mildews of corn crops are caused by three species of *Peronosclerospora*: *P. sorghi*, *P. sacchari*, and *P. sacchari-philippinensis*. Identification of these pathogens based on morphological characteristics is difficult, since extensive morphological variations exist within the same species. Isozymes from ten fungal cultures representing *Peronosclerospora* spp. were compared electrophoretically on starch gels for species identification. None of the enzymes (11) examined was influenced by host species on which the pathogen species was grown, the time of the year of collections of conidia, or growth conditions (greenhouse or growth chamber). Isozyme analysis was efficient in differentiating three species of *Peronosclerospora* (Bonde et al. 1984). The teliospores of *Tilletia indica* and *T. barclayana* present in stored wheat grains in storage facilities could be distinguished based on isozyme patterns (Bonde et al. 1989). Variations in the level of virulence may be used as the basis for identification and differentiation of strains of fungal pathogens. Isozyme analysis was applied to differentiate the isolates of *Fusarium oxysporum*. Polymorphisms in five enzymes were detected by electrophoresis technique. Twenty six electrophoretic groups were identified (Paavanen-Huhtala et al. 1999).

Cellulose acetate electrophoresis (CAE) technique was used to detect isozyme polymorphism among various isolates of closely related *Fusarium cerealis*, *F. culmorum*, *F. graminearum*, and *F. pseudograminearum* occurring in various countries across the world. The electrophoretic types (ETs) of adenylate kinase (AK), NADP-dependent glutamate dehydrogenase (NADPGDH), peptidase B (PEPB), peptidase D (PEPD), and phosphoglucosyltransferase (PGMT) were compared. PEPD alone was useful as a marker for identification and differentiation of the four taxa studied, revealing its potential for use as a rapid and simple CAE-based diagnostic tool. Irrespective of geographical origin, *Fusarium* spp. showed uniformly similar isozyme pattern for all isolates tested. Based on the similarity values, *F. graminearum* was considered to be more closely related to *F. cerealis* and *F. culmorum* than to *F. pseudograminearum*, in contrast to the conclusion arrived at based on similarity of morphologic characteristics of these taxa. Morphological similarity did not seem to reflect the genetic relatedness of *Fusarium* spp. investigated (Láday and Szécsi 2001).

Plasmopara halstedii, causative agent of sunflower downy mildew disease, has a relatively broad host range of about 35 plant species. *P. halstedii*, a homothallic species,

has frequent sexual cycles and it is likely that many new recombinants are produced over a period of time. In order to elucidate local population structure and to monitor changes over time, isozyme analysis has been employed to resolve certain taxonomic problems. Cellulose-acetate gel electrophoresis (CAE) technique was applied to analyze the properties of isozymes extracted from sporangia and sporangiophores of *P. halstedii* and to determine the genetic diversity of the isolates of this pathogen. Isolates (45) from sunflower, cocklebur, and *Helianthus x laetiflorus* comprising 10 field isolates and 35 single-spore lines of an additional 30 field isolates, representing 10 different virulent phenotypes, were analyzed. This technique required only minute amounts of material (31–100 µg sample/reaction). Sixteen isozyme systems were analyzed, of which three (isocitric dehydrogenase, malate dehydrogenase, and phosphoglucumutase) provided clear and reproducible banding patterns and revealed certain polymorphisms among the isolates (Table 2.5). Phosphoglucumutase differentiated two groups among the isolates collected from cultivated sunflower. The difference between the samples seemed to be associated with host origin rather than geographic location or virulence. All tested isolates were homozygous for the isozyme detected, regardless of their origin, suggesting that all pathogen populations were rather homozygous. The majority of the isolates (84%) from Europe and the USA belonged to MLP1 (Komjáti et al. 2008).

The biodiversity and phylogeny of *Fusarium graminearum* isolates of different geographical origins were analyzed using isozyme variation, β -tubulin, and IGS sequences. Geographically distinct groups of isolates originating from different locations in the Russia Federation, China, Germany, and Finland were investigated. The complex enzymes, α - and β -esterases, aspirated aminotransferase, and superoxide dismutase and the IGS sequences showed a high level of genetic diversity. Diversity among Asian isolates was greater than in European isolates. Correlation between genetic clusters of

Table 2.5 Variations in in vitro growth, in vitro levels of DON and zearalenone produced, and disease rating of four typed *Fusarium* isolates compared to the *F. graminearum* isolates collected in North Carolina (Walker et al. 2001).

Variables	Isolates of typed <i>Fusarium</i>				Mean
	FA5314 ^e	FC6565 ^f	FG6914 ^g	FG6925 ^h	
In vitro growth (mm/day)	3.4	3.7	7.2	5.1	4.7
DON 27-4-27°C (ppm) ^a	401	1341	952	412	64.1
DON 27°C (ppm) ^b	734	1987	4087	643	736
Zearalenone 27-4-27°C (ppm) ^c	5.6	0.2	10	1.7	26
Zearalenone 27°C (ppm) ^d	33	0	0	10	12
Disease rating (mean percent area)	13	76	69	64	51

^a In vitro DON cultures incubated at 27°C for 7 days; ^b In vitro DON cultures incubated at 27°C for 30 days;

^c In vitro zearalenone cultures incubated at 27°C for 7 days; ^d In vitro zearalenone cultures incubated at 27°C for 30 days; ^e *F. avenaceum*; ^f *F. culmorum*; ^g *F. graminearum* and ^h *F. graminearum* isolates.

IGS sequences and geographical origin of isolates of *F. graminearum* was observed. Assessment of genetic diversity and identification of phylogenetic lineages in *F. graminearum* could be useful for defining the risk of pathogen evolution, as well as development of disease management strategies (Gagkaeva and Yli-Mattila 2004). Variability in the isolates of *Fusarium avenaceum* (91) was investigated using one-dimensional sodium dodecylsulfate (SDS-) polyacrylamide gel electrophoresis (PAGE) and ITS rDNA profiles. Each isolate possessed reproducible signature protein bands. The unweighted pair group method with arithmetic averages clustering revealed that the protein profile of each group of isolates correlated with isolate virulence. SDS-PAGE technique was found to be a simple and sensitive technique for routine differentiation between pathogenic and nonpathogenic isolates of *F. avenaceum* populations (Vujanovic et al. 2009).

The seedborne nature of *Leptosphaeria maculans* (*Phoma lingam*) in crucifer seed was reported by McGuire and Gabrielson (1983). The difference in the pathogenic potential (virulence) could be the basis for identification of strains of *L. maculans*. Of these strains, 68 strains contained an isozyme of glucose phosphate isomerase (GPI) which migrated faster in starch gel (70mm) than the isozyme of GPI present in other isolates (24) that could move only a shorter distance (65mm) in 11 h. These two groups of isolates clustered into electrophoretic types (ETs) ET1 or ET2, reflecting fast or slow migration of GPI on starch gel, respectively. Highly virulent strains (ET1) had fast-moving GPI, whereas weakly pathogenic strains (ET2) had slowly migrating GPI (Sippell and Hall 1995).

A reliable procedure for identification of *L. maculans* based on electrophoretic movement of its GPI on starch gels was developed. Extracts of infected tissues of *Brassica napus* were directly subjected to electrophoretic analysis. Four different ET patterns (ET1, ET2, ET3, and ET4) of allozymes were identified and these patterns correlated well with virulence of isolates of *L. maculans*. Group A isolates with high virulence demonstrated the ET1 pattern, whereas group B isolates with weak virulence exhibited the ET2 pattern. The isolates exhibiting the ET3 pattern induced a few atypical and typical lesions. Another pathogen *Pseudocercospora capsellae* of *B. napus* produced the ET4 pattern. The allozyme produced by *P. capsellae* was the fastest, allowing the differentiation of these two pathogens based on ET patterns of allozymes produced by them or their isolates (Braun et al. 1997).

2.1.4.13 Assessment of Variations in Biological Characteristics of Fungal Pathogens

Seedborne fungal pathogens show variations in their virulence and production of toxic metabolites which may induce different types of effects on the susceptible host cultivars or genotypes. A set of host genotypes is used to differentiate the level of virulence when the pathogen isolates are inoculated on them. The level of pathogenic diversity of the isolates of *Fusarium pseudograminearum* and *F. graminearum* occurring in Australia was determined by inoculating them on 51 wheat germplasm lines, barley, triticale rye, corn, and sorghum plants. A set of nine putative wheat differentials were selected and their differential reactions were assessed by inoculating 10 and 12 isolates of *F. graminearum* and *F. pseudograminearum*, respectively. The isolates differed largely in a quantitative manner with only small differential effects, and they were statistically classified into three pathogenic groups as low, intermediate, and high. The results suggested that different resistance mechanisms might operate against each group of isolates and that three pathogenicity groups might possess different mechanisms controlling the level of

their virulence (pathogenic potential). In addition, the pathogen variability should be considered as an important factor for screening, selection of genotypes, and improvement of resistance to *Fusarium* head blight disease of wheat (Akinsanmi et al. 2006). *Fusarium* head blight (FHB) is caused by different *Fusarium* spp. individually or in combination. The possibility of within-field variability in *Fusarium* spp. was investigated. Each of FHB pathogens (*F. graminearum*, *F. culmorum*, and *F. poae*) and associated mycotoxins were quantified at each of 14 sites in 16 quadret samples. Detectable and quantifiable pathogen DNA was significantly lower in the grain than in the corresponding chaff. Deoxynivalenol was the most frequently detected toxin in the samples and its accumulation was most strongly associated with the presence of *F. culmorum* only. On the other hand, zearalenone (ZON) was strongly associated with the presence of all three *Fusarium* spp. The levels of both DON and ZON were positively related to the amount of *F. graminearum* DNA in the grain or in the chaff. The presence of a particular FHB pathogen and the amount of its DNA, as well as the associated mycotoxin(s), varied greatly among samples at each site. Within-field variability of FHB pathogen and the presence of mycotoxins produced by them have to be taken into consideration while sampling FHB-affected plants (Xu et al. 2008).

Isolates of *Stagonospora nodorum*, causal agent of *Stagonospora nodorum* blotch (SNB) disease of wheat, were evaluated for their pathogenic variability for developing cultivars with durable resistance. Variations in virulence (pathogenic potential) of 40 isolates of *S. nodorum* collected from various locations in North Dakota, USA were assessed. The isolates were inoculated on two susceptible and two resistant wheat cultivars at two-leaf stage of seedlings under controlled conditions. The virulence (aggressiveness) was characterized using two epidemiological parameters: percent necrotic leaf area (% NLA), and lesion type (LT) at 8 days after inoculation. The isolates were classified into three aggressiveness groups (AGs): low, medium, and highly virulent. Three isolates were highly virulent and could be utilized in breeding programs for the development of wheat cultivars with resistance to SNB disease (Ali and Adhikari 2008). *Rhizoctonia solani* infects several crops including peanut, cotton, and rice. It could be isolated from the roots only with some difficulty. A quantitative assay of active hyphae by using wooden toothpicks as baits was developed. The toothpicks were inserted into the soil samples collected around infected plants. After 2 days, they were placed on a selective medium. The number of colonies developing after 24 days were examined and counted under a dissecting microscope. *R. solani* could be differentiated from other fungi based on hyphal morphology. This simple and inexpensive method has the potential for use in locations where expensive molecular techniques cannot be employed (Paulitz and Schroeder 2005).

Pyrenophora teres f. *teres*, incitant of barley net form blotch, has been reported to show variations in virulence determined using a range of barley differential lines. Isolates of *P. teres* f. *teres* (91) collected in California were classified into 13 pathotypes. Isolates from Minnesota, Mexico, and England were identified as distinctly different pathotypes, with Mexican pathotypes showing maximum similarity to the Californian pathotypes (Steffenson and Webster 1992). Considerable difficulty in comparing pathotypes identified in different studies has been observed because of the use of different sets or numbers of barley differential lines by individual researchers. A population of *P. teres* f. *teres* present in North Dakota was analyzed for variation in virulence and genetic diversity, using 75 monospore isolates collected over a period of 4 years (2004 to 2007).

Pathogenicity assessment, using 22 barley differential lines at seedling stage, showed the presence of 49 pathotypes differing in their pathogenic potential. Two-way analysis of variance of disease scores indicated a significant difference in the virulence among isolates and in the level of resistance among barley lines, as well as in the interactions between the two. Forty multilocus genotypes were identified from this set of isolates by genotyping at 13 simple sequence repeat loci. Three barley lines (C15791, Heartland and Algerian) were highly resistant at the seedling stage to all isolates of North Dakota and other reference isolates, indicating they are potential sources of seedling resistance to *P. teres f. teres* (Liu et al. 2012).

Tan spot disease of wheat is caused by *Pyrenophora tritici-repentis* which exists in the form of several races, differing in their pathogenic potential (virulence). Eight races of the pathogen were identified based on their virulence on a set of wheat differential varieties. Most isolates collected from Canadian Prairies belong to races 1 and 2 of *P. tritici-repentis* and carry the *ToxA* gene. Pathogenic potential of 45 isolates of the pathogen from Alberta, a major wheat-producing province of Canada, was assessed using six differential wheat genotypes. Race 1 was more common (62%) than race 2 (36%). PCR assay and Southern blot analysis were applied for genetic characterization of the isolates using *ToxA*- and *ToxB*-specific primers and probes. *ToxA* gene was identified in isolates classified as race 1 or race 2, but not in race 3. Sequencing of *ToxA* gene from 16 isolates showed that the sequences were identical. The *ToxB* gene was not detected in any of the isolates classified as race 1 or race 2. On the other hand, race 3 isolates contained the *ToxB* homologue. The results indicated that *P. tritici-repentis* populations in Alberta followed the toxin or inverse gene-for-gene model and served to validate the race-based classification system for *P. tritici-repentis* (Aboukhaddour et al. 2013).

2.2 Detection and Differentiation of Fungal Pathogens in Propagules

Crops are cultivated using seeds, resultant of sexual reproduction, and propagules such as tubers, corms, bulbs, cuttings, or setts obtained after asexual propagation. Detection, identification, and differentiation of microbial plant pathogens present in seeds using various techniques were discussed in Section 2.1. As for true seeds, asexually (vegetatively) propagative materials also carry microbial pathogens, the infection of which may or may not result in expression of visual symptoms. The inoculum of pathogen(s) may be present in the aerial and underground plant parts of the same plant, soil, irrigation water, or air. Infected propagules are potential carriers of the pathogens over short or long distances, and also to subsequent generations if the health status of the propagules is not assessed carefully to eliminate those which are infected. When the infected tubers or corms are stored the pathogens multiply to high levels, resulting in the deterioration of their viability, quality, and market value. When infected propagules are used for raising the crops, the quantitative and qualitative losses in later generations become greater progressively if no selection for disease-free propagules is made. Use of disease-free planting materials is the primary strategy recommended for the management of diseases affecting crops such as potato, banana, onion, strawberry, sugarcane, and cassava. It is advisable and also mandatory in some crops to use certified propagules to ensure freedom from designated diseases that may ruin the crops due to their destructive nature (Narayanasamy 2011).

2.2.1 Conventional/Isolation-Dependent Methods

Asexually propagated plant materials are obtained from obviously healthy mother plants. Visual examination for the presence of disease symptoms alone may not be sufficient for selection of healthy planting materials; the symptoms may not be clearly seen if infection occurs during the late growing stages. Further, in some cases the fungal pathogens cause latent/quiescent infections or induce indistinct symptoms. In such cases, the pathogens have to be isolated on appropriate media and their morphologic characteristics have to be studied for correct identification. As in the case of seedborne fungal pathogens, some may be slow growing and it may be difficult to isolate them on media generally used. Semiselective or selective media that differentially favor the growth of the target pathogen(s) have to be employed to isolate them in pure culture. Isolation of the pathogen has to be attempted by taking appropriate plant tissues. *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* cause Petri disease in grapevines. Dark streaks were more commonly seen in the rootstock, whereas necrosis originating from buds was conspicuously noted in the trunk and cordon of older vines. The highest levels of brown-red halo symptoms (discolored areas around the pith) were seen in the early growth phase. *Phaeoacremonium* spp. and *P. chlamydospora* were usually isolated from the rooted grafts and 3 year old plants, respectively. *P. chlamydospora* strains were isolated mostly from dark streaks or dots, whereas *Phaeoacremonium* spp. were detected in tissues showing brown-red halo symptoms and other symptomatic or asymptomatic woody tissues. The highest incidence of these two pathogens was recorded in the lower part of the grapevine including the roots and rootstock (Zanzotto et al. 2007).

Phaeomoniella chlamydospora associated with grapevine Esca disease could be isolated from apparently healthy rootstock mother plants. The colonization of plant tissues by fungal pathogens can be visualized by using green fluorescent protein (GFP), which allows the progress of infection and formation of fungal structures in invaded plant tissues to be tracked. The green fluorescent protein (*gfp*) gene from the jelly fish *Aequorea victoria* encoding GFP has been widely used as a reporter gene in several organisms. The *gfp* gene has been useful for monitoring gene expression and protein localization by employing it as labels to visualize specific tissues in cells or subcellular organelles. *P. chlamydospora* isolate CBS229.95 was transformed using a pCT74 construct which contained the genetic markers for synthetic green fluorescent protein (sGFP) and hygromycin β -phosphotransferase. Nine stable *P. chlamydospora* transformed fungal lines (*Pch-sGFP* lines) were generated using polyethyleneglycol-mediated transformation of protoplasts. Grapevine cuttings of five cultivars were inoculated by immersion in a conidial suspension of selected transformed fungal lines. The highest extent of wood colonization by *P. chlamydospora* labeled with sGFP was recorded in Montepulciano and Verdicchiocultivars. The expression of the *Pch-sGFP* transformed line was localized in the xylem area, primarily around the vessels. The system allowed detection of *P. chlamydospora* plant colonization before the appearance of black streaking, the early visual symptom (Landi et al. 2012).

Verticillium dahliae causes Verticillium wilt disease of potato, which is a monocyclic and polyetic disease. The primary inoculum consists of microsclerotia that can persist in the soil for long periods. In addition, the pathogen can also survive in the infected tubers. Intratuber inoculum of *V. dahliae* is present in the vascular system of certified seed tubers. Low levels of *V. dahliae* (5% incidence) were detected in 20–30% of seed lots imported from northern Europe to Israel (Tsrer et al. 1999). Wilt severity resulting

from intratuber infection may vary among cultivars. The contribution of seed tuber infection to soilborne inoculum was assessed, using naturally infected Russet Burbank seed potato tubers from commercial production fields. A 15 mm round disk which included vascular tissue was cut from stem end of tuber and plated onto NP-10 medium. Plates were incubated at 23°C for 14 days and the development of *V. dahliae* colonies was observed. The pathogen was isolated from the vascular system of 94% plants grown from infested soils and 8% of plants grown from infected tubers in noninfested soil. Infected progeny tubers were recovered from plants grown from infested soil. The results suggested that tuber infection did not contribute to premature senescence of plants or potential inoculum production (Dung et al. 2012).

The population diversity of *Phytophthora infestans*, causing late blight disease of potato and tomato in Canada, was assessed. Novel genotypes of *P. infestans* emerged after 2009, although the US-8 genotype occurred predominantly. New US-23 and US-24 genotypes had become established in locations where they were not detected earlier. The US-11 and US-22 genotypes dominated the populations of the pathogen in certain areas of Alberta and Ontario, respectively. Clonal propagation and migration contributed to long-distance transport of *P. infestans*. In some situations, both A1 and A2 mating types of *P. infestans* were present in the same production region. This situation might increase the potential for sexual recombination within pathogen populations, leading to production of oospores that could survive in the absence of host tissue and create new strains of *P. infestans* on germination. Evidence for recombination in the pathogen populations was detected in Ontario on tomato. Segregation of *Gpi* allozymes and mating type appeared to occur independently of the RG57 loci, producing several new *P. infestans* genotypes. This highlights the need to accurately predict and effectively manage the newly emerging potentially aggressive genotypes of *P. infestans* (Peters et al. 2014).

2.2.2 Isolation-Independent Methods

2.2.2.1 Immunoassays

Soilborne fungal pathogens are able to invade propagative plant materials, facilitating their short or long-distance dispersal through infected materials. In the absence of any other modes of transmission, soilborne pathogens have only a limited rate of dispersal. Detection and identification of fungal pathogens by rapid and sensitive method(s) has become vital to prevent the rejection of consignments by quarantine regulations. Among the immunoassays, enzyme-linked immunosorbent assay (ELISA) formats have been applied more commonly. Polyclonal antibodies (PABs) generated against purified mycelial proteins of *Verticillium dahliae* infecting potato tubers were successfully employed in ELISA test. The pathogen was detected in infected seed tubers (Sundaram et al. 1991). A specific protein of pathogen origin may be employed for detection of the target pathogen. A polyclonal antiserum was produced against a 101 kDa polypeptide present in *Colletotrichum falcatum*, causative agent of sugarcane red rot disease. The PAB generated against the pathogen protein could be used for the detection of *C. falcatum* in the root eyes, buds, leaf scar, and pith region in the stalks that provided planting materials (setts) for the next crop (Viswanathan et al. 1998).

Two members of the genus *Spongospora* – *S. subterranea* f.sp. *subterranea* (*Sss*) and *S. subterranea* f.sp. *nasturtii* (*Ssn*) – are economically important pathogens of vegetables. The close taxonomic relationship of these pathogens is due to their similar cystosori morphology. Potato powdery scab caused by *Sss* could be managed primarily by planting

clean seed tubers in clean soil to reduce the disease incidence. The development of routine tests for detection of *Sss* in tubers was considered essential. The presence of *Sss* was detected in tuber extract by employing the PABs raised against the homogenates of spore balls (cystosori) in ELISA tests which had a detection limit of 0.8 spore balls equivalent per milliliter (Harrison et al. 1993). A monoclonal antibody (MAB) was generated using *Sss* cystosori as immunogen to improve the sensitivity of detection of *Sss*. The detection limit of the MAB was less than one cystosorus of *Sss* and it recognized *Sss*-infected material obtained from different countries in the world. There was no cross-reaction between the MAB and other Plasmodiophoromycetes, including *Plasmodiophora brassicae*, *Polymyxa betae*, *Polymyxa graminis*, and different *Streptomyces* spp. causing common and netted scab diseases of potatoes, respectively. A novel tuber sample test using a kitchen peeling machine was developed. The presence of *Sss* was detected in two tubers with one powdery scab lesion each in a sample containing 18 uninfected tubers. Likewise, *Sss* could be detected in soil samples spiked with different concentrations of cystosori using the MAB in ELISA tests. The absorbance values were proportional to *Sss* infestation levels. ELISA was shown to have the potential for determining the freedom of tubers and also for assessing *Sss* infestation of potato tubers (Merz et al. 2005).

In a later study, the “*Sss* AgriStrip”, a rapid and laboratory-dependent technique, was developed to overcome the problem of possible misidentification due to the difficulty of distinguishing lesions caused by *Spongospora subterranea* f.sp. *subterranea* (*Sss*) and *Streptomyces* spp. by visual examination. The *Sss* AgriStrip test was based on a lateral flow immunoassay. The accuracy and sensitivity of *Sss* AgriStrip test was compared with microscopic examination, ELISA, polymerase chain reaction (PCR), and real-time PCR assays. All techniques detected infection with *Sss* in all tubers with typical powdery scab lesions. When seed tuber lots with typical and atypical (doubtful) symptoms were tested, the presence of *Sss* was detected in all lesions by all methods. Diverse and doubtful symptoms which were difficult to differentiate from powdery or common scab tested negative with *Sss* AgriStrip and other methods, despite the microscopic examinations indicating the presence of sporosori-like structures in some sample. Isolations and molecular identification confirmed that those lesions were mostly due to *Streptomyces* spp. The *Sss* AgriStrip was as sensitive as DAS-ELISA test with a detection limit of 1–10 sporosori mL⁻¹ of buffer. *Sss* AgriStrip was found to be a rapid and selective detection tool on farms and border inspection points to prevent spread of the pathogen (Bouchek-Mechiche et al. 2011).

An indirect ELISA format was employed for the detection of *Phytophthora infestans*, causing potato late blight disease prior to symptom expression. *P. infestans* could be detected in potato shoots of 5–9 week old plants at about 39 days before disease outbreak under field conditions. However, there was no correlation between the results of ELISA and symptom development in single plants under field conditions. This could be due to infection of plants by routes other than tuber infection, such as infection by zoospores transported in soil water following heavy rainfall (Schlenzig et al. 1999).

In another investigation, mouse MABs (phyt/G147P) raised against *P. infestans* were used to determine the comparative efficacies of plate-trapped antigen (PTA-) ELISA and subtractive inhibition ELISA formats. The assays were specific to *P. infestans*, showing no or only limited cross-reactivity against other fungi tested. The MAB phyt/G1470 was incorporated in a subtractive inhibition surface plasmon resonance (SPR) immunosensor for detection of sporangia of *P. infestans*. The SPR assay involved preincubation of MAB and sporangia and a centrifugation step to remove sporangia,

requiring 75 min to obtain results. This assay seems to be more sensitive and rapid in providing the results when compared with other immunoassays (Skottrup et al. 2007).

Black Sigatoka disease caused by *Mycosphaerella fijiensis* was introduced into America and remains one of the most devastating diseases of banana. Presymptomatic quantification of fungal pathogen proteins in leaves was desirable to optimize fungicide applications. A mouse monoclonal antibody (MAb) reactive to a high-molecular-weight antigen from *M. fijiensis* mycelia of single ascospore in vitro culture was generated. This MAb did not have any cross-reaction with mycelial antigens from *M. musicola*, *M. musae*, and *M. minima* single ascospore cultures. The rabbit-specific polyclonal antibody (PAb) against *M. fijiensis* antigen, also reactive to fungus-secreted proteins, was able to detect *M. fijiensis* in naturally infected banana leaves, but it also reacted with other contaminant fungi. The MAb was employed as a capturing antibody and the PAb as a secondary antibody in triple-antibody sandwich (TAS) -ELISA test. Mycelial protein antigens could be quantified in a range of 10–40 $\mu\text{g mL}^{-1}$ and it was possible to differentiate it from healthy banana leaf proteins. The results indicated that immunoassays might be useful for quantification of the pathogen population in presymptomatic banana plants, facilitating development of an improved forecast and warning system for the management of banana black Sigatoka disease (Otero et al. 2007).

Colletotrichum acutatum infecting strawberry is an important quarantine pathogen to be intercepted in order to prevent its spread through infected plant materials. Both PABs and MABs were raised for detecting the pathogen in different plant parts such as roots, crowns, petioles, and fruits. Four types of assays – plate-trapped antibody (PTA-) ELISA, dot-blot, immunoprint, and immunofluorescence microscopy – were employed to test the specificity and sensitivities of antibodies produced. *C. acutatum* was detected by PTA-ELISA format in roots and crowns of all strawberry cultivars tested at 7 days after inoculation, when no visible symptom of infection appeared. However, the pathogen was detectable in the petioles of only one cultivar (Elsanta) (Krátká et al. 2002).

2.2.2.2 Nucleic Acid-Based Techniques

As the nature of nucleic acids of fungal pathogens is not influenced by the presence or absence of sexual spores and stages of life cycle, nucleic acid-based techniques have been preferred for the detection, identification, and differentiation of fungal pathogens present in the propagules. Further, the nucleic acid-based techniques have been shown to be more sensitive, specific, rapid, and reliable compared with other methods. These methods are therefore preferred although they are more expensive and cannot discriminate between the living and dead propagules of microbial pathogens. The gold standard procedure for identification of *Synchytrium endobioticum*, causing wart disease of potato tubers, involved visual examination by highly trained personnel. In order to detect and identify *S. endobioticum* in potato tubers reliably and efficiently, a nucleic acid hybridization technique was developed using a peptide nucleic acid (PNA) probe and a cyanine dye for color development. A segment of the 18S ribosomal RNA gene was sequenced for several *S. endobioticum* pathotypes and used to design a species-specific PNA probe. This colorimetric hybridization assay detected RNA from infected wart tissue down to 10^{-17} mol rapidly within 15 min. The protocol developed in this investigation could be employed for rapid identification of potato wart infection in nonlaboratory settings by minimally trained personnel (Duy et al. 2015).

Phytophthora infestans, incitant of the destructive potato late blight disease, could be detected by a polymerase chain reaction (PCR) -based assay. Primers designed from the

sequence of the ITS region specific to *P. infestans* were employed for detection of the pathogen in infected field samples (Trout et al. 1997). In a later investigation, potato tubers were inoculated with *P. infestans* by a reliable technique as a general basic method for greenhouse and field trials to study the development of the primary inoculum source in potato crops. The pathogen was detected in light sprouts and stem at different growth stages by employing PCR assay. The biomass of the pathogen was below the detection limit in some of the assayed stems during the course of plant growth, probably due to dilution effects. This suggested that *P. infestans* was able to grow directly up to the stem from the mycelium developed from the infected tubers, since symptoms were observed in due course. Apparently, the pathogen was able to induce symptoms in plants even at concentrations that were below the detection limit of PCR assay (Appel et al. 2001). In another study, a rapid method of detection of *P. infestans* in potato tubers was developed based on three oligonucleotide primers designed from the ITS sequences of the pathogen. The primer pair PiS-1/Pi12A-2 was employed to detect *P. infestans* in potato tuber lesions, the margin around the lesions, or the symptomless areas at various distances from tuber lesions in inoculated tubers. The primer pair generated and amplified products of the expected size (550 bp) from various tissues tested. This primer set amplified this unique DNA fragment only from *P. infestans* DNA and potato tubers infected by *P. infestans*, indicating the specificity of the assay (Jyan et al. 2002).

Phytophthora infestans is frequently found along with *P. erythroseptica* (causal agent of pink rot disease) in infected potato tubers. Both pathogens could be detected in tubers as early as 72 h after inoculation by employing primers specific to *P. infestans* or *P. erythroseptica*. Based on the unique sequences of their DNA, the primers were designed for their specific detection well in advance of development of disease symptoms on tubers (Tooley et al. 1998). In a later investigation, a primer pair specific to *P. infestans* based on sequences of ITS region amplified a 613 bp product and was used in a single-round PCR assay which detected 0.5 pg of pure pathogen DNA. This assay could detect *P. infestans* not only in potato tubers, but also in the extracts of infected leaf and stem tissues. The nested PCR format showed potential for its use for practical application for development of pathogen-free seed tubers and detection of latent infection in potato plants (Hussain et al. 2005). Skin spot disease caused by *Polyscytalum pustulans* adversely affected the market value of potato tubers. Development of a rapid and efficient method of detecting infection of tubers was therefore considered essential, since the existing procedures lacked the requirements of specificity and rapidity. A real-time PCR assay was developed for detection and quantification of *P. pustulans* in tubers. A single PCR product of size 1065 bp and sequences ITS1/ITS2 and 5.8S rDNA was amplified with universal primers ITS4/ITS5 from DNA of both *P. pustulans* isolates Pp3 and Pp14 tested. A set of real-time PCR primers PpusF1/PpusR2 and a TaqMan probe PpustPp1 was subsequently designed for unique sequences within the ITS1 regions of Pp3 and Pp14 isolates. DNA extracts of all isolates of *P. pustulans* from pure cultures produced a signal in real-time PCR assay. Likewise, DNA extracted from dried tuber peelings infected by *P. pustulans* also amplified a similar product. DNA extracted from nontarget pathogens infecting potato tubers did not produce the signal as *P. pustulans*, indicating the specificity of the assay. The procedure developed in this investigation has revealed its potential for practical applications, since it could detect *P. pustulans* in asymptomatic tubers at very low pathogen population (attogram of DNA; Lees et al. 2009).

Potato powdery scab disease pathogen, *Spongospora subterranea* f.sp. *subterranea* (Sss), is a soilborne biotrophic fungus and remains alive in the soil for several years. It is

necessary to determine the extent of tuber infection and soil infestation by the pathogen to prevent tuber infection and subsequent carryover of the disease through infected seed tubers. The presence of masses of resting spores aggregating to form sporeballs on the potato tubers is due to infection by *Sss*. Quality and marketability of infected tubers are seriously affected by the disease. Detection of *Sss* in the tubers rapidly may be helpful in eliminating the infected tubers. Primer pairs Spo8/9 and Sps1/2 were designed from the ITS sequences of the pathogen DNA and both primers were specific in amplifying the expected PCR product, facilitating its identification (Bulman and Marshall 1988). In a later investigation, *S. subterranea* was detected in potato peel and tuber washings by employing specific primers (Sps1 and Sps2) based on the sequences of the ITS regions of rDNA of the target pathogen. These primers specifically amplified a 39bp product only from *S. subterranea* and not from other fungi associated with potato tubers, indicating the specificity of the PCR assay. Detection threshold of the assay was DNA equivalent to 2.5×10^6 cystosori or one zoospore per PCR. The PCR assay developed in this investigation might be useful for disease risk assessments of potato tuber stocks (Bell et al. 1999). A semi-quantitative and sensitive real-time PCR assay was employed for the detection of *Spongospora subterranea* f.sp. *subterranea* (*Sss*) in potato tubers (van der Graaf et al. 2003).

In another study, a method to detect *Sss* directly in potato tubers was developed by combining a fast two-step automated approach to DNA extraction with a sensitive TaqMan® PCR assay for rapidly processing the samples. Based on the sequences of ITS region of the pathogen, real-time primers and probes were designed. The assay was pathogen-specific and this protocol was effective for detecting *Sss* directly on a range of potato cultivars from different locations in the UK. Furthermore, the TaqMan® PCR assay was able to detect *Sss* in the tubers at more stages in its life cycle and not just at the cystosori stage, as in the case of enzyme-linked immunosorbent assay (ELISA) test. The TaqMan® PCR assay had a higher level of sensitivity (100 fold) than that of the ELISA test or conventional PCR format (Ward et al. 2004). Specific identification and quantification of *Spongospora subterranea* f.sp. *subterranea* (*Sss*) in the potato tubers could be achieved by employing a PCR assay and primers SsF and SsR designed from the ITS region of the pathogen. The primers amplified a 434bp product from the DNA extracted from the sporeballs of *Sss* but not from DNA of healthy potato, common scab pathogen, and other taxonomically related Plasmodiophorales. *Sss* could be detected using the PCR assay in naturally infected symptomatic and asymptomatic tubers, as well as in samples of other symptomless host plants such as tomato and tobacco. The PCR procedure developed in this study has the potential for application on wider scale (Qu et al. 2006). The presence of latent infection varied over the range 27–80%, indicating the need for using disease-free seed tubers to keep the disease incidence under bay (Lees et al. 2008).

Spongospora subterranea f.sp. *subterranea* (*Sss*) present in the soil could be detected and quantified by employing a PCR-based assay, using the primers SsF and SsR designed from the ITS regions of the pathogen DNA. The PCR format was modified with improved soil DNA extraction methods to detect *Sss* in the soil. DNA was directly extracted from aqueous extract of field soil samples and noninfested field soil samples, using a Bead-beating/CTAB method and also using the UltraClean™ Soil DNA Kit. Following PCR amplification, *Sss* was detected in the infested soil samples, but not in noninfested soil sample. A competitive PCR was employed to quantify *S. subterranea* in naturally infested soil samples. The ratios of *S. subterranea* product of 434bp and

competitor product of 541 bp were determined for each reaction. The amount of amplicons (434 bp) of pathogen origin increased with increasing number of sporeballs of *Sss* in the reaction mixture. The competitor product (541 bp) showed a negative relationship. The levels of sporeballs in the soil samples determined by competitive PCR assay showed positive correlations with the extent of powdery scab disease incidence. The PCR formats have the potential for routine testing to detect and quantify *Sss* in infested soils and also in infected plant tissues (Qu et al. 2006). A real-time PCR assay capable of rapidly detecting and precisely quantifying *Sss* populations in soil was developed. This assay could reliably detect and quantify DNA from sporeballs, zoospores, and plasmodia/zoosporangia of *Sss*. This protocol could be employed to determine the viability of sporeballs in soil by combining this assay with tomato bait plant test to study the release of zoospores and infection levels in potato tubers under different environmental conditions (Lees et al. 2008).

A quantitative PCR assay was developed for determining soil inoculum level of *Spongospora subterranea* f. sp. *subterranea* (*Sss*) to find out the relationship between initial soil inoculum level of *Sss* and the incidence and severity of powdery scab disease on potato tubers at harvest. The QPCR assay detected soil inoculum in 75% of the 113 commercial potato fields across the UK, the amount of inoculum ranging over 0–148 *Sss* spore balls/gram of soil. When arbitrary soil inoculum threshold value of 0, <10, and >10 spore balls/gram of soil were set, the number of progeny crops developing powdery scab increased with the level of inoculum quantified in the field soil prior to planting. Four field trials showed that disease incidence and severity on progeny tubers increased with increase in soil inoculum level and the positive correlation between these factors was significant ($P < 0.01$). However, a cultivar effect was discernible in all years of experimentation, with disease incidence and severity scores being significantly greater in cultivars Agria and Estima than in Nicola ($P < 0.01$), possibly due to differences in the level of resistance to *Sss* under field conditions (Brierley et al. 2013).

The inoculum of *Spongospora subterranea* f.sp. *subterranea* (*Sss*) may originate from infected seed tubers or from contaminated growing media or contaminated equipment. An investigation was carried out to detect *Sss* in seed tuber production facility and identify the source(s) of contamination, and to apply necessary measures to eradicate the pathogen. Swap samples were taken at the rate of 40 samples each in August and October 2009. *Sss*-specific primers (Sps1 and Sps2) were employed in polymerase chain reaction assays to detect *Sss* in 11 surfaces in the seed tuber production facility. The presence of *Sss* was detected in 6 of 11 surfaces tested. The samples from the tunnel floor, the run-off channel, water troughs, drain pipes, the entrance floor, and cleaved crates contained the pathogen. The sporosori of *Sss* were able to survive in the tunnels in the absence of potato plants from one season to the next. The sporosori remaining in the run-off channels, water troughs, and drain pipes were the most likely sources of primary inoculum that resulted in the reoccurrence of the powdery scab disease. Corrective measures applied were effective in eliminating the pathogen from the minituber production tunnel and consequently resulted in the production of disease-free minituber stock (Wright et al. 2012).

Quantification of fungal and bacterial pathogens is essential to predict the extent of disease incidence in potato tubers. A quantitative PCR assay was developed for determining the pathogen DNA and visual assessment of disease was applied to measure tuber inoculum. The results of the tests were used to model the development of disease in potatoes grown in pathogen-free soil. Recursive partitioning and modeling using

receiver operating curves analyses showed that *Rhizoctonia solani* AG3 DNA and *Streptomyces scabies* DNA and disease symptoms induced by these pathogens were predominantly observed on potato tubers. These assessments could be the basis for predicting disease in progeny tubers. In contrast, the abundance of *Spongospora subterranea* DNA and disease symptoms on seed tubers were not consistently associated with powdery scab in progeny tubers. The relationship between *S. subterranea* DNA and seed tuber symptoms on root galling was stronger. Symptomless seed tubers that carried high levels of *S. subterranea* DNA were also associated with greater root galling than those with low pathogen DNA levels. A modest association between root galling and powdery scab in progeny tubers was recorded. The results underscored the importance of using disease-free certified seed tubers for reducing disease incidence and in subsequent crops (Tegg et al. 2015).

Synchytrium endobioticum, incitant of potato wart disease of quarantine importance, has a worldwide distribution and at least 30 biotypes have been recognized. Resting spores of *S. endobioticum* are carried by potato tubers short or long distances and are introduced into new areas where it can survive in the soil for 30–70 years (Hampson 1993). As the infected tubers form the primary sources of infection, the use of disease-free seed tubers is the basic strategy to be adopted to prevent the spread of the disease, necessitating the development of a reliable detection technique. The 18S rDNA of *S. endobioticum* was sequenced by extracting the DNA from the resting spores present in the infected tubers. DNA array technology was applied for the detection of the pathogen. Species-specific probes were designed and arrayed onto glass slides. Probes specific for viruses infecting potatoes were also designed based on nucleotide sequences of the viruses to be detected. A significant number of probes showed high specificity in detecting fungal and viral pathogens in microarray-based hybridization for identification of multiple pathogen targets (Abdullah et al. 2005). As infection of seed tubers by soilborne resting spores of *S. endobioticum* occurs, the level of soil infestation has to be determined by using a reliable technique. PCR primers based on the sequences of ITS region of multicopy gene rDNA were tested for specificity, sensitivity, and reproducibility in conventional and real-time PCR assays. The primers amplified a 473 bp product from *S. endobioticum* DNA only, but not from DNAs of other potato pathogens and related species. Standard cell disruption and DNA extraction and purification procedures were optimized for amplification of DNA from resting sporangia of *S. endobioticum*. The pathogen DNA was amplified from a single sporangium and equivalent DNA preparation from soil extracts.

The European Plant Protection Organization (EPPO) emphasized that fields infested with resting spores of *S. endobioticum* should be prohibited from growing potatoes for 20 years or more, until the absence of the pathogen is ensured by appropriate tests. A sensitive and reliable method was considered essential for detecting and quantifying the resting spores of *S. endobioticum* in the soil and plant tissues. A zonal centrifugation method known as Hendrickx centrifuge procedure was employed. Naturally infested and artificially contaminated soil samples were used for assessing the recovery percentage of and variation in numbers of winter sporangia of the pathogen. With Hendrickx centrifuge procedure, the recovery percentage was higher than the method recommended by the Dutch Plant Protection Service (van den Boogert et al. 2005; Wander et al. 2007). A real-time PCR assay was employed for the quantitative detection of *Synchytrium endobioticum* in potato tuber tissues, as well as in soil samples. Using CsCl₂ centrifugation, DNAs isolated from the sporangia in fresh wart tissues, warts,

and different plant parts of potato were tested by using specific primers and a TaqMan probe designed from the ITS region of the multicopy rDNA gene. The cytochrome oxidase gene of potato plant was used as an internal competitor DNA fragment for coamplification along with target DNA to guard against possible negative results. The TaqMan PCR assay had a detection limit of 1 fg of genomic DNA of *S. endobioticum*. The assay procedure employed in this investigation amplified a smaller product of 84bp, making it more efficient in detecting very low concentrations of the pathogen DNA, compared to the existing methods. By using this protocol, it was possible to detect the pathogen in stolons from plants with warts and also from asymptomatic plants. The sensitivity of detection of *S. endobioticum* was improved 100-fold by employing the TaqMan PCR format (van Gent-Pelzer et al. 2010).

Soilborne *Verticillium dahliae*, infecting potato tubers, also causes economically important diseases of different crops. Knowledge of the pathogen population levels in soil may help in the selection of soils that are minimally infested or free of the pathogen. A method was developed for extracting DNA from *V. dahliae* without the need for culturing the pathogen and DNA purification for use in the PCR assay. Liquid nitrogen and natural abrasives in soil were used for disruption of *V. dahliae* cells and grinding the fungal biomass. Skimmed milk powder was used for reducing losses of DNA due to degradation and adsorption. The DNA from fungal cells was then extracted with SDS-phenol and collected by ethanol precipitation. After diluting the DNA to the required concentration, it could be used directly for PCR amplification with appropriate primers. This procedure was found to be rapid, cost-effective, and could be employed for detection of *V. dahliae* directly from soil samples, after including the required internal controls. The results obtained were similar to those of conventional and nested PCR assays employed for the detection and quantification of microsclerotia of *V. dahliae* present in the soil samples (Volossiuk et al. 1995).

In a later investigation, a quantitative PCR assay based on the competitive PCR was applied to estimate *V. dahliae* DNA with a competitor DNA. This assay procedure provided accurate quantification of pathogen DNA in the range of 10^2 – 10^7 spores and 1–100 microsclerotia/gram of soil. The results of soil dilution method and PCR assay were strongly correlated ($r=0.97$). The competitive PCR format was found to be reliable and rapid and did not depend on the subjectiveness of soil dilution method (Mahuku and Platt 2002). In another study, a real-time PCR format was employed for the detection of *V. dahliae* in soil samples. The pathogen could be detected in plant tissues, soil, and irrigation water prior to symptom expression. The results suggested that screening for the presence of *V. dahliae* should be carried out before the transport of planting materials if the presence of the pathogen is suspected (Gayoso et al. 2007).

Verticillium dahliae, causing potato early drying (PED) or wilt disease, could be detected and quantified by using the primer pair VertBt-F/VertBt-R designed based on the sequence of the β -tubulin gene. The efficacy of the primer pair in real-time quantitative PCR format was shown to be greater (>95%) than the monoplex QPCR and duplex procedures which employed the primers PotAct-F/PotAct-R based on the sequences of actin gene. The real-time quantitative PCR format detected as few as 148 fg (equivalent of 5 nuclei) of pathogen DNA. The QPCR format was effective in detecting the pathogen in naturally infected, air-dried potato stems and in the fresh stems of inoculated plants. Isolation of the pathogen was not successful in 10% of the stems however, which tested positive for QPCR assay, indicating its higher level of sensitivity and reliability. The QPCR protocol developed in this investigation had the potential for application in

breeding programs for assessing the resistance levels of potato genotypes to *V. dahliae* (Attallah and Stevenson 2006; Attallah et al. 2007).

Helminthosporium solani, causing potato silver scurf disease, could be detected by a PCR-based assay. Specific primers Hs1F1/Hs2R1 were employed, which amplified a 447bp product from the DNA extracted from 20 samples of infected tubers and 54 single-spore isolates of *H. solani*. The PCR assay was rapid, giving the results in 24h, whereas the conventional plating-isolation method required about 4 weeks (Errampalli et al. 2001). In another investigation, two sets of primers Hs1F1/Hs2R1 (outer) and Hs1NF1/Hs2NR1 (nested) were designed to unique sequences of the nuclear ribosomal internal transcribed spacer (ITS1 and ITS2) regions of *H. solani*. Nested PCR format was employed to enhance the level of specificity and sensitivity of single-round PCR. Each primer set amplified a single product of 447bp and 371bp with DNA from 71 European and North American isolates of *H. solani*, respectively, and the specificity of primers was revealed by the absence of amplified product with DNA from other fungal and bacterial plant pathogens. The sensitivity of PCR for the specific detection of *H. solani* in seeded soils was determined to be 1.5 spores/gram of soil. *H. solani* was also detected in naturally infested soil and from peel and peel extract from infected and apparently healthy tubers by the PCR assay. In addition, specific primers and a TaqManTM fluorogenic probe were designed using the original primer sequences to perform the real-time quantitative (TaqManTM) PCR format. An accurate estimation of pathogen populations in tuber and soil contaminated with *H. solani* was achieved by real-time quantitative PCR assay, which might be useful for investigations on the ecology of *H. solani* and for disease risk assessments (Cullen et al. 2001).

Another fungal pathogen *Colletotrichum coccodes*, causing black dot disease, was detected using species-specific primers (CcINFI/CcNRI). The DNA was extracted from potato tubers and soil by employing a rapid method. The PCR assay had a detection limit of 3.0 spores/g or equivalent of 0.06 microsclerotia/g of soil. The pathogen was detected in the extracts of potato peel rapidly (Cullen et al. 2002). In a later investigation, tuber incubation test and PCR assay were employed for the detection of *H. solani* and *C. coccodes* in tubers from ten potato cultivars collected at harvest from three organic farms in Wisconsin, USA. The tubers were categorized as symptomatic or asymptomatic, based on visual symptoms of silver scurf and black dot and/or signs of *H. solani* and *C. coccodes* infection. Tuber incubation and PCR assays were in slight to fair level of agreement for detecting both pathogens. Most asymptomatic tubers were positive for infection by *H. solani* (75%) or *C. coccodes* (94%) when tested by both detection methods. Minituber inoculation procedure was followed to screen potato genotypes for resistance to silver scurf disease. A diploid interspecific hybrid C287 showed consistently low sporulation by *H. solani*. As the tubers from organic farms carried high levels of infection by *H. solani* and *C. coccodes*, it was suggested that acceptable management practices have to be identified for organic farms growing potatoes (Mattupalli et al. 2013).

Plasmodiophora brassicae, causing clubroot disease of cruciferous crop plant species, has a worldwide distribution. As the pathogen is soilborne and carried in the infected seedlings, the level of soil infestation by *P. brassicae* has to be assessed to select fields free of the pathogen. Conventional PCR assay was used to amplify a section of rDNA repeat and the PCR products were sequenced. Primers were designed based on the sequences of the PCR product and were directed at rRNA genes and ITS regions. The primers PbITS1 and PbITS2 amplified the predicted fragment of approximately

1100bp, whereas the nested primers PbITS6 and PbITS7 gave a product of 620bp. The primers were specific to *P. brassicae* and did not amplify the DNA from more than 40 common soil organisms and host plants. The nested PCR assay was able to detect the pathogen in all soils where the inoculum was adequate to induce the disease (Faggian et al. 1999).

In another investigation, a molecular marker for specific detection of *P. brassicae* in planta was developed using an oligonucleotide primer set from small subunit gene (18S-like) and ITS regions of rDNA of the pathogen. This primer set was employed for specific detection of *P. brassicae* in plant tissues. A 1000bp fragment was amplified by this primer set. The high copy number of rDNA gene sequences from which the primer set was developed was able to effectively detect small quantities of the target pathogen DNA in the total DNA extracts of infected Chinese cabbage plants. The DNAs of test plant and other soilborne fungi and bacteria were not amplified by the primer set, revealing its specificity of detection of *P. brassicae* (Kim and Lee 2001). In another investigation, a simple one-step PCR format was developed for the detection of *P. brassicae* using the primers TC1F/TC1R based on a *P. brassicae* partial 18S rRNA sequences from GenBank. This primer pair generated a 548bp product in the optimized PCR assay. A second primer pair TC2F/TC2R amplified a fragment of 18S and ITS1 regions of the rDNA repeat and this primer produced a 519bp product. Both primer pairs amplified the DNA of *P. brassicae* only, but not the DNA from uninfected plants, noninfested soil, or common soil fungi and bacteria tested. The assay protocol could be employed for detecting *P. brassicae* in symptomless root tissues of plants at 3 days after inoculation, indicating the reliability and possibility of early detection even before development of visible symptoms (Cao et al. 2007).

Potato smut disease caused by *Thecaphora solani* induces galls in lower stems, stolons, and tubers. DNA profiles were generated by PCR assay by employing sequence repeated primers CAG5, CTG5, TCC4, GAC4, TGTC4, and GACAC3. The DNA profiles revealed a close relationship between the teliospores and the sponge-like mat originated from the teliospores (produced in culture), indicating both structures were the same. The results of DNA fingerprinting and partial sequencing of the large subunit (LSU) rDNA region corroborated the conclusion arrived at from DNA profiles generated by PCR assay (Andrade et al. 2004). Potato stem canker and black scurf disease is caused by *Rhizoctonia solani*, is a species complex comprising 13 known anastomosis groups (AGs). *R. solani* AG-3 accounts for significant losses in potato in eastern Canada and the USA. RAPD technique was applied for rapid and reliable identification of the pathogen. Specific genetic markers of AG-3 isolates were identified. Amplification by RAPD procedure revealed the presence of a specific DNA fragment (2.6kb) in all AG-3 isolates. A PCR-based restriction mapping method was developed using restriction enzyme *XhoI* for the identification of AG-3 isolates. These virulent isolates were rapidly detected in both infected plant tissues and infested soil samples (Bounou et al. 1999). *Rhizoctonia solani* AG3-PT is the anastomosis group associated with potato black scurf disease. Primers were designed based on the sequences of rDNA ITS region of AG3-PT for use in detecting *R. solani* directly in tuber and soil samples. The assay was highly specific for AG3-PT isolate and the primers did not amplify DNA from other isolates of other AGs or subgroups of AGs. The AG3-PT was more frequently detected in tubers. AG3-PT was present in more than half of the tuber samples tested, indicating the specificity and reliability of detection of *R. solani* in tubers (Woodhall et al. 2013).

Fusarium oxysporum f.sp. *cubense* (*Foc*) causes Panama wilt disease, one of the most destructive diseases affecting banana crops and accounting for heavy losses in all countries across the world. Rapid and reliable detection of the pathogen in planting materials (suckers) is an important strategy for implementing the integrated disease management system. The pathogen exists in the form of four races that show varying pathogenic potential (virulence) on different banana cultivars. A primer set Foc-1/Foc-2 amplified a 242 bp (Foc242) DNA fragment specifically from *Foc* race 4 present in the tropical countries. The primer set Foc-1/Foc-2 was able to amplify the marker fragment Foc242 at a concentration as low as 10 pg of pathogen DNA, indicating the high level of sensitivity of the assay. The primer set Foc-1/Foc-2 could detect the *Foc* race 4 isolates in field-collected, naturally infected banana pseudostem tissues. Furthermore, the marker fragments could be detected in symptomless banana leaves. However, the bands were very faint in the gel after electrophoresis. In combination with Southern hybridization procedure, the sensitivity of the PCR assay was enhanced by a factor of 100. In addition, the *Foc* race 4-specific primer set was able to differentiate race 4 isolates from other races infecting banana (Lin et al. 2009).

Phytophthora fragariae, causing red core (root rot) disease of strawberry, is a quarantine pathogen and has spread steadily throughout Europe (only a few Mediterranean countries are free from the disease). Field inspections of nurseries were found to be inadequate for detection of red core; the bait test, although sensitive, required too long a time to be useful commercially. A PCR-based assay was therefore developed for the early detection of *P. fragariae* in infected plants. The primers P-FRAGINT and universal ITS4 were used for the detection of *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* in the roots of strawberry and raspberry, respectively. The detection efficiency was maximum when the plants were tested at 1–5 days after inoculation (Hughes et al. 1998). In another investigation by employing the primers developed based on the sequences of ITS region of ribosomal repeat (rDNA), *P. fragariae* was detected more efficiently when compared to the ELISA test (Bonants et al. 2000). Nested PCR assay was recommended because of its rapidity, sensitivity, and specificity to the required level. The assay was useful for the elimination of infected strawberry seedlings through which the disease might spread to other locations (Duncan et al. 2000).

In a later study, primers designed based on sequences of various parts of rDNA were used for the detection of *P. fragariae* var. *rubi* in infected raspberry plants. The effectiveness of detection by PCR assay was compared with bait test. PCR assay was found to be rapid, sensitive, and specific, and taxonomical expertise was not required to perform the test. The results of PCR assay corroborated that of bait test. However, PCR assay detected the pathogen in ten samples from four different stocks. On the other hand, positive results for two more samples were obtained when bait test was applied for these four rootstocks. PCR assay provided results within 3 days, whereas the bait test needed 6 weeks to yield results. Further, comparison of the sensitivity of the tests suggested that bait test appeared to be more sensitive, as only one-third of the amount of infected root tissues required for PCR assay was necessary for the bait test (Schlenzig et al. 2005).

Molecular beacon technology was successfully employed for rapid and reliable detection of *Phytophthora fragariae* in strawberry plants. A Molecular BeaconTM, with a central region complementary to the target amplicon and a 607 bp sequence labeled with a quencher at one end and a fluorescent dye at the other end, was used to detect *P. fragariae* amplicons in a quantitative manner similar to that of TaqManTM. A linear relationship with dilutions from 100 attogram (ag) to 1 pg was observed. The probe

detected amplicons in samples with as little as 100 ag pathogen genomic DNA and as few as 25 zoospores of *P. fragariae*, in addition to the pathogen DNA in water samples. Both Molecular BeaconTM and TaqManTM could provide “real-time” measurements in closed-tube system on the ABI 7700, and quantitative determination of the pathogen DNA could be accomplished. However, quantitative assessment of pathogen population in infected strawberry plants might become superfluous, as this pathogen is under strict surveillance with “nil” tolerance for quarantine clearance (Bonants et al. 2005).

Phytophthora cinnamomi, causing Phytophthora root rot (PRR) disease in avocado, is responsible for severe economic losses to the avocado industry across the world. As no known avocado rootstock resistant to *P. cinnamomi* was available, it became necessary to quantify *P. cinnamomi* in planta to evaluate tolerance of avocado genotypes to PRR disease. A real-time PCR assay was developed for sensitive detection of pathogen DNA in plant tissues. Root samples collected from a highly tolerant (Dusa) and less-tolerant (RO.12) rootstocks were tested at 0, 3, 7, 14, and 21 days after inoculation with *P. cinnamomi*. The nested primers employed were found to be sensitive and specific in detecting *P. cinnamomi* in root tissues. Tolerant avocado cultivar had lower contents of pathogen DNA compared to less-tolerant cultivar. The investigation provided a reliable molecular tool for assessment of levels of tolerance of avocado genotypes for use in breeding programs aimed at selecting PRR-resistant/tolerant rootstock genotypes/cultivar (Engelbrecht et al. 2013).

Colletotrichum acutatum infecting strawberry and other fruit crops causes yield-limiting anthracnose disease. A nested PCR assay was applied to detect the pathogen in asymptomatic leaves of inoculated plants. The leaf samples were frozen for 3 h, incubated for 2 days at 27°C, immersed in Tween-20 solution, sonicated for 30 min, and the suspension was then agitated for 1 min. Nested PCR assay detected as little as 1 fg of DNA extracted from the pathogen mycelium and as few as 1.5 conidia mL⁻¹ after sonication. When the extracts of inoculated symptomless leaves were tested, the nested PCR assay detected *C. acutatum* in the composite leaf sample containing 1 inoculated out of 50 uninoculated leaves. *C. acutatum* was also detected in field-collected asymptomatic strawberry leaves, indicating the efficiency of the procedure developed in this investigation for specific and reliable diagnosis of anthracnose infection under field conditions (Pérez-Hernandez et al. 2008).

Involvement of *Colletotrichum fragariae* and *C. gloeosporioides* in the strawberry anthracnose crown rot in several countries and *C. acutatum* in some locations such as California has been reported. Three real-time PCR (TaqMan[®]) assays were developed for specific detection, quantification, and differentiation of *Colletotrichum acutatum* and *C. gloeosporioides*. The most divergent area of ITS1 and ITS2 and 5.8S rRNA gene region was selected. A protocol for the extraction of DNA from the plant materials was optimized for use in conjunction with the new real-time PCR assays. These formats allowed detection of these pathogens directly from plant materials. The sequences selected for designing primers and probes showed identity and homologous sequences from desired target organisms. These assays were 10–100-fold more sensitive than conventional PCR format. The real-time PCR assays showed higher levels of sensitivity compared with the ELISA test, since these assays could detect the pathogen even in ELISA-negative samples. The development of *C. acutatum* was monitored using artificially infected strawberry crowns of two strawberry cultivars. The pathogen population was not affected by strawberry cultivars, but variations in population was observed at different periods (months) of testing ($P=0.394$). Diagnosis of anthracnose root rot

disease was rapid when real-time PCR format was applied. The results could be obtained in 1–2 days and the assays allowed high-throughput and inexpensive screening of strawberry plant samples directly (Garrido et al. 2009).

The importance of indexing the mother plants from which cuttings are taken for vegetative propagation for freedom from disease(s) has been emphasized by several investigations. *Phaeomoniella chlamydospora* is the important endophyte associated with a decline (esca and esca-like) symptoms or Petri disease occurring in vineyards in Europe and North American countries. The disease is responsible for serious establishment problems in newly planted vineyards. Infection by *P. chlamydospora* spreads through cuttings taken from infected mother vines and contaminated cuttings appeared to occur during nursery procedures (Fourie and Halleen 2000). Detection of the pathogen in contaminated plants at different stages during nursery propagation process was possible using nested PCR assay (Whiteman et al. 2004). A potential marker in the New Zealand isolate A21 was identified. Sequencing of the region flanking the 1010bp marker revealed a single nucleotide polymorphism in the 3' border of the marker band. Primers were designed to amplify a 487bp fragment encompassing this polymorphic site. Digestion of this product with the restriction enzyme *BsrI* produced three bands only in the isolate A21 and two bands in all other isolates of *P. chlamydospora* tested (Ridgway et al. 2005). A quantitative real-time PCR assay was developed using SYBR® Green technique that enabled detection of *P. chlamydospora* in the buds of infected grapevines (Overton et al. 2004). Later, a quantitative real-time PCR assay was applied to detect and quantify *P. chlamydospora* at different stages in the nursery. Various methods, namely PCR, nested PCR, and quantitative PCR (SYBR® Green and TaqMan®), were tested for their efficacy to detect *P. chlamydospora* during propagation. The nested PCR assay was the most sensitive followed by the quantitative PCR methods, and the single PCR assay was the least sensitive in detecting *P. chlamydospora* in the plant materials (Edwards et al. 2007). These PCR-based assays were also employed for the detection and quantification of propagules of *Phaeomoniella chlamydospora* in water samples. The pathogen was detected in water used to wash grapevine cuttings sampled at collection sites in the field. All samples taken from rainwater storage and cool-down tanks, plus water used to soak buds during grafting, tested positive for *P. chlamydospora* in all sites. The quantitative real-time PCR TaqMan® assay was able to detect *P. chlamydospora* at a concentration of as low as 10 spores per 100 mL of water. The nested PCR assay was the most sensitive for detection of *P. chlamydospora*, followed by quantitative PCR assay. The conventional (single) PCR format was the least sensitive (Edwards et al. 2007).

Eutypa lata, incitant of Eutypa dieback disease, causes appreciable losses in grapevine. Detection and identification of this pathogen was found to be difficult because of a lack of reliable diagnostic characteristics and erratic conidial production by the target pathogen. PCR-based assays employing primers designed based on sequences of ITS region and sequence-characterized amplified region (SCAR) were therefore applied for the rapid detection and identification of *E. lata*. The primers could amplify the pathogen DNA in supernatant obtained by boiling shavings of grapevine wood in water (Lecomte et al. 2000). A PCR-based restriction fragment length polymorphism (RFLP) procedure was developed, involving amplification of pathogen DNA using universal ITS1 and ITS4 PCR primers and digestion of PCR product with the restriction enzyme *AluI* (Rolshausen et al. 2004). As the PCR-RFLP-based technique employed universal ITS primers it is likely that DNAs of other fungi might also be amplified, resulting in the

production of multiple PCR products. For rapid detection of the pathogen directly in the affected grapevine wood and to identify it precisely, SCAR primers were therefore designed from the sequences of random amplified polymorphic DNA (RAPD) fragments. Primer pair EutO2F3/EutO2R2 derived from primer OPAMO2 was tested with genomic DNA from 24 isolates of *E. lata* and 12 other fungi isolated from grapevine. DNA fragments of 643bp were found in all *E. lata* isolates but not in any other fungi tested, confirming the specificity of the primers used. The presence of the 643bp PCR product was detected in grapevine canes inoculated with *E. lata* (Lardner et al. 2005).

Incidence of grapevine black-foot disease, caused by *Cylindrocarpon pauciseptatum* and different species of *Ilyonectria*, severely hampered production of healthy plants in the nurseries in Comunidad Valencia region of Spain. Samples were obtained from four sources of propagation process: pre-grafting hydration tanks; scissors used for cutting buds; Omega-cut grafting machines; and peat used for callusing. After extracting DNA from the samples, a multiplex nested PCR assay was employed for detection of black-foot disease pathogens using species-specific primers. *Ilyonectria liriodendri* and *I. macrodidyma*-complex were detected in hydration tanks, scissors, grafting machines, and peat, whereas *I. macrodidyma*-complex was frequently detected by the PCR format. In addition, ten grafted cuttings each from five grapevine scion/rootstock combinations were tested immediately after callusing and again after one growing season in a nursery field. *I. liriodendri*, *I. torrensis*, and *I. novozelandica* were detected in the root samples. Use of multiplex nested PCR format enhanced the frequency and efficiency of detection of *I. liriodendri* and *I. macrodidyma*-complex from grafted cuttings and plants in the nurseries. The results indicated that grapevine nursery propagation process should be considered as a potential source of infection for black-foot disease pathogens, affecting the grapevine nursery propagation system (Agusti-Brisach et al. 2013).

Botrytis spp. cause black mold diseases that infect several fruit and vegetable crops in the field and during storage, accounting for significant quantitative and qualitative losses. A real-time PCR assay, based on TaqMan probes, was developed for detection and quantification of *Botrytis aclada*, incitant of onion necrotic disease in onion bulbs. The DNA fragment of *B. aclada* in L45-50 region and a DNA sequence from onion serine acetyl transferase gene (SAT) as an internal control were used. The real-time PCR assay had a detection limit of $10\text{pg}\mu\text{L}^{-1}$ of pathogen genomic DNA. The assay results were not influenced by the presence of host tissues. The quantitative estimation of *B. aclada* in onion was achieved by inoculating onion disks with different concentrations of pathogen, using the real-time PCR protocol developed in this investigation. Further, the results of the real-time PCR assay could be correlated with visual observations made during the incubation period. The real-time PCR format had the potential for detecting and quantifying the pathogen population in onion bulbs during growth, harvest, and storage (Coolong et al. 2008).

2.2.2.3 Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) procedure can be performed onsite in the field, resulting in significant reduction in the time required for diagnosis of diseases caused by fungal pathogens. LAMP procedure may be carried out inexpensively using a simple water bath or heating block. It works at isothermal temperature for the synthesis of large amounts of *Bst* DNA polymerase which has strand displacement $5' \rightarrow 3'$ polymerase activity, but lacks $5' \rightarrow 3'$ exonuclease activity. Two sets of inner

(forward inner primer or FIP and backward inner primer or BIP) and outer primers (F3 and B3) are used to target specific genomic region. Initially the LAMP reaction utilizes all four primers, but later only the two inner primers are employed for strand displacement DNA synthesis. Positive reaction is recognized by the accumulation of a visible byproduct appearing as white precipitate (Notomi et al. 2000; Tomlinson et al. 2010). The LAMP method was integrated with lateral flow devices (LFDs) to improve the efficiency of detection and to become amenable for in-field application. LAMP primers based on the internal transcribed spacer (ITS) DNA sequences were used for the detection of anastomosis groups of *R. solani*. The LFD-based LAMP assay was applied for the detection of *Rhizoctonia solani* in plant tissues, seeds, and propagules such as cuttings of spathiphyllum, philodendron, diffenbachia, and impatiens. LAMP reactions were performed at 65°C for 60 min with 5 mM Mg²⁺. Clear bands representing pathogen-specific DNA could be visualized at concentration as low as 10 fg, which was equivalent to approximately one copy of genome of *R. solani*. The LAMP procedure efficiently detected *R. solani* in inoculated plant tissues and soil samples. A generic anti-biotin and anti fluorescein antibody-based LFD was used for the detection of the pathogen. The LAMP-LFD procedure was effective for the detection of *R. solani* in several infected plant species belonging to diverse families. The detection limit of LAMP-LFD protocol (10 fg) was comparable to that of RT-PCR format. The LAMP-LFD procedure was found to be easier and simpler than compared to the qRT-PCR format which requires expensive equipment. The LAMP-LFD protocol has the potential for onsite diagnosis of *R. solani* in plants, seeds, propagules, and soils (Patel et al. 2015).

2.2.3 Techniques Based on Biological Characteristics of Fungal Pathogens

Fungal pathogens may induce specific and characteristic symptoms in infected plants or in the propagative planting materials. Infection by such pathogens may be detected and even identified based on the type of symptoms caused. However, this may require a long time, experience, and critical examination. The fungal pathogens do differ in several cultural characteristics, pathogenicity, and certain genetic characteristics such as vegetative compatibility based on which the isolates/strains of a morphologic species are assigned to different groups.

2.2.3.1 Vegetative Compatibility Groups

Some fungal pathogens possess the ability to form “heterokaryons” by fusion between genetically different strains existing within a morphologic species. Strains which are vegetatively compatible with one another are considered as members of one vegetative compatibility group (VCG). The existence of vegetative compatibility system in *Verticillium dahliae* was first demonstrated by Puhalla (1979). Strains of *V. dahliae* included in one VCG were capable of forming heterokaryons with one another, but not with strains of other VCGs. Two common types of mutants that have been used to study the heterokaryons are UV-induced microsclerotial color mutants and spontaneous nitrate nonutilizing (*nit*) mutants. Using color mutants, 19 strains of *V. dahliae* were classified into four VCGs. Later, 86 strains of *V. dahliae* isolated from several host plant species and geographical locations were grouped into 16 VCGs (Puhalla and Hummal 1983). The wild-type strains (187) of *V. dahliae* from potato plants and soil from several fields in the USA were tested. Strains were assigned to VCGs based on pairings of

complementary nitrate nonutilizing (*nit*) mutants induced on a chlorate-amended medium. Two strains were assigned to VCG1, 53 strains to VCG2, and 129 strains to VCG4. The remaining 4 strains did not yield *nit* mutants and were therefore not assigned to any VCG (Joaquim and Rowe 1990). In a subsequent study, two phenotypic classes of *nit* mutants were identified among the 126 *nit* mutants characterized. Eighty-two *nit* mutants (65%) were able to utilize all nitrogen sources except nitrate. These mutants were designated *nit1* mutants, whereas the mutants belonging to the second phenotypic class were unable to utilize nitrate and hypoxanthine but retained the ability to use other nitrogen sources tested. These mutants were named *NitM* (Joaquim and Rowe 1991).

Potato tuber samples were collected from commercial seed tuber lots across North America and *Verticillium dahliae* was successfully isolated from 65 of 224 seed lots tested. The vegetative compatibility of the isolates of *V. dahliae* was assessed through complementation tests, using nitrate nonutilizing mutants. Most of the *V. dahliae* isolates belonged to vegetative compatibility group (VCG) 4. Of the 162 isolates recovered, 64% belonged to VCG4A, 33% to VCG4B, and 3% to VCG2B. All of the 39 isolates tested in the greenhouse on potato cultivar Superior were pathogenic to potato. VCG4A isolates were more virulent than VCG4B isolates. The results indicated that potato seed tubers from different locations were commonly infected with *V. dahliae* and they might serve as primary sources of pathogen inoculum. Further, VCG4A and VCG4B were distinct pathotypes of *V. dahliae* which varied in their virulence to potato (Omer et al. 2000). In a further study, *V. dahliae* isolates present in 87 fields intended for potato cultivation were subjected to vegetative compatibility analysis. Among the *V. dahliae* isolates tested 93% belonged to VCG4A group, whereas 23% of the isolates were assigned to VCG4B group. Only one isolate was related to VCG2B group. It was suggested that preplant assessment of the nature of *V. dahliae* populations might be useful for appropriate disease management decisions (Omer et al. 2008).

Verticillium dahliae has been reported to have a wide host range. However, a degree of host adaptation has been observed with different isolates exhibiting different levels of aggressiveness (virulence), depending on the host species or cultivar within a species. Further, isolates of *V. dahliae* could be separated into vegetative compatibility groups (VCGs) based on the ability to undergo hyphal anastomosis with other isolates. Distinct sub-specific groups were recognized which enclosed host-adapted pathotypes that were pathogenic to particular host plant species. Isolates of *V. dahliae* collected from potato stems, tubers, and potato fields in the USA mostly belonged to VCG4A and VCG4B. The VCG4A isolates were found to be highly aggressive on potato, compared with other groups. In contrast, isolates collected from potatoes grown in Israel were predominantly VCG2A and VCG4B. Initial inoculum of *V. dahliae* was shown to be that from soilborne microsclerotia. Tuber-borne inoculum seemed to have little effect on Verticillium wilt symptoms in infected plants and potato yields. Vascular infection by *V. dahliae* of seed tubers of moderately susceptible potato cultivar Russet Burbank had no significant effect on the development of symptoms. It did not significantly contribute to stem infection above ground level, the production of microsclerotia in debris, or progeny tuber infection. However, tuber-borne inoculum was the vital source for introducing the pathogen into the soils not known to be infested with the pathogen or in soils with low levels of pathogen population due to application of fumigants (Johnson and Dung 2010).

2.3 Appendix

2.3.1 Alkaline Blotter Method (Elwakil et al. 2007)

- i) Soak blotting papers in NaOH (0.4, 0.8, and 1.0%) or KOH (0.2, 0.4, and 0.8%); place them in petridishes; distribute 10 seed/petridish and incubate the plates at $20 \pm 2^\circ\text{C}$ for 7 days under cool white fluorescent light with alternating cycles of 12h of light and darkness.
- ii) Examine the seeds under a stereoscopic (binocular) microscope to observe the development of fungal growth.
- iii) Compare the morphological characteristics of the fungi with that of the description of fungal pathogens already known.
- iv) Prepare Czapek's agar medium amended with NaOH or KOH (final concentrations reaching 0.8 and 0.4, respectively, maintaining the pH at 12.5) and dispense the medium in the petridishes.
- v) Place mycelial disk (0.4 cm diameter) cut from the 7 day old cultures on potato dextrose agar (PDA) at the center in the petridishes and incubate at $25 \pm 2^\circ\text{C}$ for 7 days.
- vi) Determine the linear growth and sporulation using standard cultural procedures.

2.3.2 Detection of Teliospores of *Tilletia indica* in Seed by Acid Electrolyzed Water (Bonde et al. 2003)

2.3.2.1 Preparation of Acid Electrolyzed Water and Sodium Hypochlorite Solutions

- i) Acid electrolyzed water (AEW) is generated by electrolysis of water in Super Oxseed Laboratory JED020 apparatus (Proton Laboratories Inc., CA, USA); pass electric current through a dilute solution of sodium chloride (0.7 g in 4 L of water); pour equal volumes (2 L each) into the anode and cathode compartments and allow electrolysis to proceed for 15 min.
- ii) Collect AEW in anode compartment and alkaline water in cathode compartment.
- iii) Determine the pH, oxidation-reduction potential (ORP), and free chlorine content of AEW at the beginning and end phase of experiments using a pH meter, redox meter, and spectrophotometric analysis at 528 nm, respectively, using *N,N*-diethyl-*p*-phenyldiamine.
- iv) Prepare AEW afresh for each experiment.
- v) Prepare sodium hypochlorite (NaOCl) solution (0.4%, pH 10.3) for each experiment by diluting a 5.25% solution of a commercial bleach with distilled water and determine the pH and free chlorine content at the beginning and end of experiments.

2.3.2.2 Assessment of Effectiveness of AEW and NaOCl

- i) Incubate 50 g Karnal bunt-free wheat seed samples in 14 mL of water overnight at 20°C ; collect seed wash and centrifuge.
- ii) Treat the wheat seed wash pellet with 14 mL of AEW for 30 min with periodic shaking by hand; treat three samples for each lot and treat the three samples with NaOCl for 2 min in a similar manner; maintain suitable controls without any treatment.

- iii) Centrifuge wheat wash samples at $200\times g$ for 1 min; wash the pellet twice with sterile distilled water and resuspend the pellet in $200\mu\text{L}$ of distilled water.
- iv) Spread the suspension onto a square petridish ($100\times 100\times 15\text{ mm}$ with 13 mm wide grids) and pour 2% water agar amended with streptomycin sulfate and ampicillin sodium salt (AWA) at 100 mg L^{-1} each.
- v) Incubate at 20°C (with white fluorescent light and darkness for 12 h alternatively) for 14 days and determine the fungal and bacterial populations using a microscope.

2.3.3 Detection of *Sporisorium reilianum* and *Ustilago maydis* by Dot-Blot Hybridization (Xu et al. 1999)

2.3.3.1 Isolation of Smut Pathogens

- i) Suspend 10 mg of teliospores in $500\mu\text{L}$ of sterile distilled water (dH_2O); centrifuge at $9000\times g$ for 30 s; decant the supernatant and wash the precipitate containing teliospores.
- ii) Sterilize the purified teliospores with ampicillin (4 mg mL^{-1}) for 1 h at room temperature; wash the spores twice with sterile water; resuspend the teliospores in $400\mu\text{L}$ sterile water and dilute the suspension to different concentrations (1:10, 1:100, 1:1000, and 1:10,000).
- iii) Streak $200\mu\text{L}$ of each dilution on YEPS medium (1% yeast extract, 2% peptone, 2% sucrose, 2% agar) amended with $100\mu\text{g}$ ampicillin mL^{-1} ; incubate at 30°C for 3–4 days; streak single isolated colonies on plates twice more to obtain single-spore isolates.
- iv) Cultivate the single-spore isolates in 10 mL of liquid YEPS medium at 30°C for 2–3 days with constant shaking; harvest the fungal cells by centrifugation at $15,000\times g$ for 15 min; wash with 1 mL sterile water and use the fungal cells for DNA extraction by standard methods or by commercially available kits.

2.3.3.2 Nucleic Acid Hybridization

- i) Digest recombinant plasmids from selected colonies with restriction enzymes such as *SacI*, *HindIII*, *ApaI*, *BamHI*, and *EcoRI* with single restriction sites in the polylinker region of plasmid pBluescript. After digestion, a part of one or more restriction sites present in a given enzyme in an inserted fragment would be deleted. Use the subsequent self-ligated products to transform competent cells of *Escherichia coli* strain DH5 α and plate on LBamp medium to develop new recombinant plasmids with smaller insert fragments.
- ii) For colony blotting, replate white colonies containing recombinant plasmids on nylon filters (Schleicher & Schuell, Dassel, Germany) lying on LBamp medium plates and culture the colonies at 37°C for 8–10 h.
- iii) Lyse freshly grown colonies (about 1 mm diameter) with 0.4 NaOH and 10% sodium dodecylsulfate (SDS); neutralize with 0.5 M Tris-HCl and 1.5 M NaCl (pH 7.4) and fix on nylon filters by incubating at 80°C for 2 h.
- iv) For dot-blot assay, denature the genomic DNAs of smut fungi with equal volumes of 1 N NaOH; blot $2\mu\text{L}$ of DNA of each pathogen on nylon filters; adjust the concentration of fungal DNA to 100, 20, 4, 0.8, 0.16, and $0.032\text{ ng }\mu\text{L}^{-1}$ by suitable dilution and mix with an equal volume of genomic DNA ($100\text{ ng }\mu\text{L}^{-1}$); perform denaturation and dot-blot as indicated above.

2.3.4 Detection of *Phoma ligulicola* in Pyrethrum Seed by Polymerase Chain Reaction (Pethybridge et al. 2006)

2.3.4.1 Isolation of the Pathogen

- i) Surface sterilize the seeds with 1% sodium hypochlorite for 2 min; rinse in sterile distilled water; place them on 2% water agar kept in petridishes and incubate for 21 days.
- ii) Transfer individual hyphal tips to potato dextrose agar, malt extract (malt extract-Oxoid L39 and Oxoid agar No. 1) and oatmeal agar, and identify the pathogen based on morphologic characteristics.

2.3.4.2 Extraction of Pathogen DNA

- i) Prepare seed lots with varying seed infection levels (10.5, 5.25, 1.1, and 0.5%); grind seed samples (c. 100 mg) to powder using a mortar with a pestle and maintain four replicates for each seed lot.
- ii) Extract the DNA using the DNeasy Plant Min Kit (Qiagen, Germany) as per manufacturer's recommendations.
- iii) Check the presence of DNA by separation on a 1% agarose gel in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) followed by staining with ethidium bromide (0.5 mg L⁻¹) and visualizing under UV light.

2.3.4.3 PCR Amplification

- i) Perform reactions with 50 µL solution containing 2.5 mM MgCl₂, 1 mM deoxynucleoside triphosphate, 0.5 µmol of each oligonucleotide primer, and 1 U *Taq* polymerase (Qiagen) in 1 × PCR buffer (Qiagen), and use 1 µL containing 20–25 ng of DNA as the template for each reaction.
- ii) Using a thermocycler, provide the following conditions: 5 min at 94°C for initial denaturation, followed by 35 cycles of 30 s of denaturation at 94°C, 1 min of annealing at 56°C, and 2 min extension at 72°C, and conclude the reactions with a final extension at 72°C for 10 min.
- iii) Visualize the PCR products in a 2% agarose gel and 1 × TBE buffer (40 mM Tris-borate, 1 mM EDTA, pH 8.0) containing ethidium bromide under UV light.

2.3.5 Detection of *Plasmopara halstedii* in Sunflower Seed by Optimized Duplex Real-Time PCR Assay (Ioos et al. 2012)

2.3.5.1 Preparation of *P. halstedii*-Infected Seed Materials

- i) Collect the samples manually from severely infected sample (SIS) comprising different sunflower genotypes, and grind the SIS sample for 1 min in a sterilized glass bowl using a Microtron MB550 mixer mill (Kinematica, Switzerland).
- ii) Prepare 12 subsamples of 500 µL (190 ± 20 mg) using a sterile spatula; transfer aseptically to individual sterile 2 mL microcentrifuge tubes; grind the remaining seed powder for 10 s with 50 mL of TNE buffer (100 mM Tris, 2.0 M NaCl, 10 mM EDTA, pH 7.4); collect another 12 subsamples of macerate (600 µL) using a micropipette and transfer aseptically in individual 2 mL centrifuge tubes.
- iii) Use three microtubes for each replicate of each contamination level, containing 500 µL of sunflower seed powder and three microtubes containing 600 µL sunflower seed macerate as starting material for total DNA extraction.

- iv) Use commercial DNA extraction kits, for example NucleoSpin Plant II with PL1 lysis buffer, NucleoSpin Food (Macherey-Nagel, Germany), DNeasy plant mini kit (Qiagen) and PureLink Genomic Plant DNA (Invitrogen, CA, USA).

2.3.5.2 Optimized Duplex qPCR Assay

- i) Provide the following optimized duplex qPCR conditions: use 20 μ L reaction volumes employing real-time qPCR core kit No Rox (Eurogentec).
- ii) Reaction mixture contains molecular grade water, 1 $\times$ reaction buffer, 5 mM $MgCl_2$, 4 $\times$ 0.2 mM dNTPs, 0.05 μ M qPHAL-F, 0.3 μ M qPHAL-R, 0.05 μ M PHAL-P, 0.3 μ M each of the 18S-uni-F/R primers, 0.1 μ M 18S-uni probe, 0.05 μ M PHAL-P, 0.3 μ M each of the 18S-uni probe, 0.5 U of Hotgoldstar and 2 μ L of template DNA (0.8–20 ng).
- iii) Real-time PCR cycling conditions are as follows: initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s and annealing or elongation at 65°C for 45 s.
- iv) Perform all real-time PCR reaction with a Rotor-Gene 6500 (Corbett Research, Australia) set with an autogain optimization for each channel which is to be performed before the first fluorescence acquisition; determine the Ct value for each reaction using the Rotor-Gene Software, version 1.7.75, setting the threshold line at 0.02.

2.3.6 Detection of *Colletotrichum lindemuthianum* in Common Bean Seed by PCR Assay (Chen et al. 2007)

2.3.6.1 Extraction of Pathogen DNA

- i) Extract the fungal DNA using either DNeasy Plant Mini Kit as per the manufacturer's instructions or a modified cetyl trimethyl ammonium bromide (CTAB) procedure as detailed below.
- ii) For the CTAB procedure, transfer fresh filter-paper-dried fungal mycelium (50 mg) to a 1.5 mL microcentrifuge tube containing 588 μ L of 2 $\times$ CTAB buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 1% β -mercaptoethanol, 1% PVP-40, pH 8.0 preheated at 65°C) and 12 μ L 10 mg mL⁻¹ proteinase K (Sigma-Aldrich, Germany); grind the mycelium using a plastic minipestle for 3–5 min and incubate at 65°C in a water bath for 1 h with occasional gentle swirling.
- iii) Extract with a chloroform: isoamyl alcohol (24:1); dissolve the pellet in 100 μ L 10 mM Tris buffer; treat with RNase A (10 μ g mL⁻¹) (Sigma-Aldrich, Germany) at 37°C for 1 h.

2.3.6.2 Seed Powder Preparation

- i) Surface sterilize the seeds (16) with typical symptoms and seeds with healthy appearance (19) in 70% ethanol for 30 s; wash with sterile distilled water and dry the seeds using sterilized filter paper.
- ii) Grind batches of healthy and diseased seeds separately into a fine powder using a coffee grinder and mix the powder of healthy and diseased seeds in different proportions to obtain a serial dilution of pathogen concentration (100% to 0%).

2.3.6.3 PCR Amplification

- i) Manually design the forward and reverse primers based on the sequences of ITS1, ITS2, and universal reverse ITS4 regions.

- ii) Prepare the PCR reaction volumes consisting of 1 × PCR buffer, 1.5 mM MgCl₂, 125 μM each of dNTP, 250 nM of each forward and reverse primer, 0.5 units of AmpliTaq DNA polymerase (ABI, CA, USA), and 25–60 ng of sample DNA.
- iii) Perform PCR in a PTC-100 thermocycler (MJ Research Inc., MA, USA), providing the following conditions: initial denaturation at 95°C for 5 min, 12 cycles of 30 s at 94°C, 30 s from 62 to a “touchdown” (lower annealing temperature by 1°C in each subsequent cycle) at 50°C for primer annealing, 1 min at 72°C for primer extension, followed by 30 cycles with a 50°C annealing temperature and end with one cycle of 5 min at 72°C.
- iv) For nested PCR assay, use the universal primer set ITS5/ITS4 as the outer primers in the first round of PCR; use 1 μL aliquot of 10 times diluted first-round PCR product as the template in the second-round PCR, employing the selected forward primer C1F4 and ITS4 with the same “touchdown” PCR program.
- v) Separate the amplicons on a 1.4% agarose gel in 1 × TBE buffer at 100 V for 1 h; stain the gels with ethidium bromide and visualize under UV light using an AlphaImager gel documentation system (AlphaInnotech, CA, USA).

2.3.7 Detection of *Alternaria brassicae* by Conventional and Real-Time PCR Assays in Radish Seeds (Guillemette et al. 2004)

2.3.7.1 Seed Sample Preparation

- i) Disinfect the radish seeds with 1% sodium hypochlorite; rinse them with sterile water; soak in spore suspension (10⁶ spores mL⁻¹) of *A. brassicae* for 1 h and dry them at room temperature on sterile filter paper.
- ii) For confirmation of the effectiveness of disinfection and inoculation with the pathogen, place 20 seeds on malt agar in petridishes for 1 week at 25°C.
- iii) Select seed batches showing 0% (disinfected seeds) and 100% (infected seeds) and mix the infected seeds with uninfected seeds in different proportions to have 0, 5, 10, 50, and 100% contaminated seed samples.
- iv) Place 20 seeds (artificially contaminated) or 100 seeds (naturally contaminated) in a sterile tube; cover them with 0.5 mL and 3.0 mL of liquid culture MDP medium (containing 2% malt extract, 2% dextrose, and 0.1% peptone) for artificially and naturally contaminated seed samples, respectively.
- v) Incubate the tubes at 25°C for 48 h with occasional stirring for enrichment to allow increase in fungal biomass from the seeds; vortex the tubes briefly to separate mycelia and conidia from seed and collect the fungal biomass after centrifugation for 10 min at 5000 × g.

2.3.7.2 PCR-Based Assays

- i) Extract the fungal DNA from fungal mycelia and conidia harvested by scrapping the surface of cultures on petridishes by using Nucleospin Food Kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions.
- ii) Design specific nucleotides ABCsens and ABCrey complementary to the 3' end of open reading frame (ORF) 2 and 115sens and 115rev complementary to a portion of ORF1 for standard and real-time PCR assays, respectively (using the software Primer 3 online and ABI PRISM Primer Express).
- iii) Use universal primer pair ITS1/ITS2 as a positive control to assess the quality of extracted fungal DNA.

- iv) For conventional PCR assay, use 2 μ L of undiluted DNA preparations under the conditions detailed below: Tris-HCl, pH 9.0, 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01% Tween 20 (w/v), 1.5 mM MgCl_2 , 200 μ M each of deoxyribonucleotide triphosphate and 1 unit of thermostable DNA polymerase (Goldstar Red DNA polymerase, Eurogentec, Belgium).
- v) Use Thermojet thermocycler (Equibio, Belgium) with an initial step of 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 50 s at 60°C and 1 min at 72°C, and a final step of 10 min at 72°C.
- vi) Visualize amplification results after electrophoresis of an aliquot (10 μ L) of reaction mixture on a 1.2% agarose gel.
- vii) For real-time PCR assay, carry out reactions in a 25 μ L final volume containing 2.5 μ L DNA, 12.5 μ L SYBR Green PCR Master Mix 2 $\times$ (Applied Biosystems, France), 300 mM forward and reverse primers and H_2O up to 25 μ L.
- viii) Use an ABI PRISM 700 Sequence Detection System (Applied Biosystems) with steps mentioned below: 2 min at 50°C, 10 min at 95°C, and 40 cycles consisting of one step at 95°C for 15 s followed by one step at 60°C for 1 min.
- ix) Quantify nucleic acids in unknown samples by direct comparison to a standard; extract the DNA to be used as a standard from fungal cultures with concentrations assessed by fluorometric assay (Turner Design Institute, Sunnyvale, CA) before dilution.

2.3.8 Assessment of Genetic Diversity of *Fusarium fujikuroi* by UP-PCR Analyses (Cumagun et al. 2011)

2.3.8.1 Extraction of DNA from Pathogen Mycelium

- i) Inoculate potato dextrose broth (250g potato, 20g dextrose, 1000 mL water) placed at 500 μ L/tube, in 1.5 mL Eppendorf tubes and incubate for 72 h at room temperature.
- ii) Centrifuge for 5 min at 13,000 rpm; wash the pellet in 500 μ L TE buffer (10 mM Tris, 1 mM 50.5 EDTA, pH 8.0, 800 mL distilled water); decant the TE buffer solution; add 300 μ L extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and crush the mycelia using a sterilized toothpick followed by the addition of 150 μ L 3 M sodium acetate, pH 5.2.
- iii) Place the tubes at -20°C for 10 min; centrifuge and transfer the supernatant to another tube.
- iv) Add 450 μ L isopropanol; place the tubes at room temperature for precipitation of fungal DNA.
- v) Pellet the DNA using the microfuge; wash the pellet with 70% ethanol; air dry the pellet and resuspend the DNA in 50 μ L TE buffer.
- vi) Check the presence of DNA by performing gel electrophoresis, followed by staining with ethidium bromide and visualization of the specific bands using UV transilluminator.

2.3.8.2 UP-PCR Amplification

- i) Generate genomic fingerprints of isolates of pathogen through UP-PCR assay as indicated in the following.
- ii) Mix 1 μ L of DNA extract with 1 μ L AA2M2 primer (5'-CTGCGACCCAGAGCGG-3') (Invitrogen), 0.4 μ L 2 U i-Taq polymerase (INtRON, Biotechnology), and 17.6 μ L of

- reaction buffer (i-Taq1 × PCR buffer (INtRON Biotechnology), 2 mM i-Taq MgCl₂, and 0.8 mM deoxynucleoside triphosphate (dNTP) mixture (INtRON, Biotechnology).
- iii) Perform UP-PCR in a MyCyclerTM thermal cycler (Bio-Rad Laboratories, CA, USA) with following conditions: 30 cycles with DNA denaturation at 92°C for 50 s (first denaturation step at 94°C for 3 min), primer annealing at 56°C for 70 s and primer extension at 72°C for 60 s (final extension step at 72°C for 3 min).
 - iv) Analyze PCR products by electrophoresis in two agarose gels as detailed below: mix 85 g agarose with 50 mL of 0.7 × TBE buffer; heat in a microwave oven for 1 min and 15 s; pour into a mold with 15-well comb; allow to solidify before placing it in electrophoresis tank with 0.7 × TBE buffer; load PCR products (3 μL of each UP-PCR reaction) with 1 μL blue juice each into individual wells from the third to the twelfth to enable 10 PCR reactions to be electrophoresed per run; load gels with 1000 bp ladders as molecular size standards on the second and thirteenth well; carry out two runs to accommodate 20 PCR reactions and visualize the bands by staining with ethidium bromide for 10 min.

2.3.9 Detection and Quantification of *Verticillium dahliae* in Spinach Seed by Real-Time PCR Assay (Duressa et al. 2012)

2.3.9.1 Extraction of Pathogen DNA from Infected Spinach Seed

- i) Collect 1000 seed samples (three replicates) from individual seed lot; grind seeds for c. 45 s to a fine powder using an IKA10 analytical mill (IKA Laboratory, Germany).
- ii) Extract DNA from 200 mg fractions of ground seeds using a FastDNA Kit following manufacturer's recommendations (MP Biomedicals, OH, USA); further purify DNA extracts using AMPure magnetic beads and a SPRI 96-Ring Magnet Plate (Agencourt, Beverly, MA) for cleansing DNA of potential PCR inhibitors and determine concentration using a Qubit fluorimeter and Quant-iT dsDNA BR assay Kit (Invitrogen, CA, USA).
- iii) Dilute all DNA samples to 1 ng μL⁻¹ in nuclease-free water; use aliquots of 5 μL of diluted DNA for quantitative (q) PCR assay and prepare six 200 mg fractions of ground seed from each of three replicates.

2.3.9.2 Quantitative PCR Assay

- i) Perform the assay in the LightCycler 480 model II (Roche Diagnostics, USA) in 96 well plates with total reaction volumes of 20 μL containing 10 μL of 1 × IQ SYBR Green Supermix (Bio-Rad, CA, USA), 0.4 μL of forward VertiBt-F and reverse VertiBt-R primers at 200 nM each, 5 μL of template DNA (1 or 5 ng total), and 4.6 μL of nuclease-free water, and load each DNA sample in triplicate in a 96 well plate.
- ii) Provide the following conditions: 95°C for a 5 min denaturation step, followed by 40 cycles of amplification at 95°C for 10 s and 65°C for 35 s and melt curve analysis of heating to 95°C, cooling to 65°C for 1 min and heating to 97°C at a rate of 1°C/5 s.
- iii) Use actin gene of spinach as a reference gene and amplify in each sample using primer pair S0385-F/S0489-R at a concentration of 300 nM under the same qPCR cycling conditions given above.

2.3.10 Assessment of Variation Among Isolates of *Fusarium graminearum* Using Random Amplified Polymorphic DNA (RAPD) (Walker et al. 2001)

- i) Prepare single-spore isolates from each original field isolate; transfer to 1.5 mL microcentrifuge tubes containing 500 μ L yeast-malt (YM) broth (3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone, 10 g dextrose L⁻¹) and incubate for 7 days.
- ii) Add 500 μ L sterile Tris-EDTA, pH 8.0 (TE); boil the contents in the tubes at 100°C for 10 min; cool on ice for 10 min; centrifuge at 13,000 rpm for 10 min; transfer the aqueous fraction containing DNA to fresh tubes and dilute by mixing 3 μ L of aqueous DNA fraction with 97 μ L of sterile water.
- iii) Perform RAPD reaction with 15 μ L mixture consisting of 5 μ L diluted DNA, 1.5 μ L *Taq* polymerase buffer containing 0.9 μ L 25 mM MgCl₂, 1.2 μ L of 1.25 mM dNTPs, 1.2 μ L of 100 $\times$ unacetylated bovine serum albumin (BSA), 30 ng primer (Operon Technologies, CA), and 0.2 μ L of *Taq* polymerase (Promega, WI, USA) and use 96 well plates and a PTC 100 thermocycler (MJ Research, MA, USA).
- iv) Provide the following cycle parameters: 1 min at 92°C, 1 min at 35°C followed by 2 min at 72°C repeated for a total of 40 cycles.
- v) Visualize the bands on 1.5% agarose gels at 100 V for about 4 h stained with ethidium bromide under UV fluorescence.
- vi) Screen the isolates twice with each primer and score repeatable polymorphisms as 1 for presence of band and 0 for absence.

2.3.11 Differentiation of *Tilletia* spp. by PCR-RFLP Technique (Levy et al. 2001)

2.3.11.1 Extraction of Pathogen DNA

- i) Soak the teliospores from bunted wheat kernels in 5 mL distilled water or Tween-water (1–2 drops of Tween-20 in 100 mL distilled water) for 24 h at 22–25°C; pellet the teliospores by centrifugation at 1000 $\times$ g for 3 min; resuspend the pellet in 10 mL 0.42 NaClO (8% commercial bleach, w/v); centrifuge immediately for 1 min as before; wash the pellet twice with 14 mL sterile water followed by centrifugation for 1 min and resuspend the pellet in 1.8 mL sterile water.
- ii) Divide the spore suspension into two equal quantities; spread one on water agar (WA) supplemented with streptomycin and ampicillin (100 mg L⁻¹ each); incubate at 19°C; remove aseptically small blocks of agar with single germinating teliospores; affix to the lid of a petridish containing potato dextrose broth allowing discharge of secondary basidiospores into the broth; remove from the petridish the mycelia developed from the secondary sporidia after 7–14 days; blot on filter paper; vacuum-dry in 1.5 mL microcentrifuge tubes and store at –20°C until DNA extraction is to be carried out from them.
- iii) Freeze the vacuum-dried mycelium in liquid nitrogen; pulverize using a microtube pestle; isolate the pathogen genomic DNA using the PureGene DNA isolation kit (Gentra Systems, Inc., NC, USA) as per the manufacturer's instructions.

2.3.11.2 PCR Amplification

- i) Dilute purified DNA to 1:10 concentration with sterile water; perform amplification reactions using pathogen-specific primer pairs Ti17M1/M2 and Ti57M1/M2, and amplify the ITS1 region using primer pair ITS5/2 as positive control.
- ii) Amplify the fungal DNA in 50 μ L reactions using a DNA amplification kit (Perkin-Elmer, Applied Biosystems, CA) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl,

- 1.5 mM MgCl₂, gelatin at 10 µg mL⁻¹, 200 µM each dNTP, 20 pmol each primer, and 1.25 units of AmpliTaq.
- iii) Provide the following thermocycler schedule: one denaturation cycle of 94°C for 3 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min.
 - iv) Electrophorese in 5% polyacrylamide in 1 × TBE buffer (89 mM Tris, 89 mM borate, and 2.5 mM EDTA at 110 V for 2 h and visualize after silver nitrate staining.
 - v) For sequencing, amplify the PCR products under the same conditions described above using the primer pairs ITS5/4 for complete ITS region or ITS5/2 and ITS3/4 to amplify the region in two parts.

2.3.11.3 DNA Sequencing and Analysis

- i) Separate the PCR products in 1.2% agarose gels in 1 × TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) at 70 V for 1.2 h, followed by staining with ethidium bromide.
- ii) Excise the bands; crush them and incubate at 35°C overnight with elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 400 µL per tube; elute the DNA and filter through 0.45 µM Spin-X columns (CoStar), and concentrate the precipitated DNA by centrifugation.
- iii) Carry out further purification of DNA using the Wizard PCR Preps DNA purification kit as per the manufacturer's instructions (Promega, WI, USA).
- iv) Sequence the purified PCR products in both directions using dye-terminator sequencing with an ABI 373A automated sequencing system.

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3

Biology of Fungal Pathogens

Plant diseases are caused by abiotic and biotic agents. Abiotic agents include different environmental and soil conditions that may be either above or below optimal conditions required for plant development. Microbial plant pathogens such as fungi-like and true fungi, bacteria, phytoplasma, viruses, and viroids constitute the major group of biotic agents. Microbial pathogens are responsible for a large number of economically important diseases that lower the quality and quantity of produce from agricultural and horticultural crops. Successful crop production depends on several interdependent and interacting factors or operations such as choice of crop/cultivar, planting materials, method of planting/sowing, fertilization, tillage, type and frequency of irrigation, method of harvesting, and method and duration of storage, in addition to the effectiveness of plant protection strategies applied. The incidence and spread of diseases caused by fungal plant pathogens may be influenced by these factors and may consequently affect the quantity and quality of the produce obtained. Among the microbial plant pathogens, fungal pathogens are more numerous and induce different and distinct types of symptoms characteristic of the diseases caused by them.

Knowledge of the biology of fungal pathogens is important since the effective management of crop diseases induced by them depends largely on the requirements for development of the fungal pathogens both in and outside the crop hosts. Nutritional requirements, temperature and pH optima for growth and reproduction, host range, existence of physiologic races/biotypes, variations in the phenotypic and genotypic characteristics, and factors that contribute to the development of epidemics are studied to provide a sound and dependable basis for formulations of suitable disease management tactics. A large proportion of fungal pathogens causing seedborne diseases can lead a saprophytic life (ability to grow in the absence of living host plant species) for short or longer periods. In contrast, some fungal pathogens such as sunflower downy mildew pathogen *Plasmopara halstedii* need living plant hosts for their existence; all attempts to cultivate them on cell-free media have met with failure so far. These pathogens are known as obligate pathogens. The ability/inability to develop on cell-free media is an important factor that allows or restricts the possibility of gathering information on the nutritional requirements of fungal pathogens associated with seedborne diseases.

Among the microbial pathogens, the fungus-like and fungal pathogens are highly evolved; they belong to the Eukaryotes, which include plant hosts and animals. They have a well-developed body (thallus) consisting of innumerable filamentous thread-like hyphae weaving into a mycelium. The hyphae may be septate (with a cross-wall, referred to as a septum) or nonseptate/coenocytic (without cross-walls, as in fungi-like

microorganisms). The cells may be uninucleate or binucleate with well-defined cell walls. The coenocytic (nonseptate) mycelium of Phycomycotina is like a continuous, tubular, branched or unbranched multinucleate cell or multinucleate hyphae in which septa are formed later, when spore-bearing structures are formed. The mycelium is formed by the elongation of hyphal tips produced following germination of spores/conidia. Members of Plasmodiophorales do not have a true mycelium but form naked amoeboid, multinucleate plasmodia, whereas Chytridiales produce a system of strands, known as rhizomycelium, that vary in diameter. Populations of fungal pathogens increase rapidly by means of both asexual and sexual reproduction. Specialized asexual reproductive structures such as spores, conidia, sporangia, and pycnidiospores are produced by fungi in large numbers. They germinate and grow into a new independent colony, when optimum conditions are available. Generally sexual spores are produced late in the life cycle of fungal pathogens or when nutrients become restrictive. Sexual spores such as oospores, zygospores, ascospores, and basidiospores are formed after completion of the sexual process.

3.1 Biological Characteristics

3.1.1 Disease Cycles of Fungal Pathogens

3.1.1.1 Reproduction of Fungal Pathogens

Fungus-like and true fungal pathogens are carried in and/on seeds and propagules. Further, these pathogens may reach the seeds or propagules from soil, irrigation water, and air. The pathogens may infect roots, stem, and floral parts and subsequently infect the seeds. Fungal pathogen populations increase primarily in three ways. Vegetative cells may be cut off from the hyphae and grow into new individual colonies. Some cells may develop thick walls and become separated from the hyphae. These thickened cells, known as chlamydospores, are resistant to adverse conditions and germinate when optimal conditions for growth become available, forming new thallus. In the anamorphic (asexual) phase of life cycle, asexual spores such as sporangia or conidia are produced in large numbers. These spores germinate under optimal environmental conditions to form new individual colonies in the substrate. Later in the sexual phase of the life cycle, thick-walled oospores are formed after mating between oogonia (–) and antheridia (+) in Oomycetes. Ascospores are produced in asci enclosed in ascocarps. They are ejected out at maturity and infection occurs when they reach appropriate tissues in the susceptible host plants. Likewise, the basidiospores formed from basidia infect plants, causing diseases such as smuts and bunts in cereal crops. Sexual reproduction is only possible in some of the fungus-like and all members of Basidiomycotina when two morphologically similar, but physiologically different gametangia (+ and –), come into contact with each other. Gametangia from the same (+ or –) isolate cannot unite to produce sexual spores. This phenomenon is known as heterothallism, in which thalli of two different compatible isolates (+/–) are required for sexual reproduction as they are individually isogamous in nature. Heterothallism is common in most of the higher Ascomycotina. Basidiospores or ascospores are formed, following a union between compatible isolates which show no morphological differences.

Infection by *Phytophthora capsici* is considered as a limiting factor in the production of pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita pepo*),

and watermelon (*Citrullus lanatus*). *P. capsici* produces many infectious propagules such as oospores, sporangia, zoospores, and encysted zoospores. The viability of *Phytophthora* spp. propagules may be evaluated by germination tests, infectivity tests, or vital stains. The vital staining is less time-consuming, quantitative, and has the potential to assess viability without affecting the biological activity of propagules. The fluorescent vital dye FUN[®]-1 (2-chloro-4-(2-3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl-methylidene)-1-phenylquinolinium iodide) was evaluated as a tool to determine the viability and metabolic activity of *P. capsici*. Under aerobic conditions, mycelia, sporangia, and zoospores were cultured on agar medium and stained with FUN-1 dye. The decline in the percentage of metabolically active sporangia and increase in the number of zoospores fit both a linear and log regression model. The FUN-1 dye was useful to differentiate live and dead sporangia and also for effectively monitoring the changes in the metabolic activity of sporangia over time (Lewis Ivey and Miller 2014).

3.1.1.2 Survival and Dispersal of Fungal Pathogens

Fungal pathogens may exist as saprophytes on dead organic matter present in the soil or on dead plant tissues attached to the weakened plants or plant debris left over in the field after harvest. They may colonize seeds sown in the subsequent season, causing seed rot or seedling blight diseases. The ability to survive during the absence of crop hosts is a crucial factor for the survival of the fungal pathogens. On the other hand, obligate pathogens such as *Plasmopara halstedii* and *Peronosclerospora sorghi* remain as dormant mycelium in the seeds of their respective crop hosts, sunflower and sorghum (Zad 1979; Ahmad and Majumder 1987). The oospores of *Peronosclerospora sorghi* can overwinter in the infected plant debris or in the soil during the absence of susceptible sorghum plants. Thick-walled teliospores of *Sporisorium sorghi* are released from the smut sori by the rupture of the silvery membrane that covers the sorus formed by fungal structures in place of healthy earhead. The teliospores fallen on the soil remain dormant and infection occurs when the next sorghum crop is sown. *Colletotrichum lindemuthianum*, causative agent of anthracnose diseases of common bean and other leguminous crops, was able to survive for up to 22 months in plant debris in the northern latitudes (Dillard and Cobb 1993).

Ustilago maydis has a dimorphic pattern alternating between a budding haploid form and a filamentous dikaryon resulting from the mating of two haploid cells (+ and -). This morphological switch plays a vital role in pathogenicity, since the filamentous dikaryotic hyphae alone can infect corn plants. A role for the cAMP signal transduction pathway in dimorphism and pathogenicity has been recognized. Repression of a subset of genes in filamentous cells may be critical for programming virulence. Suppression subtractive hybridization procedure was applied to identify the filament downregulated genes and to understand better the role of wild-type budding cells in the disease cycle of *U. maydis* in nature. A library of approximately 5500 cDNA clones was arrayed. The reverse Northern blot analysis showed that most clones were downregulated during filamentous growth. More than 88% of the clones detected as differential in the Northern blot screening harbored sequences corresponding to 48 different genes. Differential expression was confirmed for 37 of these genes by Northern blot analysis. For eight of these confirmed differential genes, expression could only be detected in budding cells. In the case of genes expressed in both budding and filamentous cells, levels of differential expression varied from as much as 65-fold to only two-fold higher levels in budding cells. The differential genes had similarity to database sequences (García-Pedrajas and

Gold 2004). *Ustilago tritici* produces teliospores that can survive for long periods. Under experimental conditions, the strains of *U. tritici* were maintained at 2–4°C which required large amounts of labor and physical resources. The survival of *U. tritici* race T2 was therefore determined as mycelium in infected seed stored at –15°C for up to 32 years. A significant decline in the percent viability of inoculated seed was observed over time. In contrast, the percent infection of viable seed showed significant increase as the interval increased. The results suggested that either the cold treatment could enhance the survival of the pathogen mycelium or the survival of wheat seed was enhanced by the presence of pathogen when stored at lower temperatures. Storage of *U. tritici* as mycelium in infected seed at –15°C was found to be an effective method of maintaining the *U. tritici* strain T2 (Menzies et al. 2010).

Tilletia tritici, incitant of wheat Karnal bunt or partial bunt disease, is a soilborne pathogen. Teliospores of *T. indica* may fall on the soil surface during harvest and persist there for long periods. The teliospores on the soil surface germinate and produce primary and secondary sporidia which infect the earheads under favorable environmental conditions. Survival of teliospores of *T. indica* was investigated using soils differing in texture and other characteristics collected from four locations, equilibrated to –0.3 MPa. The soil samples were infested with teliospores to have a density of 10^3 spores g⁻¹ of dry soil. The teliospores enclosed in polyester bags placed in polyvinyl chloride tubes were buried vertically in the ground. The rates of teliospores recovered from soil samples were 90.2, 18.7, 16.1, and 13.3% after 1 day and 8, 20, and 32 months, respectively, after burying the teliospores in the soils. The percentage of teliospores recovery was greatest in loamy soil and lowest in silt loamy soil. Depth of burial did not influence the survival of teliospores. A negative correlation between soil temperature (–18°C to 22°C) and percentage of teliospores germination was observed after 37 months of incubation, the maximum being at –5°C. Microscopic observations revealed that disintegration of teliospores commenced after breakdown of the sheath covering the teliospores. The results indicated that *T. indica* teliospores were able to remain viable for more than 32 months in Montana (Babadoost et al. 2004).

Formation of dikaryotic hyphae is the prerequisite for infection of susceptible plants by fungal pathogens belonging to the genera *Ustilago* and *Tilletia*. Mating of two genetically opposite (+ and –) promycelia from basidiospores produced by teliospores is essential for formation of dikaryotic hyphae. Mating is initiated by sexual pheromones produced by the two mating types of *Ustilago hordei*, causal agent of barley-covered smut disease. The binding of pheromone to its complementary receptor induces formation of conjugation tubes which follow pheromone gradients to locate each other to form dikaryotic mycelia (Martinez-Espinoza et al. 1993). *U. hordei* produced mating pheromones that break down to smaller peptide compounds that might act as potent inhibitors of mating and germination in other fungi. The pheromones are members of the farnesylated family of proteins. Synthetic peptide analogs of the pheromone derivatives ranging in size from 4-mers to full length pheromones were farnesylated, methyl esterified, or both, and tested for mating or teliospore germination inhibition with *U. hordei* or *Tilletia tritici*, respectively. The most potent inhibitors were methionine methylester and modified 4-mer peptides. The observation that methionine and cysteine methyl esters could inhibit germination of *T. tritici* teliospores suggested that these compounds might be nonspecific. Use of pheromone antagonistic to mating or teliospore germination might be a promising novel strategy of the management of smut and bunt diseases of wheat and small grain crops (Kosted et al. 2002). The pseudosclerotia

and conidia of *Phoma ligulicola* could survive in the soil in the absence of several host plant species of this pathogen. Pyrethrum and chrysanthemum are seriously affected by *P. ligulicola*, which is transmitted through seeds of pyrethrum. Epidemics of ray blight disease caused by *P. ligulicola* were frequently observed in Tasmania (Pethybridge et al. 2006). *Verticillium dahliae* is a soilborne pathogen with a wide host range, causing economically important diseases in a diverse range of crops worldwide. Microsclerotia of *V. dahliae* survive for several years in the soil and may be dispersed via farm equipment, soil, and irrigation water (Klosterman et al. 2009). Spinach seeds produced in Europe and the USA were found to be almost invariably infected by *V. dahliae* (du Toit et al. 2005). *V. dahliae* in the spinach crop residue that was returned to soil harbored the pathogen inoculum for crops such as lettuce that were grown in rotation with spinach. Infected seeds of spinach might also introduce exotic strains of *V. dahliae* to new areas of crop production (Duressa et al. 2012).

Pyricularia oryzae (= *Pyricularia grisea*), the causal agent of the devastating rice blast disease, infects seeds which may play an important role in the disease dispersal and perpetuation. The importance of seedborne infection by *P. oryzae* in rice seeds and its transmission to seedlings was studied quantitatively with naturally infected seeds of three rice cultivars. Disease severity in panicles was positively related to the extent of seed infection. Healthy-looking panicles also contained infected seeds. Seed transmission rate was generally low. When seed samples with 21% seed infection were planted, 4% of the seedlings exhibited blast lesions. An increase of 10% neck blast infection resulted in a 22% increase in seed infection, and panicles with neck blast symptoms had significantly higher level of seed infection than seeds from panicles without neck blast symptoms. Sporulation of *P. oryzae* at the embryonal end of seeds alone was observed at a higher frequency than in nongerminating seeds on which sporulation occurred all over the seeds. Recovery of *P. oryzae* from seeds sown in sterilized soil was greater than that from seeds sown in unsterilized soil. Latent infection of rice seedlings raised at low temperature (15–20°C) was higher than in seedlings raised at higher temperatures (25–30°C). Seedlings with latent infection could be a potential source of inoculum under field conditions (Manandhar et al. 1998).

Pyricularia grisea could sporulate on infested seed left on the soil surface under field conditions and the dissemination of conidia by wind or rain could initiate infection on seedlings which may act as a source of inoculum. A marked strain of *P. grisea* was used to determine whether the inoculum from infested rice grain could cause primary infection and sustain a rice blast epidemic for infection of aerial plant parts. A sulfate nonutilizing (*sul*) mutant of *P. grisea* was used to infest rice grains applied at different inoculum densities (infested seeds) on the soil surface at the time of seedling emergence. Leaf blast symptoms were first observed in plots incorporated with infested rice grains at 35 days after plant emergence in both years of experimentation (1996 and 1997). The transmission of *P. grisea* from infested seeds indicated that under field conditions the dissemination of conidia to rice seedlings continued up to 2 weeks from an inoculum source on the soil surface. This inoculum was important in the early-season epidemiology of rice blast. The marked strain of *P. grisea* could be recovered throughout the season from leaf, collar, and neck infections under field conditions. The results indicated that naturally infested seed on the soil surface (commonly found) could be potential sources of primary inoculum. Sporulation in artificially infested grain occurred for up to 2 weeks after application under field conditions. Conidiophores and conidia formation were predominantly associated with the embryonic end of the rice seed for both artificially

and naturally infested seed. Sporulation was usually confined to the embryonic end. The *sul* mutant of *P. grisea* could be easily differentiated from the wild-type strain recovered from blast lesions by a differential medium containing sodium selenate. The *sul* mutant predominated in all leaf, collar, and neck blast lesions sampled in 1996 and 1997. Furthermore, blast disease development progressed in a similar pattern in leaves inoculated with *sul* mutant and wild-type strain (Long et al. 2001).

Cylindrocladium parasiticum, a soilborne fungal pathogen, causes Cylindrocladium black rot (CBR) disease of peanut (groundnut). The pathogen infects hypocotyls, pegs, pods, and seeds. Seed transmission of *C. parasiticum* occurred when speckled seeds (with dots or flecks) were planted. Sixty-three commercial seed lots of peanut were examined for the presence of speckled seed at rates ranging over 40–96%. Ambient temperatures during winter seed storage (–10 to +28°C in 1999 and –8 to +33°C in 2000) substantially reduced pathogen recovery percentage from speckled seed stored for 16 or 24 weeks. On the other hand, drying in commercial drying trailers at temperatures of up to 35°C greatly enhanced the percentage of recovery of the pathogen from speckled seeds. The speckled seeds were present in all 15 samples from field plots where plants showed symptoms of CBR disease. A significant linear relationship ($r^2 = 0.70$) existed between CBR incidence in the field and the percentage of speckled seed produced. *C. parasiticum* survived well in speckled seed stored at recommended temperatures (1–5°C) for commercial seed storage. The wide variation in pathogen survival in speckled seed among seed lots makes it difficult to predict the role of these seeds in pathogen dispersal. The results suggested that the percentage of speckled seed in commercial seed lots might be reduced if fields with high CBR disease incidence could be avoided (Glenn et al. 2003). Ramularia leaf spot (RLS) disease caused by *Ramularia collo-cygni* (*Rcc*) has become an economically important disease in barley crops grown in Europe, Ireland, the UK and South Africa. The presence of *Rcc* DNA could be detected in both embryo and nonembryo tissues of infected barley seeds. Transmission of *Rcc* from infected seeds to seedlings was investigated. The pathogen DNA was detected using qPCR assay in DNA extracted from all leaves of plants emerging from infected seeds under growth chamber conditions. Application of Raxil (tebuconazole + triazoxide) as seed treatment did not prevent the movement of *Rcc* from seeds to seedlings. Samples of winter and spring barley plants grown under field conditions were analyzed by qPCR assay. Colonization of barley plants by *Rcc* commenced from lower leaves and then moved up to apical plant parts. DNA contents of *Rcc* reached up to 10,000 pg g⁻¹ in the leaves of spring barley. A linear correlation between visual symptoms and *Rcc* DNA levels was observed in both years (2008 and 2009) of experimentation (Fig. 3.1) (Havis et al. 2014).

Fusarium graminearum (teleomorph *Gibberella zeae*), causing Fusarium head blight (FHB) disease of wheat and other small grain crops worldwide, is the most devastating pathogen of the 17 organisms associated with FHB or scab disease. This pathogen produces macroconidia in sporodochia on infected heads and crop debris in the asexual stage. The macroconidia are splash-dispersed by rain and have an important role in the secondary infection of wheat heads. Sexual fruiting bodies, perithecia, are formed in the spring on infected crop residue. Ascospores, the primary sources of inoculum, are forcibly discharged from the perithecia and are dispersed by the wind 3–5 days after rainfall. The survival of the pathogen in *Fusarium*-damaged kernels (FDKs) was investigated under field conditions. The FDKs were left on the soil surface or buried at 5 or 10 cm and monitored for 24 months. The pathogen could be isolated from FDKs

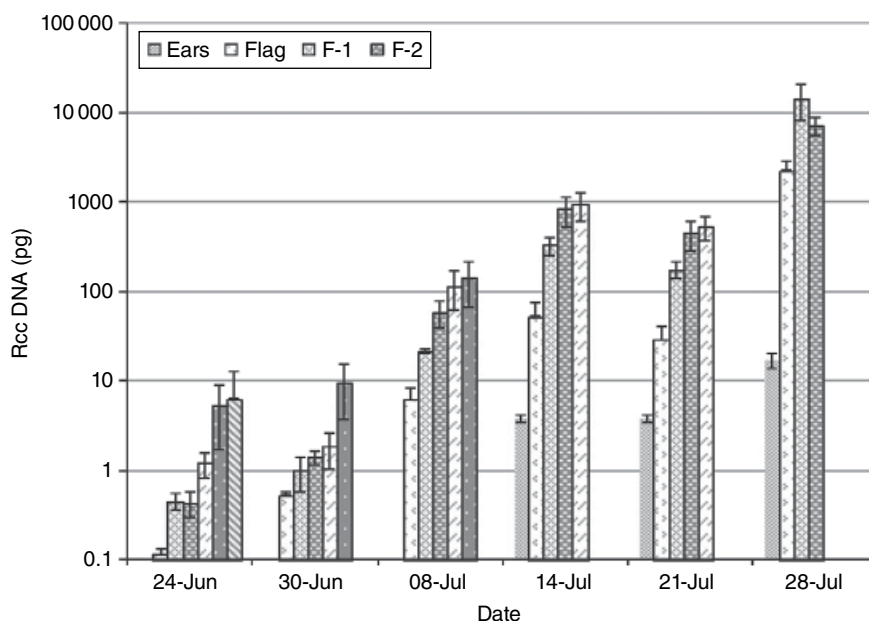


Figure 3.1 Determination of DNA contents of *Ramularia collo-cygni* in different layers of leaves and ears of barley cultivar Lanark 2009 at different sampling dates, using quantitative assay. Source: Havis et al. (2014). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

under all conditions, with isolation frequency ranging over 85–99% kernels. Although perithecia were formed at all locations, ascospores were produced only from the perithecia present in soil surface. The pathogen survived in 76–100% of kernels in the soil at -10 , 2 and 20°C . Perithecia developed on kernels incubated at 20 and 2°C , but ascospores developed only in perithecia on FDKs at 20°C on soil surface. The results indicated that the perithecia found on FDKs present on the soil surface could function as sources of inoculum for *G. zeae* to cause infection on wheat earheads (Inch and Gilbert 2003).

In addition to wheat and barley, the host range of *F. graminearum* has been reported to expand from cereals to noncereal crops such as canola (Chongo et al. 2001), potato (Ali et al. 2005), soybean (Martinelli et al. 2004), and sugar beet (Hanson 2006). Isolates of *F. graminearum* (21) obtained from potato, sugar beet, and wheat were inoculated onto wheat earheads. They induced typical symptoms of FHB disease on wheat-susceptible cultivar, and were able to produce a wide range of trichothecene mycotoxins in wheat grains. All isolates possessed the *Tri5* gene involved in the biosynthesis of trichothecenes. Potato isolates exhibited greater variability in producing different mycotoxins than sugar beet and wheat isolates. The results indicated that infected potatoes and sugar beet could be potential sources of inoculum for FHB epidemics in wheat (Burlakoti et al. 2007). *Fusarium graminearum* also causes a seriously damaging pod blight, seed, and root rot disease in soybean. Seeds infected by *F. graminearum* appear pink to reddish in color, but seed infection may sometimes be asymptomatic. The effect of inoculum density of *F. graminearum* on disease development was investigated. Individual soybean seed was inoculated with $100\ \mu\text{L}$ of suspension of 2.5×10^2 , 2.5×10^3 , 2.5×10^4 , or 2.5×10^5 macroconidia mL^{-1} in a rolled-towel assay at temperatures

of 18, 22, and 25°C. Mycelia developed within 2–3 days after inoculation. For optimum disease development, inoculum concentration of 2.5×10^4 macroconidia mL⁻¹ or higher was required, irrespective of the temperatures tested (Ellis et al. 2011).

It is necessary to determine whether the seedborne fungal pathogens are transmitted to the emerging seedlings and further invade other plant parts/organs such as leaves, stem, and inflorescence in due course. Transmission of *Stagonospora nodorum* and the effect of seedborne inoculum on the incidence of *S. nodorum* blotch in the field were investigated. Infected wheat seeds were sown to serve as a source of primary inoculum in the epidemics of cereal disease. Oxall agar medium, yeast malt extract medium, and potato dextrose medium were used for detection of seedborne *S. nodorum*. The effect of seed infection with *S. nodorum* on subsequent leaf and glume blotch severity in the field were assessed using triticale and wheat. Planting highly infested seed led to more severe disease incidence than planting healthy or only slightly infected seed. The correlation coefficient for the level of seed infestation and disease severity in individual varieties was low and only significant for spring triticale (Arseniuk et al. 1998). Transmission of *Stagonospora nodorum* from four naturally infected wheat seed lots was assessed quantitatively in controlled environment germination chambers at 9, 13, 17, 21, and 25°C. The presence of lesions caused by *S. nodorum* in coleoptiles and the first leaves of seedlings was examined. The first leaves were incubated in Bannon's medium for 2 weeks, after which they were examined for pycnidia of the pathogen. Transmission of *S. nodorum* to the coleoptiles was observed at all temperatures of incubation, but the percentage transmission was reduced from 37% to 2% as the temperature was increased from 9 to 25°C. The first leaves (44%) were symptomless at all temperatures tested, but at 25°C the percent of first leaves showing no symptoms increased to 96%. Transmission to seedling leaves occurred over a broad temperature range. If wheat is sown with high seed densities, it is likely that a significant number of infected seedlings per unit area may originate from relatively low initial seed infection levels and transmission efficiencies (Shah and Bergstrom 2000).

Survival of sporangia of *Phytophthora infestans* under ambient, relatively dry atmospheric conditions was investigated. The viability of sporangia was assessed by determining the proportion of potato tuber slices or leaflets that were infected after inoculation with sporangia exposed to daily weather conditions for different durations and to direct or indirect sunlight. The maximum survival time of sporangia was 24 h under moderately cool (mean of 15°C) and dry conditions (relative humidity or RH < 25%). Sky conditions were sunny during the day. Only 25 of 566 (4.4%) groups of sporangia caused infection. Of these 25 groups, 23 (92%) had been exposed to mean temperatures below 33°C for <4 h. Binary logistic regression analysis of the data showed that duration of exposure, direct sunlight, and type of day were all significant factors affecting survival of sporangia. Long exposure in direct sunlight limited the survival of sporangia. Sporangia was more likely to survive on cool, cloudy days and cool, rainy days than on other types of day (Sunseri et al. 2002).

The seed transmission of *Phytophthora infestans*, causing late blight disease of tomato, was investigated by inoculating tomato fruits at green mature stage with a mixed sporangial suspension of A1 and A2 isolates of the pathogen. Tomato fruits were also inoculated either with A1 or A2 isolate. Seeds extracted from blighted fruits were sown in soil or on agar media to test for the transmission of the pathogen to the emerging seedlings. All blighted seedlings (23 of 25,000 seedlings) originated from fruits inoculated with mixed (A1 + A2) sporangia. Isolates of *P. infestans* recovered from the

emerging blighted seedlings were apparently of oosporic origin, as they differed phenotypically (mating type, virulence, sensitivity to metalaxyl) from parent isolates used to inoculate the fruits. The results suggested that transmission of *P. infestans* might be possible through seeds extracted from fruits carrying oospores which are resistant to adverse environmental conditions (Rubin and Cohen 2004).

Spinach crops are infected by leaf spot diseases caused by *Cladosporium variable* and *Stemphylium botryosum*; these diseases seriously reduce the crop value, resulting in significant economic losses, when cool and moist conditions prevail. The seedborne nature of these two pathogens in spinach seeds was investigated by applying freeze-blotter seed health assay. Seed lots (77) of spinach from the USA, Denmark, the Netherlands, and New Zealand showed infection of all seed lots by *S. botryosum* with a mean incidence of 29.1% per lot. The presence of *C. variable* or morphologically similar *C. macrocarpum* was detected in 37 of 77 seed lots with a mean incidence of 1.8% per lot. Some isolates of both fungi were pathogenic to spinach. Nonpathogenic isolates were identified as *C. macrocarpum*. Survival of *C. variable* and *S. botryosum* in spinach seeds was assessed under storage. Pathogenic isolates of *S. botryosum* were detected in each of 12 seed lots stored for up to 11 years at 4.4°C and 60% relative humidity. *C. variable* or *C. macrocarpum* was only detected in 2 of 11 seed lots which had been stored for 3 and 8 years. The results indicated the potential difficulty in eliminating these seedborne pathogens, as they were able to invade embryos of seeds (Hernandez-Prez and du Toit 2006).

Fungal pathogens infecting vegetatively propagated crops survive in different organs of mother plants such as tubers, corms, stem pieces, setts, and suckers that are used for planting in the subsequent seasons. In addition, these pathogens may survive in the soil on the organic matter added as manures or crop residues left over in the field after harvest. *Phytophthora* spp., *Colletotrichum* spp., and *Fusarium* spp. may remain in planting materials as dormant mycelium which escapes detection by conventional isolation methods. Fungal pathogens present in the seeds and propagules are primarily dispersed in two ways. The developing seedlings may be infected by inoculum present in the seeds. The seedlings may be killed either in the nursery or after transplantation in the main fields. The spores produced in the infected seedlings may be dispersed by wind or irrigation water. Alternatively, the fungal pathogens may reach the soil and perpetuate on the organic matter or crop residues available in the fields. In the case of downy mildew, smut, or bunt diseases, the pathogens occupy the growing points of plants following infection of seedlings and remain without inducing any visible symptoms of infection. The symptoms are produced after a long incubation periods. Whitish mildew growth may be seen on the lower leaf surfaces, whereas infection by smut and bunt diseases may be revealed in the heading stage only. Individual flowers or the entire inflorescence may be transformed into sac-like sori containing millions of teliospores which are dispersed by the wind, when the sorus wall is ruptured.

Fusarium oxysporum f.sp. *vasinfectum*, a soilborne pathogen causing the devastating Fusarium wilt disease of cotton, has a worldwide distribution. Although the seed transmission of the pathogen has been reported in several countries, attempts to detect it in seed from California, an important seed production region, were not successful. Occurrence of the wilt disease in isolated spots in previously uninfested fields suggested the possibility of the pathogen dispersal through infected seed. *F. oxysporum* f.sp. *vasinfectum* race 4 occurring in the San Joaquin Valley was highly virulent on most commercial cotton cultivars. The pathogen could be isolated from Pima cultivar DP744

acid-delinted seeds using Komada's agar or a selective liquid medium. DNA of race 4 of the pathogen was isolated from pure cultures and amplified with race-4-specific primers. Amplicons were consistently detected from the positive controls (infected seed sample) and no amplification was seen in the negative controls (healthy seed sample) (Section 3.5.1). The investigation indicated that the potential dangerous consequences of distributing cotton seed originating from areas infested with race 4 need to be considered carefully until effective management strategies have been agreed upon (Bennett et al. 2008).

3.1.2 Host Range of Fungal Pathogens

The host range of microbial plant pathogens is one of the critical epidemiological factors contributing to the low or high levels of incidence and slow or fast spread of crop disease(s). Some fungal pathogens have the ability to infect only one or a limited number of plant species under natural conditions. Fungal pathogens with a wide host range pose a serious threat to crop production, since the inoculum may be supplied by another crop plant species cultivated along with the primary crop which has to be protected. In addition, the pathogen may be able to infect different weed plant species that may be inside or outside the field. Furthermore, seed infection may occur in a fewer plant species, but the inoculum from other infected plant sources may enhance the chances of seed infection making the management of the disease very difficult.

Verticillium dahliae, causal agent of Verticillium wilt disease, has a worldwide distribution and infects a wide range of crop plant species including cotton, lettuce, potato, strawberry, and forest trees. Spinach seeds produced in Europe and the USA invariably carried *V. dahliae* (du Toit et al. 2005). *V. dahliae* infects potato and its presence in certified potato seed lots was detected in Israel and in Washington (Tsrur et al. 1999; Omer et al. 2000). *V. dahliae* carried in the seeds may be introduced into the new areas at short or long distances. New or more virulent strains may also be dispersed through infected seeds to areas where the pathogen may be currently unimportant or may be limited in its distribution. *V. dahliae* is transmitted through the spinach seeds. Infested seed has been implicated in the introduction of *V. dahliae* into lettuce fields. In this investigation, 100 commercial spinach fields in four coastal CA counties of USA were surveyed. Regardless of the county, *V. issacii* was the most predominant species from spinach, followed by *V. dahliae* and less frequently by *V. klebahnii*. Transmission of *V. dahliae* from infested spinach seeds to lettuce was investigated in microplots. Verticillium wilt developed on lettuce following two or three plantings of *Verticillium*-infested spinach in independent experiments. Polymerase chain reaction (PCR) assays confirmed the identity of the pathogen recovered from infected lettuce plants from microplots as *V. dahliae*. Further, the transmission of *V. dahliae* through seeds to lettuce roots was demonstrated using a green fluorescent protein (GFP-) tagged mutant strain of *V. dahliae* after two cycles of incorporation of infected spinach residue into the soil. The results clearly showed that lettuce is another crop plant species functioning as additional host of *V. dahliae* (Short et al. 2015).

Fusarium graminearum, causal agent of Fusarium head blight (FHB) disease of wheat and barley, is transmitted through infected seeds. Inoculum of *F. graminearum* consisting of an infected wheat floret was inoculated onto wheat, barley, rye, triticale, canary seed, flax, canola (*Brassica napus* and *B. rapa*), mustard, bean, field pea, lentil, and chickpea. Reduction in emergence in all crops (except canola, mustard, and field pea)

due to infection was observed as root rot severity was also enhanced. At low temperature (10.5°C) however, seedling emergence was not affected and no root infection occurred. However, as the temperature increased from 10 to 30°C, root severity increased in addition to reduction of seedling emergence. These susceptible crops could be potential alternative sources of inoculum of *F. graminearum*, as they are grown in rotation with wheat (Chongo et al. 2001). *F. graminearum* was reported to infect noncereal crops such as canola, potato, soybean, and sugar beet. The inoculum from these noncereal crops may contribute significantly to the development of FHB epidemics in wheat (Burlakoti et al. 2007).

Infection in the inflorescences of weed plants belonging to 67 graminaceous and nongraminaceous species was examined by isolation of the FHB pathogen throughout the year. Fifty-four of the weed species belonging to 19 families were identified as alternative hosts of *F. graminearum*. Further, the tricothecene chemotype of a group of isolates was analyzed. Strains belonging to 15-acetyldeoxynivalenol (15-ADON) and nivalenol (NIV) chemotypes were differentiated among the isolates obtained from weed plant species which might act as additional sources of inoculum for infection of wheat crops, indicating the epidemiological significance of the weed plants (Mourellos et al. 2014). *Phytophthora capsici* causes Phytophthora blight disease in many vegetable crops such as pepper, cucurbits, and eggplant. The pathogen produces sporangia and zoospores under favorable environmental conditions, and they are important in pathogen dissemination and repeated initiation of infection in one season. On the other hand, sexual spores (oospores) formed later in the life cycle are required for pathogen survival in the absence of susceptible host plant species. The development of resistance to commonly applied chemicals was shown to be another mode of survival mechanism operating in *P. capsici* (Jackson et al. 2012).

3.2 Physiological Characteristics of Fungal Pathogens

3.2.1 Variations in Virulence of Fungal Pathogens

Isolates of some of the fungal pathogens show variations in the physiological activities that contribute to their growth, reproduction, and pathogenic potential (virulence). Responses of isolates/species of fungal pathogens to the environment, such as light, temperature, pH, moisture (humidity), availability of nutrients in the substrate, and the ability to produce spores/vegetative structures (sclerotia/chlamydospores) or sexual spores that are resistant to adverse environmental conditions, differ significantly. The efficiency of production of toxic metabolites such as mycotoxins by different isolates of *Fusarium* spp. may also vary and this factor is very crucial for the acceptance of cereal grains for human and animal consumption (Narayanasamy 2002). Within a morphological species, differences in the pathogenic potential may be recognized by using a set of differential varieties/genotypes of host plant that show a range of responses from susceptibility to high resistance to the isolates or strains of a fungal pathogen. The levels of pathogenic diversity have been determined for many fungal pathogens associated with seedborne diseases of wheat, barley, corn, and sorghum using sets of differential cultivars/genotypes (Table 3.1). This aspect is discussed in detail in Chapter 2.

Colletotrichum capsici causes pepper (chilli) fruit rot in tropical and subtropical countries. Isolates (37) of *C. capsici* were placed into five groups, namely Cc-I, Cc-II,

Table 3.1 Assessment of variability in pathogenic potential of fungal pathogens using differential cultivars/genotypes of crop plants.

Crop/disease	Fungal pathogens	References
Wheat/Fusarium head blight (FHB)	<i>Fusarium graminearum</i> , <i>F. graminearum</i> , <i>F. culmorum</i> , <i>F. poae</i>	Akinsanmi et al. (2006); Xue et al. (2008)
Wheat/Stagonospora nodorum blotch (SNB)	<i>Stagonospora nodorum</i>	Ali and Adhikari (2008)
Wheat/tan spot disease	<i>Pyrenophora tritici-repentis</i>	Aboukhaddour et al. (2013)
Barley/net form net blotch	<i>Pyrenophora teres</i> f. <i>teres</i>	Steffenson and Webster (1992); Liu et al. (2012)
Pepper/fruit rot disease	<i>Colletotrichum capsici</i>	Sharma et al. (2005)

Cc-III, Cc-IV, and Cc-V, based on differences in the cultural and morphologic characteristics. The size and shape of conidia did not show significant variations. By inoculating a differential set of *Capsicum* cultivars, differences in the virulence of 37 isolates were recorded and used for distinguishing 15 pathotypes in *C. capsici* populations prevalent in Himachal Pradesh, India. Pathotype Cc-I was the most virulent, capable of infecting all differentials used. The results of random amplified polymorphic DNA (RAPD) analysis corroborated the results of virulence assessment by inoculating differential cultivars of pepper (Sharma et al. 2005). *Plasmopara halstedii*, causal agent of sunflower downy mildew disease, exists as several races with differing aggressiveness (virulence). The assessment of aggressiveness of two races 100 and 710 of *P. halstedii* was accomplished by applying the method developed in this investigation. Two sunflower lines exhibiting different levels of quantitative resistance were used to determine their aggressiveness based on two criteria: latent period and sporulation density. A strain of race 100 required a shorter latent period and attained greater sporulation density than that of a strain of race 710. The inbred line, BT, needed a shorter latent period to express visible symptoms and allowed greater sporulation than the inbred line, FU, which showed a higher level of resistance to these races of *P. halstedii*. The results indicated that race 100 was more aggressive than race 710 on the susceptible sunflower inbred line under field and laboratory conditions (Sakr et al. 2009).

The aggressiveness (virulence) of isolates, representing eight species of *Fusarium* involved in Fusarium head blight (FHB) disease of wheat, was investigated. Six wheat lines and cultivars were artificially inoculated with twelve isolates of *Fusarium graminearum* and six isolates each of *F. acuminatum*, *F. avenaceum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. poae*, and *F. sporotrichoides* at 50% anthesis. Symptoms of FHB were rated as disease severity on a scale of 0–9 at 4, 7, 14, 21, and 28 days after inoculation and as a percentage of infected spikelets (IS) after 21 days. *F. graminearum*, *F. crookwellense*, and *F. culmorum* were able to induce severe disease symptoms (>35% IS). The results indicated that variations in the pathogenic potential of all isolates of *Fusarium* spp. should be considered during screening of wheat lines and cultivars (Xue et al. 2004).

Variability in the virulence of pathogens is assessed by inoculating a set of host plant differentials (genotypes) that exhibit differing responses with various isolates of the pathogen. It is essential to determine whether the host–pathogen interaction is differential or nondifferential for determining the levels of resistance of host genotypes.

A differential interaction occurs when the severity of the disease induced by the pathogen isolates (genotypes) is dependent on the host genotypes. In contrast, a non-differential association is seen when the severity of the disease induced by the pathogen isolate is not influenced by host genotype (Crute 1986). *Fusarium graminearum* and *F. pseudograminearum* cause crown rot disease of wheat. Pathogenic variability of *F. graminearum* and *F. pseudograminearum* existing in Australia was investigated. Overall, *F. pseudograminearum* was more aggressive than *F. graminearum*. When seedlings of 60 wheat lines were inoculated with four pathogen isolates, a lack of specialization was indicated. The first principal component representing nondifferential aggressiveness explained a large part (up to 65%) of the variation in crown rot (CR) severity. The differential components were small and more pronounced in seedlings than in adult plants. The results indicated that a range of analytical tools have to be employed to investigate pathogenic specialization (Chakraborty et al. 2010).

The relationship between phenotypic traits (mycelial growth, sporulation, and spore germination) and pathogenicity (in vitro glume infection and in vivo central floret inoculation) to two Brazilian wheat cultivars, Guamirin (moderately susceptible) and BRS194 (susceptible), was investigated using *F. graminearum sensu stricto* (*Fgss*) (either a 3-DON/15-ADON chemotype) and *F. meridionale* (*Fmer*) (NIV chemotype). Results showed significantly higher growth rates, greater spore production, and quicker germination for the *Fgss* isolates compared to *Fmer* isolates. The *Fgss* isolates were less sensitive to tebuconazole than *Fmer* isolates. All isolates infected glume tissues of both varieties with an overall higher infection frequency for *Fgss* than for *Fmer* isolates when inoculated on Guamirin than on BRS194, which exhibited similar infection frequency between species. The results suggested that phenotypic traits and host resistance might play a role in the selection of more aggressive populations under field conditions (Spolti et al. 2012).

Nitrogen application favors vegetative growth of plants and increases susceptibility of plants to pathogens. The gene expression patterns of nitrogen-responsive gene FGSG-03881 and role of nitrogen in virulence and toxin production in *Fusarium graminearum*, incitant of Fusarium head blight (FHB) of wheat, were investigated. Using transcriptional fusion pFGSG-03881::GFP, it was observed that FGSG-03881 exhibited differential expression profiles in 22 different nitrogen compounds. A mathematical equation was derived to normalize the growth with the expression of FGSG-03881. Expression of FGSG-03881 increased in the nonpreferred sources of nitrogen and decreased in the preferred nitrogen sources such as glutamine. In addition, this gene was not linked to the biosynthesis of the mycotoxin DON or regulated by the global regulator *Tri6*. Disruption of FGSG-03381 occurred when levels of infection and intensity of symptoms increased. The results suggested that FGSG-03881 might be responsive to nitrogen availability resulting in increased virulence of *F. graminearum* (Walkowiak and Subramaniam 2014).

Occurrence of false smut (green smut) caused by *Ustilaginoidea virens*, first reported from Tamil Nadu State, India (Ou 1972), has been observed to cause serious losses in several rice-growing countries including China and the USA. Isolates of *U. virens* (59) obtained from 46 rice hybrids in 14 counties of China were evaluated for their pathogenicity by inoculating them on three rice hybrids. Six isolate groups, differing in their pathogenicity, were differentiated. The resistance of the rice hybrids to the pathogen isolates differed significantly. In addition, there were significant differences in the responses of different female and male parents compared with isolates of the same male

and female parent origin, indicating that the parents of different hybrid combinations may also influence the pathogenicity of different isolate groups. Significant differences in the sporulation of 59 isolates of *U. virens* were also noted. Sporulation capacity might also be related to the pathogenicity, but not significantly ($P < 0.05$). Pathogenicity data suggested specialization was dependent on the site of origin of the isolates, the original host (rice hybrids), and the parentage of the original host (Lu et al. 2009).

3.3 Genotypic Characteristics of Fungal Pathogens

3.3.1 Pathogenicity of Fungal Pathogens

Two groups of microorganisms existing in the environment have been recognized based on their ability or inability to cause disease(s) in plants and other organisms. The microorganisms with unique genes for pathogenicity involved in the production of characteristic symptoms are the pathogens of different host plant species. In contrast, the other group of microorganisms, lacking pathogenicity genes, is unable to induce any disease with or without symptoms in other living host(s); they are saprophytes, involved in the degradation of dead organic matter. The pathogens have specific genes for adhesion to plant surface (or receptors), production of toxins and enzymes, resistance to oxidative stress or chemicals, degradation of plant cell walls, in plant nutrition, and in breaching defense strategies of plant hosts. The microbial pathogens possess highly specialized tactics to overcome plant defense systems and to promote development of disease. The pathogen factors capable of inducing susceptibility in host plants include host-selective toxins and small molecule suppressors from fungal pathogens. Susceptibility or resistance of plants to a pathogen is observed as a result of expression of virulence and avirulence gene(s), respectively (Narayanasamy 2008).

Fusarium graminearum, causing corn ear rot disease, produces trichothecene mycotoxins which act as virulence factors on some host plant species. Phylogenetic analyses of the sequences of a 19 kb region of trichothecene gene cluster showed that polymorphism within these virulence-associated genes has persisted through multiple speciation events (Ward et al. 2002). The trichothecene synthase gene (*TRI 5*) was characterized and shown to be a virulence factor for *F. graminearum*. Although the *tri5* deletion mutants had normal growth and development, they exhibited reduced virulence on Wheaton wheat, common winter rye, and corn (Bai et al. 2002). Mitogen-activated protein kinases (MAPKs) are involved in the transduction of extracellular signals. *Fusarium MAP1* gene was identified in *F. graminearum* by employing a PCR approach by its homology to a known pathogenicity gene of *Magnaporthe grisea*, the rice blast pathogen, the mitogen-activated protein kinase gene (*PMK1*). Gene-replacement *F. graminearum* mutants were nonpathogenic on wheat flowers and roots, and could not infect wounded wheat floral tissues or tomato fruits. *Map1* mutants lost their ability to form perithecia in vitro, but their rate of conidial production was not affected (Urban et al. 2003). A conserved mitogen-activated protein kinase (MAPK) cascade homologous to the yeast Fus3/Kss1 mating/filamentation pathway is involved in the regulation of vegetative development and pathogenicity in *Fusarium graminearum*. FgGpml (orthologue of Fus3/Kss1) was shown to be involved in vegetative differentiation and virulence-related functions (Zhao et al. 2007). The transcription factor Ste12 functions downstream of the MAPKs Fus3/Kss1. The whole-genome examination showed the

presence of the ortholog FgSte12 in *F. graminearum*. For the functional analysis of FgSte12, gene deletion mutants were generated using homology recombination strategy. The *FgSTE12* mutant ($\Delta FgSte12$) did not show recognizable phenotypic changes compared with the wild-type HN9-1 strain of *F. graminearum* in PDA or minimal medium (MM). The pathogenicity of $\Delta FgSte12$ was evaluated by point mutation (individual spikelets) with conidial suspensions on flowering wheat earheads. Scab symptoms did not develop in spikelets inoculated with conidia of $\Delta FgSte12$ mutant. In contrast, the wild-type HN9-1 and the complemented strain induced scab symptoms on inoculated wheat earheads. Although the mutant did not exhibit any detectable defect in vegetative development on wheat head medium compared to the wild-type HN9-1 strain, it was unable to infect wheat earheads. The results indicated that *FgSte12* was required for pathogenicity in *F. graminearum* (Gu et al. 2015).

The *CMK1* gene encoding a MAP kinase was reported to have a critical role in conidial germination, formation of melanized appressoria, penetration of plant surface, and subsequent colonization of tissues in cucumber infected by *Colletotrichum lagenarium*, causative agent of anthracnose disease. *CMK1* could complement appressorium formation of the *PMK1*-MAP kinase mutant of *Magnaporthe grisea*. Loss of *CMK1* led to reduction in conidiation and complete loss of pathogenicity on cucumber, since the conidia did not germinate and form appressoria in contrast to *pmk1* mutants of *M. grisea*. MAP kinase signaling pathways appeared to be important for the formation of infection structures by fungal pathogens (Takano et al. 2000). The darkly melanized appressoria formed at the end of germ tubes from the conidia of *Colletotrichum lagenarium* penetrate host plant surface. The *CMK1* gene regulates conidial germination, appressorial formation, and invasive growth. The *CST1* gene from *C. lagenarium* was characterized and the *cst1* Delta strains were nonpathogenic on cucumber leaves, but they could form lesions in wounded leaf surfaces. Infection hyphae were not formed from appressoria of *cst1* Delta strains, suggesting that *CST1* was required for penetration of host surface by infection hyphae. The rapid disappearance of lipid droplets during appressorium formation in *cst1* Delta strains might have a bearing on their ability to penetrate host plant surface (Tsuji et al. 2003).

The expression of genes of *Stagonospora nodorum*, causing wheat net blotch disease, was investigated. *Gna1*, a gene coding for a GI subunit, a key component of signal transduction pathways, was characterized. A slight decrease in transcription levels was observed shortly after spore germination, after which levels steadily increased until sporulation. A defect in direct penetration of host plant surface and consequent reduction in pathogenicity of the *Gna1* mutants was revealed. Furthermore, *Gna1* mutants did not sporulate and secrete one or more brown pigments into growth medium; no such secretion from the wild-type strain was detected, however. *Gna1* seemed to be the first signal transduction gene to be cloned and characterized from *S. nodorum* (Tan et al. 2004).

Sorghum head smut, caused by the soilborne facultative biotrophic fungal pathogen *Sporisorium reilianum* (anamorph *Sphacelotheca reiliana*), accounts for appreciable losses in grain yield. It is very difficult to eliminate the pathogen from the soil, even by adopting crop rotation to prevent the annual reinoculation of the pathogen teliospores. *S. reilianum* infects emerging sorghum seedlings, and visible symptoms of infection are expressed only at the flowering stage when the entire earhead is converted into a mass of countless teliospores covered by a thin whitish-gray membrane, resulting in total loss of grains from the infected plant. Changes in pathogen virulence were assessed by

applying four inoculation procedures, soil and teliospore mixture, seed coating, media placement, and syringe injection. The most effective method of inoculation was syringe injection. The syringe inoculation involves growing sporidial colonies for each of the 32 isolates of *S. reilianum* on potato dextrose broth (PDB) for 4 days on a rotary shaker set at 150 rpm. The sporidial inoculum (milky color) was filtered through cheese cloth and the concentration was adjusted to 1×10^5 spores mL⁻¹. The sporidial suspension was used to inoculate by injection below the apical meristem of 18–20 day old seedlings between 16:00 and 19:00 h to avoid drying due to heat. Each plant was injected with 0.5–1.0 mL of sporidial suspension using a Precision Glid Needle #229G × 1 attached to a 30 mL hypodermic syringe (Becton-Dickinson Co., NJ, USA). By inoculating six differential genotypes, 23 of 32 Texas isolates were identified as race 4. The presence of race 1 and new virulent races 5 and 6 was also recognized among the isolates from south Texas. Race 2 and race 3 were absent from the isolates of *S. reilianum* examined (Prom et al. 2011).

3.3.2 Production of Mycotoxins by Fungal Pathogens

Seedborne fungal pathogens may adversely affect production of grains both quantitatively and qualitatively. The reduction in grain weight and poor filling of grains due to infection reduces the amount of produce obtained. In addition, because of the contamination of seeds with toxic metabolites produced by the fungal pathogens, the grains become unfit for consumption as food and feed. The fungal secondary metabolites occurring naturally as contaminants of agricultural and other consumable products cause toxicity symptoms in humans and animals when administered via the natural route (oral consumption). These compounds are called mycotoxins and the ailments induced are referred to as mycotoxicoses. Various species of *Alternaria*, *Aspergillus*, *Fusarium*, and *Penicillium* infect seeds, resulting in poor seed quality and contamination of seeds with different kinds of mycotoxins. The genes involved in the biosynthesis of mycotoxins by fungi have been identified and their expression has been shown to have a bearing on the production of mycotoxins in seeds and other plant tissues.

The fungal pathogens infecting grains when grown on suitable media produce mycotoxins. The effect of carbon sources on the production of mycotoxins, emodin, catenarin, and islandicin by *Pyrenophora tritici-repentis*, causing wheat red smudge disease, was investigated using high-performance liquid-chromatography technique. The highest emodin concentration was observed for the isolate Alg3-24 on Fries medium supplemented with fructose, whereas the maximum concentration of catenarin was produced by the isolate TS93-71B in Fries medium supplemented with starch. Islandicin was not produced by any isolate of *P. tritici-repentis* under the experimental conditions provided. The dynamics of production of emodin and catenarin by isolate 331-2 on V8 PDA medium were determined. Rapid accumulation of the mycotoxins was noted during the first week of incubation, followed by a large decline by 14 days. As the optimal conditions and carbon sources required for mycotoxin production may vary widely, optimization of growth medium has to be taken up for evaluating the potential of fungal pathogens to produce mycotoxins (Bouras and Strelkov 2010).

Pyrenophora tritici-repentis produces the toxin inducing necrosis of infected leaf tissues. The proteinaceous effector ToxA was found to be responsible for foliar necrosis on ToxA-sensitive wheat genotypes. The single-copy *ToxA* gene was deleted from wild-type race 1 of *P. tritici-repentis* via homologous recombination of a knockout construct.

Expression of the *ToxA* transcript was found to be absent from transformants (*tox a*), resulting in the absence of ToxA protein production from culture filtrates. Plant bioassays showed that *toxa* strains were unable to induce necrosis on ToxA-sensitive wheat genotypes. This report appeared to be the first demonstration of a targeted gene knockout in *P. tritici-repentis* (Moffat et al. 2014).

Fusarium moniliforme (*Gibberella fujikuroi*) and other species of *Fusarium* produce fumonisins that contaminate corn. *FUM5*, the putative polyketide synthase gene involved in fumonisin biosynthesis, has been characterized (Proctor et al. 1999, 2004). *Fusarium verticillioides* also produces fumonisin in corn earhead. The presence of fumonisin produced by *F. verticillioides* could be detected in a single corn kernel by using reflectance and transmittance visible and near-infrared spectroscopy. The color and chemical constituents of infected kernels formed the basis for the detection of fumonisin in single kernels in a nondestructive manner (Dowell et al. 2002). *Fusarium graminearum*, causing wheat Fusarium head blight (FHB) disease, produces the mycotoxins deoxynivalenol (DON) and nivalenol (NIV) with the possibility of inducing mycotoxicoses in humans and animals. The gene *Tri3* involved in the production of DON was targeted for detection of *F. graminearum* in wheat grain sample by a PCR-based assay. The isolates of *F. graminearum* were grouped into three chemotypes based on the nature of DON produced by them (Jennings et al. 2004). Disruption of the trichodiene synthase gene *Tri5* of *F. graminearum* resulted in the loss of the ability to produce DON. Further, the spread of the mutant from the inoculated spikelet to other spikelets was also inhibited, indicating the vital role of DON in the progress of the disease in infected wheat ears (Bai et al. 2002).

Fusarium head blight (FHB) or scab disease of cereals is primarily induced by *Fusarium graminearum*, *F. culmorum*, and *F. cerealis* which elaborate trichodiene mycotoxins, mainly nivalenol (NIV) and deoxynivalenol (DON). Genes *Tri13* and *Tri7* from the trichothecene biosynthetic gene cluster convert DON to NIV (*Tri13*) and NIV to 4-acetyl-NIV (*Tri7*). The positive-negative PCR assays based on *Tri13* and *Tri7* sequences accurately indicated DON or NIV chemotype in *F. graminearum*, *F. culmorum*, and *F. cerealis*. The risk of trichothecene contamination could be assessed by employing the PCR assays developed in this investigation. All NIV chemotype isolates possessed functional copies of both *Tri13* and *Tri7*, while in all DON-producing isolates both genes were either disrupted or deleted. Several mutations in these genes were recognized and were conserved across *F. graminearum* lineage, RAPD and SCAR groupings, and between the three species of *Fusarium* (Chandler et al. 2003).

Expensive and laborious chemical analyses are required to estimate trichothecene content in wheat grains infected by *Fusarium graminearum* and other *Fusarium* spp. A real-time PCR assay was employed for quantification of trichothecene-producing *Fusarium* species (TMTR1 assay) and *F. graminearum* (TMFg12) present in barley grain and malt. The DNA was extracted from ground kernels using a commercial DNA extraction kit, and analyzed in a LightCyclerR system using specific primers and fluorogenic TaqMan probes. The TMTR1 and TMFg12 assays were effective in quantification of trichothecene-producing *Fusarium* DNA and *F. graminearum* DNA in barley grain and malt samples naturally and artificially contaminated with the respective pathogens. These two assays were sensitive and reproducible. The amount of *Fusarium* DNA analyzed with TMTR1-trichothecene assay could be employed for estimating the DON contents in barley grains. On the other hand, TMFg12 assay for *F. graminearum* provided reliable estimates of DON contents in the North American barley and malt

samples, while the assay gave poor results with Finnish samples which generally had low contents of DON (Sarlin et al. 2006).

A constitutively green fluorescent protein (GFP-) expressing wild-type strain and its knockout mutant, preventing trichothecene biosynthesis, was used to study the role of trichothecene synthase gene in the pathogenicity of *F. graminearum*. Trichothecenes were found to be virulence factors during infection through fruit coat of wheat grains. However, the pathogen spread was blocked in the absence of trichothecenes by the development of heavy cell-wall thickening in the rachis node of Nandu wheat, a defense breached by the mycotoxin. On the other hand, in barley the development of pathogen hyphae was inhibited at the rachis node and rachilla, limiting infection of adjacent florets through the phloem and along the surface of the rachis (Jansen et al. 2005). The genes responsible for production of zearalenone by *Gibberella zeae* (*F. graminearum*) were identified. In addition, a complete set of 15 PKS genes were also identified and characterized genetically. The functions of the products of these PKS genes in the life cycle of *G. zeae* were determined by genetically disrupting each of these PKS genes and characterizing the mutant isolates. Five of the 15PKS genes were involved in the biosynthesis of the mycotoxins zearalenone, aurofusarin, and fusarin and the black perithecial pigment. Diverse patterns of expression during grain colonization, plant colonization, sexual development, and mycelial growth could be recognized (Gaffoor et al. 2005).

Contamination of corn kernels with deoxynivalenol (DON), a trichothecene mycotoxin, increased in proportion to the incidence of *F. graminearum* (Ngoko et al. 2001). Disruption of the gene *FUM1* encoding a polyketide synthase required for fumonisin biosynthesis did not affect the pathogenicity of the mutant *Fusarium verticillioides*, as the corn inoculated with the mutant produced ear rot disease symptoms, in the same way as the wild parent strain of *F. verticillioides* (Desjardins et al. 2002). Synthesis of fumonisins by *Fusarium verticillioides* was shown to be directed by a cluster of 15 genes among which *FUM1* is the largest, encoding a polyketide synthase. This enzyme might catalyze the synthesis of a polyketide that forms a larger portion of the fumonisin structure. A total of 27 strains possessing the *FUM1* gene, as determined by PCR assay, were analyzed. A portion of the *FUM1* gene was amplified and sequenced from 6 of 27 Brazilian strains isolated from corn and sorghum. Six strains of *F. verticillioides* showed sequence similarity to almost 100% (da Silva et al. 2007).

Fusarium populations were sampled in 12 small commercial wheat fields (1–3 ha) in Germany at three spots per field with 12 heads each. PCR assays were employed using generic primers to confirm the identity of 338 isolates of *F. graminearum sensu stricto* (s.s.). Populations of *F. graminearum* s.s. in Germany had three types of trichothecenes with a dominance of 15-acetyldeoxynivalenol chemotype (92%) followed by 3-acetyldeoxynivalenol chemotype (6.8%) and a few isolates of nivalenol chemotype (1.2%). All these isolates were genotyped using 19 microsatellite loci. The 12 populations showed high genetic diversity within the small-scale sampling areas, resulting in 300 different haplotypes. Genetic diversity within the populations (71.2%) was appreciably greater than among population (28.8%), as indicated by molecular variance analysis. The composition of haplotypes of a population followed over 2 years changed considerably. The results showed that populations of *F. graminearum* s.s. in Germany exhibited a very high level of genetic variation on a local scale with a restricted diversity among populations (Talas et al. 2011).

Populations of *Fusarium graminearum*, causing Fusarium head blight (FHB) on wheat and barley, were considered to produce trichothecene mycotoxin deoxynivalenol

(DON) contaminating the grains. However, the genetically distinct Gulf Coast populations of *F. graminearum sensu strict*, demonstrated to produce nivalenol (NIV) from small grain-growing regions of Louisiana, were found to be NIV-producers. The isolates from Louisiana (237) were identified as distinct genotypes using the newly developed PCR-RFLP markers and multiplex primers that distinguished among three trichothecene types, namely two DON-types (15- and 3-ADON) and one NIV-type. Isolates classified as belonging to southern Louisiana population were predominantly of the NIV-type (93.6%). Aggressiveness and toxigenic potential were determined for 24 isolates that encompassed the genetic variations present in the *F. graminearum* clade from Louisiana (Fig. 3.2). Statistical analysis showed that overall spread in spikes and mycotoxin accumulation in inoculated spikelets was compared between NIV- and DON-producers. The DON-producers elaborated approximately four times as much toxin as the NIV-producers, while the DON-producers also spread significantly more (although that difference was not as pronounced) (Table 3.2). Further, aggressiveness and toxigenic potential were compared between NIV-types of *F. graminearum* and *F. asiaticum*. Infections with *F. asiaticum* isolates spread more and accumulated more NIV in plant tissues, compared with *F. graminearum*. The existence of NIV-type populations of *F. graminearum* s.s. and *F. asiaticum* in the USA was reported for the first time by Gale et al. (2011).

The regulatory mechanism of fumonisin B1 (FB1) production by *Fusarium verticillioides* in corn was studied. The *GBP1* gene, encoding a monomeric G-protein, and its

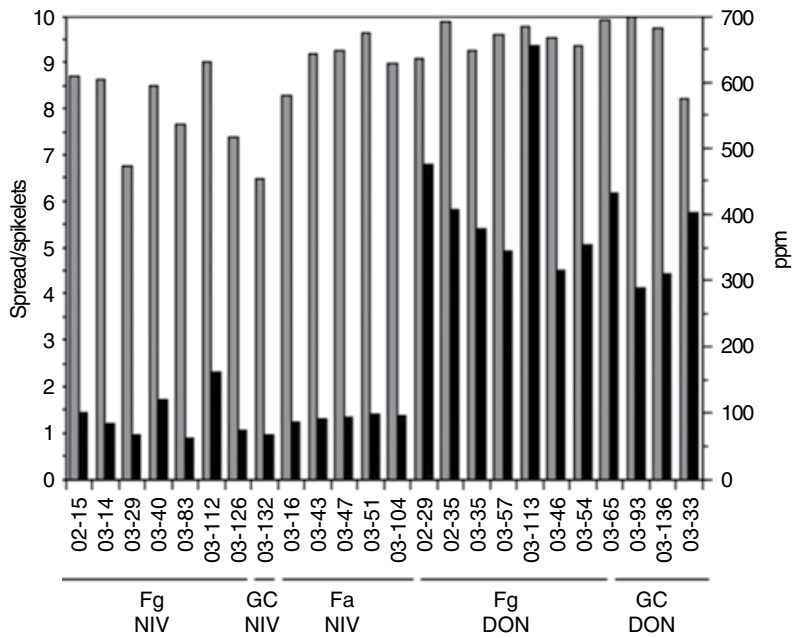


Figure 3.2 Assessment of virulence and toxigenic potential of isolates representing genetic variation in *Fusarium graminearum* present in Louisiana State, USA. Gray columns: average spread in spikelets in point-inoculated wheat spikes of susceptible cultivar Norm. Black columns: average trichothecene mycotoxins accumulation in ppm in inoculated spikelets; Fg: *Fusarium graminearum*; GC: Gulf coast population of *F. graminearum*; Fa: *F. asiaticum*; NIV: nivalenol; DON: deoxynivalenol. Source: Gale et al. (2011). Reproduced with permission of the American Phytopathological Society, MN, USA.

Table 3.2 Average spread in wheat spikelets and toxin accumulation in inoculated spikelets in cultivar Norm after inoculation with NIV- or DON-type isolates of *F. graminearum* clade from Louisiana (A) or NIV-type isolates of *F. graminearum* and *F. asiaticum* from Louisiana (B) (Gale et al. 2011).

Isolates of <i>Fusarium</i>	Trichothecene type ^a		NIV (ppm)
	NIV (13) ^c	DON (11)	
A. Isolates of <i>F. graminearum</i> clade			
Spread ^b	8.38	9.48	
Toxin (ppm)	99.1	388.8	
B. <i>F. graminearum</i> clade species ^a			
Spread ^b , <i>F. graminearum</i> (8)	7.96		86.6
Spread, <i>F. asiaticum</i> (5)	9.05		119.3

^a Numbers in parenthesis indicate number of isolates tested; ^b Symptoms assessed in 10 spikelets located above and below and including the inoculated spikelets at 14 days after inoculation (dai); ^c NIV types, DON for DON types estimated at 14 dai.

role in FB1 biosynthesis was investigated. *GBP1* was found to be an expressed sequence tag (EST) upregulated in the *F. verticillioides fcc1* mutant that showed reduced conidiation and no FB1 biosynthesis when grown on corn kernels. Sequence analysis showed that *GBP1* encodes a putative 368-amino acid protein with similarity to the DRG and Oby subclasses of G-proteins that are involved in development and stress responses. When grown on corn kernels, a *GBP1* knockout mutant ($\Delta gbp1$) exhibited normal growth but increased FB1 production (>58%) compared with wild-type. The complementation of $\Delta gbp1$ with the wild-type *GBP1* gene restored ability to produce FB1 to the levels in the wild-type strain. The results indicated that *GBP1* was negatively associated with FB1 biosynthesis, but not with conidiation in *F. verticillioides* (Sagaram et al. 2006). The gene *GBB1* encoding a putative subunit of a heteromeric G-protein was disrupted and the effects on fumonisin biosynthesis and virulence were evaluated. *GBB1* deletion mutant ($\Delta gbb1$) did not show significant differences in radial growth and mycelial mass, but it produced significantly less fumonisin B1 (FB1) compared with the wild-type strain. Reduced expression of FB1 biosynthetic genes *FUM1* and *FUM8* in the mutant $\Delta gbb1$ provided further evidence that *GBB1* was involved in FB1 regulation. The heteromeric G-protein β subunit played an important role in regulation of FB1 biosynthesis and hyphal growth, but not in virulence of *F. verticillioides* (Sagaram and Shim 2007).

The nature of mycotoxins may vary depending on the species of *Fusarium* and crop plant species infected. The main groups of mycotoxins produced by *Fusarium* spp. in corn are trichothecenes, zearalenones, fumonisins, and moniliformin. In *Fusarium*-infected corn ears, beauvericin and fusaproliferin have also been detected. Further, nivalenol and fusarenone-X were found to be associated with infection of corn ears by *F. cerealis* and diacetoxyscirpenol and T-2 toxin with the occurrence of *F. poae* and *F. sporotrichioides*, respectively. The presence of moniliformin was detected in corn ears infected by *F. avenaceum* and *F. subglutinans* (Logrieco et al. 2002).

High year-to-year variations in the corn ear rot disease caused by *Fusarium verticillioides*, *F. graminearum*, *F. cerealis*, *F. subglutinans*, and *F. proliferatum* and the level of mycotoxin contamination were observed in 84 field samples from Germany during 2006–2007. The growth season characterized by high temperature and low rainfall during anthesis and early grain filling resulted in 75% of the corn samples being contaminated with deoxynivalenol, 34% with fumonisins, and 27% with zearalenone. On the other hand, in the growth season with moderate temperature and frequent rainfall during 2007, none of the 40 corn samples had quantifiable levels of fumonisins, whereas deoxynivalenol and zearalenone were detected in 90% and 93% of the fields, respectively. Further, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), moniliformin, beauvericin, nivalenol, and enniatin B were detected in both growth seasons (2006 and 2007) with different environmental conditions as indicated above. The results indicated the differential influence of environmental conditions on different *Fusarium* spp., producing mycotoxins (Goertz et al. 2010).

The role of aggressiveness (virulence) of *Fusarium graminearum* and *F. culmorum* in the resistance of wheat cultivars to Fusarium head blight (FHB) disease was investigated. Aggressiveness of the pathogens depended on their capacity to produce deoxynivalenol (DON) and nivalenol (NIV). However, cultivar resistance influenced DON production significantly. In the highly resistant genotypes toxin contamination remained near zero, whereas very high concentrations of toxins were detected in susceptible cultivars. The toxin levels were correlated with the ratio of *Fusarium*-damaged kernels (FDK), and this ratio was very low in highly resistant cultivars. The results indicated that the DON-producing ability of pathogen isolates could be decisive in inducing disease incidence, and resistance cultivars might be a major factor in suppressing disease development and accumulation of DON in resistant cultivars (Mesterházy 2002).

In another investigation, the relationship between aggressiveness (virulence) and deoxynivalenol (DON) production by isolates of *F. graminearum* (24), half of which were 3-acetyldeoxynivalenol (3-ADON) and other half being 15-acetyldeoxynivalenol (15-ADON) chemotypes, were tested for their ability to produce DON and to induce Fusarium head blight (FHB) disease in spring wheat cultivars. Field tests of all isolates were conducted with three replicates each at two locations in Canada and Germany in 2008, with three host genotypes with varying levels of FHB resistance. The resistant host genotypes were resistant, irrespective of the pathogen chemotype or location. The differences between mean FHB indices of 3-ADON and 15-ADON isolates were not significant for any genotype. In contrast, average DON production by 3-ADON isolates (10.44 mg kg^{-1}) was significantly higher than for the 15-ADON isolates (6.95 mg kg^{-1}) at three of four locations where moderately resistant lines were tested, indicating the greater risk posed by 3-ADON isolates. Breeding for lines with high levels of resistance to FHB diseases appears to be the most effective disease management strategy for containing the disease incidence and restricting the mycotoxin contamination of grains (von de Ohe et al. 2010).

The degree of aggressiveness of *Fusarium graminearum sensu stricto* (s.s.) isolates and its relationship with trichothecene (mycotoxin) production was studied. Disease severity was visually assessed based on the percentages of spikelets bleached per ear as a parameter for aggressiveness (virulence). Disease severity induced by the pathogen isolates ranged over 27.19–84.73% and yield reduction (grain weight) caused by the isolates varied over 35–85%. In addition to loss of grain weight, grains infected

by *F. graminearum* contained significant levels of mycotoxins, trichothecenes. No correlation could be established between aggressiveness and the toxins in the kernels at maturity. Amplified fragment length polymorphism (AFLP) analysis showed that the isolates (18) of *F. graminearum* were grouped with the two *F. graminearum* s.s. reference isolates, with a similarity coefficient greater than 75%. Other reference strains of *F. graminearum* complex were clearly separated with similarities ranging over 55–73%. The AFLP groups did not show any relationship with the ability to produce mycotoxin on kernels or with geographical origin of the isolates (Alvarez et al. 2010).

The ability of mycotoxin production by *Gibberella zeae* (*Fusarium graminearum*) and the existence of vegetative compatibility groups (VCGs) were assessed. The populations of *F. graminearum* were obtained from wheat in Argentina. Production of deoxynivalenol (DON), nivalenol (NIV), and 3-acetyldeoxynivalenol (3-ADON) by 70 strains of the pathogen were determined using nitrate nonutilizing (*nit*) mutants. Out of 367 *nit* mutants generated, 41% utilized both nitrite and hypoxanthine but not nitrate (*nit3*); 9% utilized nitrite, but not hypoxanthine (*NitM*); and 5% utilized all the nitrogen sources (*crn*). Fifty-five different VCGs were identified and the overall VCG diversity (number of VCGs/number of isolates) averaged over the three locations was 0.78. Forty-eight strains were incompatible with all others and each of these strains constituted a unique VCG. Analysis of the nature of mycotoxins produced by 70 strains of *F. graminearum* showed that only 90% of the strains produced DON, and 10% produced DON and very low concentrations of 3-ADON. None of the isolates produced NIV. As high diversity in VCGs was noted in *F. graminearum* populations occurring on wheat germplasm, entries have to be screened using a mixture of all VCGs in breeding programs (Ramirez et al. 2006).

Competition effects among isolates of *Fusarium culmorum*, causing seedling diseases, foot rot, and head blight of cereals, were estimated under field conditions. Either four isolates individually or four 1:1 mixtures of the same isolates were tested on two winter rye single crosses under six field environments. Two isolates FC46 and FC64 were highly aggressive deoxynivalenol (DON) and 3-acetyldeoxynivalenol producers, while the isolates FC 30 and FC71 were medium aggressive nivalenol (NIV) producers. Rye heads were inoculated during flowering with conidia of pairs of isolates, expressing similar (FC46 + FC64, FC30 + FC71) or contrary (FC46 + FC71, FC30 + FC64) levels of aggressiveness and similar or different concentrations and chemotypes of mycotoxins, respectively. Aggressiveness was determined based on head blight disease rating and effect on yield components. The identity of isolates obtained from inoculated rye earheads was established using RAPD-PCR molecular markers in one environment. Aggressiveness of the isolate mixtures was significantly lower than that of the isolates applied individually on both rye genotypes. Likewise, mycotoxin combinations were significantly lower in earheads inoculated with isolate mixtures in seven out of eleven comparisons (Miedaner et al. 2004).

The adverse effects of mycotoxins on seed germination and seedling development were determined for *Fusarium* spp. causing Fusarium head blight (FHB) of oats in Norway. Oat genotypes were screened by spawn- and spray inoculation under field conditions during 2007 to 2010. The relationship between reduction in germination capacity (GC) and deoxynivalenol (DON) contamination was investigated. Correlation coefficients between GC and DON level varied from -0.412 to 0.711 ($P < 0.001$). High DON levels were associated with low GCs, but the reverse was not always related. DON did not inhibit initiation of germination, but it did retard seedling growth in a

germination assay performed on DON-amended water agar (WA). Abnormal seedling morphology indicated the negative effect of DON on seedling development. Seed infection was high (>60%) for both types of inoculations (Tekle et al. 2013).

Fusarium graminearum, the principal pathogen causing Fusarium head blight (FHB) disease of wheat in Canada, produces deoxynivalenol (DON) and its acetylated forms 3-ADON or 15-ADON. The 3-ADON chemotype increased in the number of isolates of *F. graminearum* collected in Manitoba between 1998 and 2004, whereas prior to 1998 the 15-ADON chemotype was considered to be the only significant cause of FHB in the North America. The ratio of 3-ADON to 15-ADON chemotypes was monitored by recovering them from field plots artificially inoculated, usually in a 1:1 ratio of 3-ADON and 15-ADON isolates. The majority of isolates recovered from two locations (Carman and Glenlea) were of the 3-ADON chemotype in 2008. In the subsequent years however, the ratio was closer to 1:1 or sometimes with a slight predominance of 15-ADON isolates. Under controlled conditions, recovery of 15-ADON isolates predominated at lower incubation temperatures (18–22°C), whereas at 28°C, 3-ADON isolates were more prevalent. At moderate temperature of 24°C, the chemotypes were found at different ratios. Analysis of weather variables over the 5 year period of field studies did not indicate any correlation between the recovery of chemotype and temperature or rainfall (Gilbert et al. 2014).

The effects of infection timing on disease development and toxin accumulation were assessed using eight characterized isolates of *F. graminearum*, *F. culmorum*, and *F. poae* involved in FHB disease under controlled conditions. The fungal pathogens were inoculated on winter wheat spikes before or at another extrusion or at 8, 18, and 28 days later. Disease levels were estimated by AUDPC and 1000-kernel weight (TKW), while the fungal biomass and toxin concentration were determined by qPCR assay and UPLC-UV-MS/MS, respectively. Toxin content correlated well with fungal biomass in kernels independently of inoculation date. The AUDPC was correlated with fungal DNA, but not for early and late infection dates. The highest disease and toxin levels were associated with inoculation around anthesis, but early or late infections resulted in detectable levels of pathogen biomass and toxin for the most aggressive isolates. Development of the pathogens seemed to be faster in kernels than in the chaff in plants inoculated at anthesis, while the opposite trend was seen in plants inoculated later. The results confirmed the earlier view that the most susceptible stage of wheat was anthesis, but early and late infections might also lead to higher levels of disease severity and toxin accumulation which might be difficult to estimate by visual examination (Siou et al. 2014).

Aspergillus flavus, infecting maize (corn) grains, produces aflatoxins that are a series of highly toxic polyketides with carcinogenic and teratogenic properties. Aflatoxin contamination leads to reduced crop value and adverse health hazards to humans and domestic animals when contaminated grains are consumed. *Aspergillus flavus* has several morphotypes (strains) among which the L and S strains have been investigated frequently. L strain isolates produce few large sclerotia and highly variable amounts of aflatoxins, with some isolates (atoxigenic) entirely lacking the ability to produce aflatoxins. On the other hand, S strain isolates produce numerous small sclerotia and higher concentrations of aflatoxins (Cotty 1989). *A. flavus* isolates (>3,400) were recovered from 263 ground maize samples collected from four provinces of Kenya during 2004–2006. A total of 96 atoxigenic isolates lacking the capacity to produce aflatoxins were identified. The atoxigenic isolates were classified into 53 vegetative compatibility groups (VCGs) through complementation of nitrate nonutilizing mutants. Atoxigenic

isolates were tested for their potential to reduce aflatoxin concentrations in viable maize kernels that were coinoculated with highly toxigenic S strain isolates. Twelve isolates reduced aflatoxin levels by more than 80%. These atoxigenic *A. flavus* isolates have the potential for practical exploitation for reducing aflatoxin contamination of maize grains (Probst et al. 2011).

3.3.3 Production of Enzymes and Other Compounds by Fungal Pathogens

Seedborne fungal pathogens produce various enzymes and diverse compounds during pathogenesis (process of disease development). The enzymes may be produced in cultures and in planta by the fungal pathogens that may be required for their growth and reproduction, and also for the breakdown of complex compounds in the plants into simpler forms that can be absorbed by the pathogens. The fungal pathogens may produce growth-regulating compounds that are responsible for the formation or enlargement of plant cells or tissues. Of the several enzymes produced by fungal pathogens, cell-wall-degrading enzymes (CWDEs) are important for fungal invasion of different plant tissues. The importance of CWDEs produced by *Fusarium graminearum* during infection of wheat spikes was elucidated by observations under scanning electron microscope (SEM) and transmission electron microscope (TEM), coupled with enzyme-gold and immuno-gold labeling techniques. The results indicated that the polysaccharide-degrading enzymes might be important pathogenicity factors of *F. graminearum* during infection of wheat spikes (Wanjiru et al. 2002).

Subcellular localization of an acidic β -1,3-glucanase and acidic chitinase in *Fusarium culmorum*-infected wheat spike was visualized by employing the antisera raised against these enzymes from tobacco and immunolabeling technique. Distribution of β -1,3-glucanase and chitinase was very similar in the uninoculated healthy and infected wheat spikes. The enzymes were primarily localized in the cell walls of different tissues, including the lemma, ovary, and rachis of wheat spike, while the cytoplasm and organelles of cells in these tissues were without any immunogold label. However, accumulation of β -1,3-glucanase and chitinase in the infected wheat spikes differed distinctly between resistant and susceptible wheat cultivars. The results indicated that β -1,3-glucanase and chitinase accumulation in the pathogen-infected wheat spike might be involved in resistance to pathogen spread in the host tissue (Kang and Buchenauer 2002). Cell-wall-degrading enzymes (CWDEs) secreted by *Fusarium graminearum* have been shown to be important pathogenicity factors. FgGpmk1 (involved in virulence of *F. graminearum*) regulates the activities of extracellular glucanases and xylanolytic and proteolytic enzymes and secretion of lipolytic enzymes (Jenczmionka and Schafer 2005). The Δ FgSte12 mutant of *F. graminearum* could not penetrate a cellophane sheet, probably due to its reduced proteolytic and cellulase activities, compared to the wild-type HN9-1 strain. However, the mutant strain did not show any reduction in lipolytic activity. Quantitative real-time PCR assay showed that the expression levels of four cellulase genes decreased significantly in Δ FgSte12 mutant strain. The *FgGPMK1* mutant also exhibited defects in pathogenicity, penetration of cellophane and secretion of protease and cellulase as the *FgSte12* mutant strain. FgGpmk1 controlled the nuclear localization of FgSte12. The results indicated that FgSte12 played an important role in the pathogenicity in *F. graminearum* (Gu et al. 2015).

Fusarium moniliforme produces endopolygalacturonase (endo-PG) and pectate lyase (PAL) in liquid medium containing 1% citrus pectin and 0.2% glucose at pH 5.0

and pH 8.0, respectively. About 10 h after completion of conidial germination, production of the enzyme into the medium reached its peak. The addition of 0.2% glucose enhanced endo-PG production, while enzyme production was completely repressed by 1% glucose. The similar effects of glucose were observed on the production of pectate lyase. These two enzymes produced by *F. moniliforme* were quantified by enzyme-linked immunosorbent assay (ELISA) and immunolocalization. The results suggested that glucose might control the expression of both enzymes at transcription level (Niture et al. 2006). Factors governing the regulation of *Sclerotinia sclerotiorum* cutinase A (SsCUTA) and polygalacturonase 1 (SsPG1) during disease development were investigated. *SsCutA* transcripts were detected within 1 h postinoculation of leaves and its expression was primarily governed by contact of mycelia with solid surfaces. The expression of *SsPg1* was moderately induced by contact with solid surfaces including leaf, and its expression was restricted to the expanding margin of the lesion as the infection progressed. Glucose supported a basal level of *SsPg1* expression, but accentuated expression when provided to mycelia used as inoculum. Further, disruption of calcium signaling affected *SsCutA* and *SsPg1* expression and decreased the virulence of *S. sclerotiorum*, whereas cAMP levels reduced virulence without affecting gene expression (Bashi et al. 2012).

Ustilago maydis causes corn smut disease, characterized by tumor formation in place of healthy heads. The pathogen has yeast-mycelium dimorphic phases during its growth. The intracellular aspartylacid proteinase (pumiA) has been shown to be associated with the yeast-mycelium dimorphic transition. The proteinase was purified from a cell-free extract by ammonium sulfate fractionation and chromatographic steps. The enzyme had a mass of 35.3–36.6 kDa, a pH and temperature optimum of 4.0 and 40°C, respectively, and a pI of 5.5. The enzyme degraded hemoglobin, gelatin, albumin, and casein but not collagen, and the enzymatic activity was strongly inhibited by pepstatin K with aspartyl proteinase-specific inhibitor. The biochemical properties of pumiA were similar to other fungal intracellular aspartyl proteinases (Mercado-Flores et al. 2005). *Ustilago maydis*, in addition to enzymes, produced indole-3-acetic acid (IAA) efficiently from tryptophan. In order to understand the role of IAA in induction of tumors in maize plants, the pathways leading to fungal IAA biosynthesis were studied. In addition to *iad1* gene, another indole-3-acetaldehyde dehydrogenase gene *iad2* was also identified. The mutants $\Delta iad1$ and $\Delta iad2$ were also blocked in the conversion of both indole-3-acetic acid and tryptamine to IAA, although the reduction in IAA formation from tryptophan was not significantly different from $\Delta iad1$ mutants. The influence of indole-3-pyruvic acid on IAA formation was assessed by deleting the aromatic amino acid aminotransferase genes *tam1* and *tam2* in the mutants. This resulted in a further reduction in IAA levels by five- and ten-fold in mutants, harboring the $\Delta tam1$ and $\Delta tam2$ deletions, respectively. The results showed that indole-3-pyruvic acid served as an efficient precursor of IAA formed in *U. maydis*. The enhanced levels of host IAA following infection by *U. maydis* were reduced significantly in corn tissues infected by $\Delta iad1\Delta iad2\Delta tam1$ or $\Delta iad1\Delta iad2\Delta tam2$ mutants, whereas induction of tumors was not affected. The results showed that fungal IAA production critically contributed to IAA levels in infected tissue, but IAA production by the pathogen was not apparently important for triggering host tumor formation (Reineke et al. 2008).

Plasmopara halstedii, causal organism of sunflower downy mildew disease, is a homothallic species and has several sexual cycles in one season, resulting in possible formation of new recombinants. It is essential to determine the structure of local populations of *P. halstedii* and to monitor changes occurring in its populations over time.

Isozyme analysis has been shown to be useful to resolve some taxonomic problems, identify unknown isolates, analyze the extent of genetic variability in pathogen populations, and trace geological origins of pathogen strains/isolates. Isozyme analysis by cellulose-acetate gel electrophoresis technique was employed for assessing the variability in 45 isolates of *P. halstedii* from sunflower, cocklebur, and *Helianthus x laetiflorus*. Of the 16 isozyme systems analyzed, three enzymes – namely isocitrate dehydrogenase, malate dehydrogenase, and phosphoglucumutase – produced clear, reproducible banding patterns and revealed some polymorphisms among the isolates tested. Phosphoglucumutase differentiated two groups among the isolates collected from sunflower, while other enzymes were polymorphic between isolates from different hosts. Polymorphisms were not related to virulence phenotypes. Differences among isolates appeared to be associated with host origin rather than geographic location or virulence. All tested isolates were homozygous for the isozymes detected, regardless of their origin. Isozyme analysis was found to be robust, easy to score, and reproducible across laboratories (Komjáti et al. 2008).

Accumulation in and contamination of vegetables with mycotoxins produced by fungal pathogens infecting asexually propagated crops have been a great concern for producers and consumers. *Fusarium proliferatum* infects onion and garlic plants. Isolates of *F. proliferatum* obtained from roots and bulbs were characterized for their sexual fertility, and the sexual stage was identified as the mating population D of *Gibberella fujikuroi* complex. Eleven isolates from onion and garlic were also tested for their pathogenicity and toxigenicity. All isolates were pathogenic to both hosts; onion plants were significantly more susceptible, showing a higher disease severity index. Six isolates produced fumonisin B1 from 25–3000 $\mu\text{g g}^{-1}$ and beauvericin from 400–550 $\mu\text{g g}^{-1}$; ten isolates produced fusaric acid (80–950 $\mu\text{g g}^{-1}$) and moniliformin (50–520 $\mu\text{g g}^{-1}$); and all isolates produced fusaproliferin up to 400 $\mu\text{g g}^{-1}$. The results indicated that there was a potential risk due to contamination of mycotoxins produced by *F. proliferatum* for onion and garlic crops and bulbs (Stankovic et al. 2007).

Fusarium graminearum, causing dry rot of potato tubers, produces trichothecene mycotoxins in the infected tubers. Accumulation and diffusion of trichothecenes in potato tubers were monitored. Potato tubers of cultivar Russet Burbank were inoculated with 14 *F. graminearum* isolates from potato, sugar beet, and wheat, followed by incubation at 10–12°C for 5 weeks. Twelve isolates were deoxynivalenol (DON) genotype, while two isolates were nivalenol (NIV) genotype. DON was present up to a concentration of 39.68 $\mu\text{g mL}^{-1}$ in the rotted potato tissue, whereas accumulation of NIV up to 18.28 $\mu\text{g mL}^{-1}$ was recorded. Potato tubers were inoculated with two isolates of *F. graminearum* producing DON, and incubated up to 7 weeks at 10–12°C. The recovery of *F. graminearum* from the rotted tissue was 60% after 3 weeks of incubation, which was significantly different from the adjacent asymptomatic tissue 1, 2, and 3 cm away from the lesion margin. A recovery of 25% was obtained from 1 cm distal to the lesion, but was significantly different from the rotted tissue and the section adjacent to asymptomatic tissue 3 cm away from the lesion margin. After 7 weeks of incubation, colonization and penetration of *F. graminearum* were 82, 68, 64, and 53% in the rotted tissue and adjacent asymptomatic tissue 1, 2, and 3 cm distal to the lesion respectively. DON was not detected in the surrounding tissue (Delgado et al. 2010).

Sugar beet crops can be infected by *Fusarium acuminatum*, *F. avenaceum*, *F. culmorum*, and *F. graminearum*, resulting in significant reduction in root yield and sucrose concentration in the roots. During storage, a different species composition was observed.

F. redolens was predominant in freshly harvested beet root, whereas *F. culmorum*, *F. cerealis*, and *F. graminearum* comprised 50% and 84.8% in 2006 and 2007, respectively, of the sugar beet mycoflora in long-term pile storage. However, *F. graminearum* and *F. sambucinum* could cause severe root symptoms. The potential of 34 isolates of different species selected for mycotoxin production was assessed. A total of 26 *Fusarium* mycotoxins were detected by liquid-chromatography tandem mass spectrometry, including trichothecenes, zearalenone, and especially high concentrations of beauvericin, enniatins, and moniliformin. As many *Fusarium* spp. were associated with beet roots in storage, it is likely that many other compounds of pathogen origin might be present in beet roots for which further investigations may have to be pursued (Christ et al. 2011).

3.3.4 Sensitivity of Fungal Pathogens to Fungicides

Management of seedborne fungal pathogens relies primarily on the use of disease-free seeds and propagules. Crop production system is based on integration of agricultural practices and plant protection measures applied prior to and after sowing. Application of fungicides and chemicals to the seeds, propagules, plants, and soil has been practiced to reduce the pathogen population and to restrict the incidence and spread of the diseases. Frequent and indiscriminate application of fungicides has resulted in insensitivity in fungal pathogens, necessitating the use of higher concentrations of fungicides to which they have developed resistance. In addition, enhanced levels of pollution of the environment and retention of chemical residues in grains, fruits, vegetables, and other food products have also been observed. Seed treatment with fungicides is the preferred method of chemical application against fungal pathogens present in seeds, propagules, and soil, since this method is easier and more economical than soil and spray application on plants under field conditions. Loss of sensitivity of fungal pathogens to different groups of fungicides has been observed. Knowledge of decreasing sensitivity of fungal pathogens to fungicides may be useful to develop effective integrated disease management systems.

Carbendazim is one of the major fungicides used for the management of wheat *Fusarium* head blight (FHB) disease. The effectiveness of carbendazim was threatened by the emergence of pathogen populations resistant to carbendazim in the field. Different levels of sensitivity to carbendazim of field isolates of *Gibberella zeae* (*Fusarium graminearum*) infecting wheat in China were observed. A total of 56 isolates were characterized by their ability to grow on potato sucrose agar amended with different concentrations of carbendazim. The field isolates were classified into sensitive (S, inhibited at $1.4 \mu\text{g mL}^{-1}$), moderately resistant (MR, completely inhibited at $100 \mu\text{g mL}^{-1}$), and highly resistant (HR, only partially inhibited at $100 \mu\text{g mL}^{-1}$). Inheritance of carbendazim resistance was studied by analyzing the sensitivity of hybrid F1 progenies of six isolates, representing three sensitivity-level phenotypes selected randomly. The nitrate nonutilizing mutant (*nit*) was used as genetic marker to confirm that individual perithecia were resultant of out-crossing of $S \times S$, $MR \times S$, $MR \times MR$, $HR \times S$, and $HR \times MR$ isolates. In crosses between parent with sensitivity levels $MR \times S$, $HR \times S$, and $HR \times MR$, the progeny fit a 1:1 segregation ratio of two parental phenotypes. In crosses of $S \times S$ and $MR \times MR$, no segregation occurred. The results indicated that the MR and HR phenotypes in *G. zeae* were conferred by different allelic mutations within the same locus. Resistance to carbendazim in these isolates was not affected by modifying genes or cytoplasmic components (Yuan and Zhou 2005).

Fusarium graminearum (*Gibberella zeae*), causing wheat scab (*Fusarium* head blight), became insensitive to carbendazim after about 30 years of application to wheat crops in China. Pathogen populations on the eastern coast and along areas of the Yangtze River commonly showed MBC resistance. EC_{50} (effective concentration based on the inhibition of mycelial growth by 50%) values of resistant isolates of *F. graminearum* averaged $7.02 \pm 1.86 \mu\text{g mL}^{-1}$, while the minimum initiative concentration values were less than 0.9 and $1.4 \mu\text{g mL}^{-1}$, respectively. Both field-resistant and sensitive MBC strains shared similar temperature sensitivity, fitness, and virulence on ears. Field-resistant strains and UV-induced mutants exhibited cross-resistance to other benzimidazole derivatives and were mainly at intermediate MBC resistance level. High resistance to MBC in field isolates was rarely observed. No mutation in β -tubulin was found in *F. graminearum*, in contrast to mutation in this tubulin which resulted in MBC resistance in other plant pathogens (Chen et al. 2007). In a further study, five isolates of *F. graminearum* representing three phenotypes of different carbendazim sensitive levels (S, MR, and HR) were randomly selected to study the inheritance of carbendazim resistance by three genetic crosses under field conditions. Each parent in all crosses was marked with a different class of nitrate nonutilizing (*nit*) mutation. Results revealed that 5.7–20.9% outcrossing frequency occurred in the three crosses and confirmed sexual recombination under field conditions. No significant differences in mycelial growth and pathogenicity between selected recombinants and their parents were noted, but their sporulating ability and capacity to produce perithecia showed differences. However, most of the sexual recombinants possessed fitness levels comparable with those of their parents. The results indicated that sexual recombinations could play a significant role in the development of carbendazim resistance in the field (Chen and Zhou 2009).

Single-nucleotide polymorphisms (SNPs) are associated with resistance of fungal pathogens to the MBC-group fungicides such as carbendazim; a method for SNP analysis therefore has to be employed to detect mutations related to carbendazim resistance in fungal pathogens. Primer-induced restriction analysis (PIRA-) PCR was developed to detect isolates of *Fusarium graminearum* with moderate resistance to carbendazim. Isolates of *F. graminearum* (26) collected from infected spikelets from different fields in China were evaluated for their sensitivity to carbendazim, assessed (i) by the conventional method of measuring the mycelial growth on fungicide-amended agar; and also (ii) by the PIRA-PCR format, using the outer primer pair NoF+NoR and using the inner primer pair Hind3F+Hind3R. The results of conventional method and PIRA-PCR format were similar. However, the conventional method required 5–7 days to identify the genotype of *F. graminearum* with moderate resistance to carbendazim, whereas PIRA-PCR assay could provide the results in 7–8 h, indicating the rapidity of providing the results of tests (Luo et al. 2009).

Point mutations in the β -tubulin gene (FSG6_066113) were shown to be responsible for the development of resistance in *F. graminearum* to the benzimidazole fungicide. Point mutation at codon 167 (TTT→TAT, F167Y) was detected in more than 90% of field isolates in China. It was therefore considered necessary to develop an effective method for detection and quantification of this point mutation in *F. graminearum* populations. A pair of primers, codon 167F/codon 167R, was designed to amplify a fragment containing the mutation site. Two cycling probes labeled with different fluorescent reporters were employed to detect occurrence of mutation. Frequency of the point mutation was assessed in 282 *F. graminearum* perithecia collected from different fields in Jiangsu Province. The mutation frequency in ascospores from perithecia to carbendazim was

6.0% as determined by spore germination assay, while the mutation frequency at codon 167 was only 3.9% as shown by the cleave real-time PCR assay. The cleave real-time PCR method determined point mutations in the β -tubulin gene in the populations of *F. graminearum*, conferring resistance frequency in the fungal pathogen (Hou et al. 2011).

Sensitivities of wheat head blight pathogens *Fusarium graminearum* and *Microdochium nivale* to strobilurin fungicides showed distinct differences. Strobilurin fungicides are known to inhibit the activity of complex III in the mitochondrial electron transport chain. In the medium amended with azoxystrobin (AZ), *F. graminearum* had higher oxygen consumption. On the other hand *M. nivale*, with a greater level of sensitivity to azoxystrobin, did not show any difference in AZ-amended medium. Treatment with azoxystrobin enhanced the generation of intracellular H_2O_2 in both fungal pathogens. *M. nivale* consistently registered higher activities of antioxidant enzymes than *F. graminearum* following AZ treatment. The quantification of alternative oxidase (AOX) transcripts by real-time PCR indicated that transcription of AOX in *F. graminearum* and *M. nivale* was induced by AZ, suggesting regulation of AOX activities at transcription level in both pathogens. AZ induced AOX transcription rapidly in *F. graminearum*, reaching the peak by 60 min after treatment. In contrast, slow and low rate of AOX induction characterized the response of *M. nivale* to AZ. This differential ability of *F. graminearum* and *M. nivale* to activate AOX transcription might be responsible for the variation in their sensitivities to azoxystrobin (Kaneko and Ishii 2009).

Isolates (55) of *Fusarium graminearum* (*Gibberella zeae*) isolated in Belgium, Canada, Germany, Italy, Luxembourg, and the USA belonged to three chemotypes: 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and nivalenol (NIV). These isolates were screened for their sensitivity to the fungicide trifloxystrobin using the liquid culture method. None of the isolates was entirely inhibited by the fungicide up to 3 mM concentration. On the other hand, prothioconazole completely inhibited the mycelial growth to a level of 14–65% of the strain tested. The extent of inhibition was not significantly affected by the country of origin or by the chemotype. As the strains of *F. graminearum* were isolated before the market introduction of strobilurins, the high level of resistance of isolates of *F. graminearum* might be largely a case of natural resistance and not primarily related to strobilurin use for the crop disease management (Dubos et al. 2011).

Isolates of *Ustilago maydis* were exposed to UV-irradiation and the resistant mutants were selected using the media containing $75 \mu\text{g mL}^{-1}$ fenpropidin, the piperidine fungicide. Genetic analysis of 15 mutants resistant to fenpropidin resulted in the identification of two unlinked chromosomal loci, U/fpd-1 and U/pfd-2. Mutations in these loci were responsible for moderate resistance levels to fenpropidin. Haploid strains carrying both U/fpd mutations did not exhibit higher levels of resistance to fenpropidin, indicating a lack of additive gene effect between nonallelic genes. Isolates of U/pfd mutants showed a positive cross-resistance to the piperidine, piperalin, and to the related morpholine fungicides fenpropimorph and tridemorph, but not to the inhibitors of C-14 demethylase and squalene epoxidase. Progenies of crosses between U/fpd-2 and U/fpm-2 mutants were analyzed. No recombinants with wild-type sensitivity could be recognized. A 1:1 progeny segregation was however noted at the minimal inhibitory concentrations (MIC) for the U/fpd-2 isolates, indicating that these genes were alleles of the same locus. The results indicated that the U/fpd mutations did not influence the virulence fitness-determining characteristics like growth in liquid culture and pathogenicity on young corn plants (Markoglu and Ziogas 2001).

Development of insensitivity to azoxystrobin in the isolates of *Didymella bryoniae* (anamorph *Stagonospora cucurbitacearum*), causing cucurbit gummy stem blight disease, was observed in the eastern USA 2 years after first commercial use in 1998. Sixty-one isolates of *D. bryoniae* collected before 1998 were sensitive. Median EC₅₀ was 0.55 mg L⁻¹ azoxystrobin (range of 0.005–0.81). Forty isolates collected after exposure during 1998 were also sensitive. On the other hand, 53 of 68 isolates collected from South and North Carolina between 2000 and 2006 were insensitive to 10 mg L⁻¹ azoxystrobin. Sensitive and insensitive isolates were differentiated by their development on *Cucumis melo* seedlings treated with azoxystrobin (20 or 200 mg L⁻¹). The presence of insensitive isolates of *D. bryoniae* was detected in fields with a history of strobilurin applications (Keinath 2009).

Resistance to fungicides may develop in fungal pathogens due to exposure to high-risk fungicides or the introduction of resistant isolates from external source(s). In order to assess the possibility of watermelon seeds being a potential source of fungicide-resistant inoculum of the fungal pathogen *Didymella bryoniae*, causing gummy stem blight (GSB) disease of watermelon and other cucurbits, direct plating, sweat box, and blotter assays were employed. Seeds from commercial watermelon seed lots were visually examined for symptoms of GSB disease. Three isolates of *D. bryoniae* were isolated from two seed lots and were evaluated for their levels of sensitivity/insensitivity to boscalid, tebuconazole, azoxystrobin, and thiophanate-methyl, which were commonly used for GSB disease management. The isolates were resistant to thiophanate methyl, indicating the existence of fungicide-resistant isolates of *D. bryoniae* in naturally infested commercially distributed watermelon seeds. The results highlight the serious situation, demanding a new approach for fungicide resistance management in watermelon production (Thomas et al. 2014).

Magnaporthe grisea, the causative agent of rice blast disease, is inhibited by Qo- inhibiting fungicides which act as respiration inhibitors by binding to the Qo center of cytochrome *b*. Sensitivities of fungal pathogens can be influenced by the induction of alternative respiration by mutational changes of the cytochrome *b* target site. The expression and impact of both resistance mechanisms operating in *M. grisea* during conidial germination were investigated. In the absence of a host plant, alternative respiration provided a >500-fold rescue from azoxystrobin during germination of conidia derived from four wild-type isolates of *M. grisea*. The pronounced effectiveness of alternative respiration during conidial germination was not apparent, when barley leaves were protected by azoxystrobin prior to inoculation with conidia. A wild-type strain and an alternative respiration-deficient mutant were compared. Azoxystrobin efficacies in suppressing symptom development differed by a factor of two, with full disease suppression achieved for both genotypes at 1 µg mL⁻¹ of azoxystrobin. In contrast, conidia derived from two QoI-resistant target-site mutants of trifloxystrobin are fully capable of infecting leaf surfaces protected with 10 µg mL⁻¹ of azoxystrobin. Both target-site mutants emerged spontaneously in the presence of high azoxystrobin doses when residual mycelial growth was supported by alternative respiration (Avila-Adame and Köller 2003).

Development of resistance to scytalone dehydratase inhibitors of melanin biosynthesis (MBI-D) in *Magnaporthe grisea*, the rice blast pathogen, was investigated. The distribution of MBI-D-resistant isolates of *M. grisea* was mainly limited to Saga Prefecture in Japan, where MBI-Ds have been used since 1998. In 2002, resistant isolates were detected in all prefectures of Kyushu. DNA fingerprinting analysis of

resistant isolates revealed that mutation causing resistance to MBI-Ds had arisen independently in each area. The results suggested that resistant isolates might emerge in any area and become dominant under continuous selection pressure for MBI-Ds. Resistance to isolates of *Magnaporthe grisea* may be due to a single-point mutation in the scytalone dehydratase gene. This mutation results in a replacement of valine with methionine at codon 75 of the fungicide target protein (Sawada et al. 2004). PCR-Luminex, a novel system developed for high-throughput analysis of single-nucleotide polymorphisms (SNPs) was effectively employed for diagnosis of MBI-D resistance, using specific oligonucleotide probes coupled with fluorescent beads. In addition, the PCR-Luminex system was tested for its potential in identifying fungi involved in wheat Fusarium head blight disease. *Fusarium graminearum* (= *F. asiaticum*), *F. culmorum*, *F. avenaceum*, and *Microdochium nivale* were subjected to PCR-Luminex analysis successfully, and their involvement in FHB was established in this disease complex in Japan (Ishii et al. 2008).

Cruciferous crops suffer appreciable losses due to black spot disease caused by *Alternaria* spp. The development of resistance to fungicides in the fungal pathogens is inferred to be a result of the excessive application of fungicides. Field isolates of *Alternaria* spp. pathogenic to crucifers were evaluated in vitro for their sensitivity to various fungicides. Effective concentration (EC), representing toxicity responses, was determined based on the inhibition of mycelial growth by 50% (EC₅₀). Three sterol biosynthesis-inhibiting fungicides – flutriafol and difenconazole (triazole group), and prochloraz (imidazole group) – were all equally inhibitory to mycelial growth of *A. brassicae*, *A. brassicola*, and *A. japonica*. Among the two selected dicarboximides, iprodione was more effective than procymidone. Fludioxonil (phenylpyrroles family) was inhibitory to most isolates of *Alternaria* spp. tested. The in vitro tests indicated the existence of *A. brassicola* isolates highly resistant (EC > 100 mg L⁻¹) to iprodione, procymidone, and fludioxonil. Sporulation and aggressiveness of the resistant isolates were not affected when compared to sensitive isolates of *A. brassicola*. The presence of high cross-resistance to dicarboximides and phenylpyrroles in *A. brassicola* was revealed by the in vitro assessments (Iacomi-Vasilescu et al. 2004).

The development of resistance to pyrimethanil in *Aspergillus flavus* infecting peanuts in China was observed. The potential risk of resistance development in *A. flavus* was assessed using five highly pyrimethanil-resistant (Pyr^R) strains generated through UV-mutagenesis, tested for fitness parameters and aflatoxin B1 production. All five mutants had similar cultural characteristics such as mycelial growth rate, sporulation, and aflatoxin production as the wild-type parent strain. This indicated that pyrimethanil possessed a high risk of developing resistance in *A. flavus*. In addition, the mutant did not show any difference in the amino acid sequences of the four key enzymes – cystathionine β-lyase (CBL), cystathionine γ-synthase (CGS), methionine sulfoxide reductase (MsrB), and sulfate permease (SP2) – involved in the biosynthesis and metabolism of methionine and sulfate assimilation, as compared with wild-type parent strain. The results suggested that these enzymes might not be related to the aniline-pyrimidines (APs) resistance in *A. flavus* (Liu et al. 2014).

The frequent and indiscriminate application of fungicides to reduce the incidence and spread of fungal diseases transmitted through propagules has resulted in the development of strains/isolates with reduced sensitivity to fungicides which were found earlier to be inhibitory to the target pathogens. This aspect has to be taken into consideration when strategies for the management of diseases of crops are being formulated.

Different *Fusarium* spp. – *F. oxysporum*, *F. equiseti*, *F. sambucinum*, *F. avenaceum*, *F. cerealis*, *F. solani*, *F. acuminatum*, *F. sporotrichoides*, *F. torulosum*, *F. tricinctum*, and *F. graminearum* – were found to be associated with the dry rot disease of potato tubers obtained from Michigan seed production storage facilities. Representative isolates of all *Fusarium* spp. were pathogenic to potato tubers when inoculated onto seed tubers of Dark Red Norland potato. Isolates of *F. sambucinum* were more virulent than other isolates of *Fusarium* spp. The sensitivity of 228 isolates of *Fusarium* spp. to difenconazole, fludioxonil, and thiabendazole was tested. The effective fungicide concentration which caused 50% inhibition of mycelial growth (EC_{50}) ($<5 \text{ mg L}^{-1}$) was determined for each fungicide. Insensitivity to fludioxonil ($EC_{50} > 100 \text{ mg L}^{-1}$) was detected in the isolates of *F. sambucinum* and *F. oxysporum* at 8.9 and 20.4%, respectively. All isolates were sensitive to thiabendazole ($EC_{50} < 5 \text{ mg L}^{-1}$), except for those of *F. sambucinum* ($EC_{50} > 100 \text{ mg L}^{-1}$). The results indicated that the identity of the fungal pathogen(s) has to be precisely established and the sensitivity to the commonly used fungicides has to be determined to develop effective disease management strategies for propagative materials during storage (Gachango et al. 2012).

3.4 Influence of Storage Conditions

The effects of storage conditions on the development of *Fusarium culmorum* and mycotoxin contents in wheat seed were studied. Wheat seed samples with different initial infection levels of *F. culmorum* were stored for 36 weeks. Samples taken before storage and at intervals of 6–8 weeks were analyzed for seed health, seed quality, and mycotoxin contents. Zearalenone (ZEA) reached higher concentrations towards the end of storage when the seeds were stored under warm and humid conditions (25°C, 90% RH). On the other hand, the deoxynivalenol (DON) contents of severely infected kernel samples ($>50\%$) did not show significant changes under any of the storage conditions. However, in seeds with slight (4%) or moderate (15%) *Fusarium* infection level, DON contents showed significant increases under warm and humid conditions. Although nivalenol was not detected in the preharvest samples, its concentration increased in seeds later on during storage under cool or warm, but very humid, conditions (15°C, 84% RH or 25°C, 90% RH). The percentage of kernel infection by *Fusarium* did not correlate with mycotoxin contents. Under warm but dry conditions (25°C, 62% RH) *Fusarium* infection level of kernels decreased rapidly. The results indicated that warm and humid conditions were not suitable for maintaining the quality of both seed and grain products (Homdork et al. 2000).

The seeds may carry fungi that gain access to the seeds either during the heading stage or after maturity, but before harvest. Known as field fungi, these will have inflicted all the damage before harvest time and cease to develop when seeds are dried. The field fungi do not have the capacity to adversely affect the seeds during storage since they require moisture contents in equilibrium with relative humidity (RH) of 95–100%, which will provide free liquid water. Such conditions are not generally available during storage, resulting in their elimination during storage. In contrast, storage fungi which cannot colonize actively metabolizing plant tissues tend to invade dying, dead, or naturally dried seeds. These fungi are adapted to grow without free water, unlike field fungi. The storage fungi have a significant role in seed deterioration in storage, whereas the field fungi are responsible for different kinds of discoloration and poor filling, reduction of size of seeds, and other parameters that are essential for producing healthy

and vigorous seedlings from seeds. Different fungal pathogens infecting crop plants and seeds generally remain dormant during storage and until the seeds are sown in the field or glasshouses. The storage fungi associated with grains mostly belong to the genera *Aspergillus* and *Penicillium*. Various types of adverse effects are caused by storage fungi, such as reduction in germination, discoloration, musty or sour odors, caking, altered nutritional status, and reduction in processing quality. In addition, contamination with mycotoxins produced by fungal pathogens such as *Fusarium* spp. could be the most serious danger, resulting in health hazards to humans and animals following consumption of contaminated grains/feed (Narayanasamy 2006).

3.5 Appendix

3.5.1 Isolation of *Fusarium oxysporum* f.sp. *vasinfectum* from Cotton Seed (Bennett et al. 2008)

3.5.1.1 *Fusarium* Selective Liquid Medium

- i) Dispense the following constituent: D-glucose (20.7 g), L-asparagine (1.2 g), $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ (1.2 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), yeast extract (0.5 g), NaCl (0.1 g), and distilled water (1000 mL).
- ii) Autoclave and cool to 50°C.
- iii) Add the following to the medium: ampicillin (0.25 g), pentachloronitrobenzene (10 mL, 5 mg mL⁻¹ EtOH stock solution), streptomycin sulfate (2 mL, 0.15 g mL⁻¹ stock solution).

3.5.1.2 Isolation of the Pathogen

- i) Transfer 120 mL of selective medium to 250 mL flasks and place 50 surface sterilized acid-delinted cotton seeds.
- ii) Shake the contents of the flasks gently for 10–14 days at room temperature.
- iii) Harvest the mycelia in each flask by filtration through Miracloth (EMD Biosciences, CA) and rinse with sterile distilled water.
- iv) Transfer the mycelia aseptically to plates containing potato dextrose agar medium for multiplication.

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4

Process of Infection by Fungal Pathogens

Fungal pathogens associated with seeds and propagules may reach their primary sites of infection through different routes or agencies. Fungal pathogens present in the soil or carried through irrigation water may infect the roots or basal portions of the stem and grow through the vascular system, ultimately reaching the floral parts or seeds. Fungal spores or conidia discharged into wind currents may reach different aerial plant parts or finally inflorescence. The floral parts of susceptible plants such as anthers, pistil/ovary, or other seed tissues are invaded by the fungal pathogens. In some cases, spores or conidia may have access to the floral parts directly through the agencies of wind or insects attracted by sweet nectar in the flowers. Seed infection is the result of the successful establishment of a pathogen within any part of seed following penetration of the ovary wall, seed coat, or natural openings. The process of seed infection constitutes different stages, commencing from contact between pathogen propagule and plant cell surface, penetration into the susceptible cell, colonization of plant tissues and proliferation of fungal structures, production of new progenies of pathogen propagules, and symptom expression. This process of infection leading to the development of symptoms characteristic of the disease is known as pathogenesis. Different stages in the process of infection may follow in quick succession or there may be short or long intervals between stages, depending on the routes through which the pathogen may reach the seed tissues from infected mother plants.

4.1 Invasion Paths of Seedborne Fungal Pathogens

The nature of the pathogens and invasion (infection) routes may determine the location of the seedborne pathogens and their ability to survive in plant tissues, particularly in seed tissues (Table 4.1). Pathogenesis or the process of infection and disease development represents the dynamic interaction between the pathogen and host plant, depending on the levels of host plant resistance, pathogenic potential of the fungal isolates/strains, and the existing environmental factors. The different stages of pathogen invasion may be monitored and visualized by applying various techniques.

4.1.1 Direct Infection of Floral Parts

Direct infection of seeds occurs more commonly in the case of viruses which are transmitted directly through the tissues of mother plants to the developing seeds.

Table 4.1 Invasion paths of seedborne fungal pathogens.

Fungal pathogen	Plant tissue penetrated	References
A. Systemic infection through floral organs		
<i>Diaporthe phaseolorum</i> var. <i>sojae</i>	From systemically infected mother plants to soybean seed	Crittenden (1968)
<i>Fusarium moniliforme</i>	Maize seed	Cordero et al. (1992)
<i>Fusarium oxysporum</i> f.sp. <i>lagenarium</i>	From vascular bundles of fruits of bottle gourd to tomato seed	Kuniyasu and Kishi (1977)
<i>F. oxysporum</i> f.sp. <i>lycopersici</i>	From systemically infected mother plants to cotton seed	Baker (1952)
<i>F. oxysporum</i> f.sp. <i>vasinfectum</i>	From systemically infected mother plants to pea seed	Rudolph and Harrison (1945)
<i>F. oxysporum</i> f.sp. <i>pisi</i>	From systemically infected mother Plants to seed	Baker (1952)
<i>Peronospora parasitica</i>	Entry through hilar region from vascular tissues of infected radish; through stigma and ovary leading to embryonic infection	Jang and Saffeuilla (1990)
<i>Phytophthora capsici</i>	Oospores in seed embryos of squash; penetration through cuticle and integument stratum	Melo et al. (1992)
<i>Plasmopara halstedii</i>	From systemically infected mother plants to sunflower seed	Cohen and Sackston (1974)
<i>Verticillium dahliae</i>	From systemically infected mother plants to spinach sugar beet seed	van der Spek (1972)
B. Penetration through stigma		
<i>Alternaria alternata</i>	Entry through style and ovary of pepper	Lang (1917)
<i>Cercospora kikuchii</i>	Entry through hilar groove into the tracheids of soybean	Sakai and Ogawa (1983)
<i>Colletotrichum capsici</i>	Entry through seed coat or remnant outer layer of endosperm of pepper	Sangchote and Juangbhanich (1984)
<i>C. dematium</i> f. <i>spinaciae</i>	Entry through cuticle of seed coat of sugar beet	Chikuo and Sugimoto (1989)
<i>C. lagenarium</i>	Entry through seed coat of watermelon	Rankin (1954)
<i>Phomopsis longicolla</i>	Entry through funiculus and hilum or directly through seed coat	Roy and Abney (1988)
<i>Ustilago nuda</i>	Penetration through ovary wall of barley	Malik et al. (1960)
<i>U. segetum</i> var. <i>tritici</i> (loose smut)	Entry through style and ovary of barley and wheat	Lang (1917)
<i>Verticillium albo-atrum</i>	Entry through styles and ovary of alfalfa	Huang et al. (1985)
C. Entry through natural openings/injuries		
<i>Phoma betae</i>	Entry through basal pore in the pericarp/apical pore in the seed cap	El-Nashaar and Bugbee (1981)

A fewer number of fungal pathogens infect seeds by a direct route. *Fusarium oxysporum* f.sp. *lagenarium* was found to invade seeds from the vascular bundles of fruits (Kuniyasu and Kishi 1977). Ascospores of *Gibberella zeae* (anamorph *Fusarium graminearum*) forcibly discharged into the still air are transported through the atmosphere to the spikes of wheat and barley and the silks of corn ears where they induce *Fusarium* head blight and *Gibberella* ear rot on susceptible cultivars (Schmale et al. 2005). The ability of three *Fusarium* species – *F. graminearum*, *F. verticillioides*, and *F. subglutinans* – to infect maize ears as the silks and kernels mature was assessed by inoculating a moderately resistant and two susceptible hybrids at two points of entry (silk channels and kernels). Irrespective of the *Fusarium* spp., the ears inoculated at early stages of silk development showed maximum disease severity, with a peak in the level of susceptibility around 1–6 days after silk emergence followed by a rapid decrease in disease severity thereafter. When kernel inoculation was compared, a general decrease in disease severity was observed with lapse of time after inoculation for all *Fusarium* spp. Disease severity was the lowest in earheads inoculated with *F. verticillioides*. On the other hand, *F. glutinans* induced greatest disease severity when silk channels were the point of entry. In contrast, *F. graminearum* was able to induce maximum disease severity following inoculation of kernels. The results indicated that disease severity might be influenced by the species of *Fusarium*, time of inoculation, and tissue through which infection is initiated (Reid et al. 2002). In a later investigation, infection of wheat spikes by *F. graminearum* was found to occur at anthesis, when ascospores and/or conidia could directly penetrate exposed anther and ovary. The hemibiotrophic hyphae colonized floral tissues and developing grains to induce premature ear senescence (Urban et al. 2003).

The early events of colonization of wheat floral tissues by *Fusarium graminearum* were studied by employing a strain of *F. graminearum* transformed to constitutively express the green fluorescent protein (GFP) from the jelly fish. Wheat cultivars Rubin (susceptible) and Sumai 3 (resistant) raised in a greenhouse were point-inoculated at anthesis with a spore suspension of GFP-*Fusarium* transformed strain and then maintained under intermittent misting conditions until harvest. The heads harvested at different intervals were examined under the microscope. Hyphae of *F. graminearum* were visible inside the florets of both cultivars at the point of inoculation within a few hours after inoculation. The pathogen exhibited notable affinity for pollen and anthers of both cultivars. Prolific growth of *F. graminearum* was observed in the ovaries and stigmas of both wheat cultivars at all intervals after inoculation. In general, the softer tissues of ovaries and stigmas were preferentially colonized in both cultivars and the pathogen invasion to other tissues, rachis, and exterior of the floret commenced in this manner. Later the pathogen spread along the spike both internally through rachis and across the external surfaces of the rachis and florets. The spread of the pathogen through the parenchyma and vascular tissues was slower and less extensive in Sumai 3 compared to the susceptible cultivar Rubin (Miller et al. 2004).

The primary site of infection of wheat spikelets by *Fusarium graminearum* could not be visualized by conventional microscopical methods. A strain of *F. graminearum* was therefore transformed with green fluorescent protein (*gfp*) gene to study the infection patterns in barley and *Arabidopsis* model system. Infected tissues were examined for fluorescence under the blue light with a dissecting microscope equipped with a 470 nm excitation filter and 535 nm emission filter. The most rapid route of infection involved the brush hairs at the extruded seed tip. Colonization of seed tissue followed a pattern

of a rapid basipetal growth along the pericarp epithelium, followed by a slower growth inward through the pericarp and testa. This technique provided the possibility of tracking the invasion of barley seed tissues by *F. graminearum*, without any misinterpretation of observations due to contaminating fungi present on the seeds. It was also possible to determine the development of *F. graminearum* in barley genotypes with differing levels of resistance (Skadsen and Hohn 2004).

In another investigation, the route of infection of wheat spikes by *F. graminearum* and the role of trichodiene synthase gene in its pathogenicity were examined using a constitutively GFP-expressing wild-type strain and its knockout mutants, lacking trichodiene synthesis. Trichothecenes did not have any function as a virulence factor during infection through the fruit coat. However, the pathogen was blocked in the absence of trichothecenes by the development of heavy cell-wall thickening in the rachis node of Nandu wheat, a defense strategy, inhibited by the mycotoxin. In contrast, in barley the hyphal development was inhibited at the rachis node and rachilla, limiting infection of adjacent florets through the phloem and along the surface of the rachis (Jansen et al. 2005).

Infection patterns of *Fusarium graminearum* were studied in barley by employing a strain of *F. graminearum* transformed with green fluorescent protein (*gfp*) gene for monitoring the progress of development of Fusarium head blight (FHB) disease. Inoculation of intact barley spikes resulted in rapid colonization of the brush hairs (ovary epithelial hairs) at the extruded seed tip within 7 h after inoculation. Colonization followed a distinct pattern of rapid basipetal growth along the pericarp epithelium (interior to the lemma and palea), accompanied by slower growth inward through the pericarp and testa. Interestingly, at 16 days after inoculation, the aleurone and starch endosperm were not invaded by the pathogen, despite heavy colonization of the pericarp. Outer lemma was colonized at a much slower rate. The investigation revealed the usefulness of employing *gfp*-transformed pathogens to trace their development in various parts of the infected plant (Skadsen and Hohn 2004). In another investigation, the importance of *Fusarium*-infested seed as the source of inoculum of Fusarium head blight (FHB) disease was assessed, using *F. graminearum* strain transformed with *gfp* gene. Six treatments, using mixtures of *Fusarium*-free and *Fusarium*-infested seed in the ratios of 100:0, 90:10, 75:25, 50:50, 25:75, and 0:100 of free to infested seeds were evaluated for three years. Yield was significantly reduced (up to 20%) in the 100-infested seed treatment, which might be due to lower stand establishment and lower plant density. Grain weight was reduced in two of three years. Seedling blight was not observed during all the years. *F. graminearum* was isolated from infected spikes only in Manitoba, while *F. graminearum* was isolated from Vauxhall material. The severity of FHB was not significantly different across treatments. The results showed that *Fusarium*-infested seed was not a major source of inoculum of FHB during the experimental duration, since treatment effects were not associated with the development of FHB in wheat (Miller et al. 2004).

Ten different species of *Fusarium* including *F. moniliforme* were detected as seedborne fungi in soybean seeds, causing wilt, root rot, and foot rot diseases (Richardson 1979). The process of infection of soybean seeds by *F. moniliforme* was studied by inoculating surface-sterilized seeds with the pathogen and incubating them at $20 \pm 2^\circ\text{C}$. Samples were examined under scanning electron microscope (SEM) at 6, 12, 24, and 48 h after inoculation. Conidial germination was initiated at 6 h after inoculation and infection hyphae penetrated directly through the cuticle via cell juncture without forming appressorium.

The mycelium grew subcuticular and intercellularly between and below cuticular layer of the seed coat. Sporulation was seen frequently on the seed surface. Mycelial growth increased rapidly, covering the whole seed surface which turned into a rotten mass of tissue 96 h after inoculation (Muzahid-E-Rahman et al. 2010). The infection process (optimal time of infection and infection pathways) of *Fusarium graminearum* in oats and its subsequent effects on kernel infection, deoxynivalenol (DON) content and germination capacity were studied. Oat cultivar Morton was spray-inoculated at different developmental stages. The pathogen entered primarily through the floret apex into the floret cavity, where it could infect via the internal surfaces of the palea, lemma, and caryopsis. The appearance of visual symptoms and fungal infection commenced at the apical portions of the florets and progressed to the basal portions. Hyphae of *F. graminearum* developed more rapidly on the anthers than on other floret parts at the early stages of infection. Disease progress within oat panicle was slow due to physical contact primarily between adjoining florets, indicating that long pedicels might contribute to resistance to *F. graminearum* in oats (Tekle et al. 2012).

Loose smut pathogens *Ustilago nuda* and *U. tritici*, infecting barley and wheat respectively, are seedborne. Fluorescence microscopy technique was employed to follow the establishment of the loose smut pathogens in seed of barley and wheat. Severely infected seeds were used to prepare hand-cut sections and were stained with fluorochrome Blankophor®. The presence of pathogen hyphae was traced and these hyphae invaded the shoot apical meristem and leaf primordia during the first days after the commencement of seed germination. At the first node stage, the ear and leaf primordia were generally extensively colonized by the respective pathogens. Hyphae of *U. nuda* were frequently seen in high density in the nodes. The results of microscopical observation were corroborated by those of real-time polymerase chain reaction (PCR) assays (Wunderle et al. 2012).

Six cereals (bread and durum wheat, triticale, rye, barley, and oats) were subjected to point-inoculation with two isogenic strains of *Fusarium graminearum*, a wild-type strain (*Tri5*⁺) producing trichothecene and a mutant (*Tri5*⁻). Trichothecene-producing strains were generally more aggressive than the nonproducing strains, but this characteristic varied according to crop species. High resistance levels of oat were due to the large space between florets. The results confirmed the role of trichothecenes in FHB pathogenesis (Langevin et al. 2004). In another investigation, four inoculation treatments were followed. A conidial suspension was sprayed on flowering ears of wheat and the effect of application of moisture during kernel setting and filling with mist-irrigation system was assessed. Mist irrigation after inoculation resulted in a higher mean disease severity but in a lower toxin contamination as compared to nonirrigated treatment. Genotypic differences in deoxynivalenol (DON) accumulation were recognizable. In one wheat line DON contamination significantly increased when irrigated, whereas two genotypes allowed accumulation only to a significantly lower level. The closest relationships $r = 0.73\text{--}0.89$ between visual symptoms and the DON content were obtained under moderate mean infection pressure. However, at higher disease severity the relationship between symptoms and DON content became nonsignificant (Lemmens et al. 2004). Barley genotypes with different levels of resistance to FHB were inoculated using three different methods (point or spray inoculation into the greenhouse and grain spawn in a field spray) and rated for disease severity and deoxynivalenol (DON; a mycotoxin produced by the pathogen) contents. A positive correlation between FHB rating and DON levels was observed when point inoculation

and grain spawn inoculation was followed. Indoor spray inoculation provided the most reproducible results (Geddes et al. 2008).

Rice false smut pathogen *Ustilaginoidea virens* infects developing spikelet at the booting stage and transforms individual grains into smut balls. When rice booting and heading stages coincided with rainy days, severe symptoms on a high percentage of rice plants appeared in epidemic proportions. A green fluorescent protein (GFP-) labeled *U. virens* isolate colonized leaves of plants belonging to various families including Poaceae (*Oryza sativa*, *Echinoachloa crusgalli*, *Digitaria sanguinalis*, and *Leptochloa chinensis*), the Brassicaceae (*Arabidopsis thaliana*), and the Solanaceae (*Nicotiana benthamiana*) without symptoms. Some conidia were able to germinate on leaves of these plants over several days. In the absence of water, some conidia could either bud to form new conidia or were converted into chlamydospores. The results indicated that *U. virens* could exist as an epiphytic microbe until the rice plants at the susceptible stage are available (Fan et al. 2014).

Various species of *Fusarium* produce different mycotoxins, differing in their chemical constitution, and they are differentiated based on the nature of the mycotoxins produced by them. The isolates/strains of a morphologic species producing one mycotoxin are grouped into one chemotype and there may be different chemotypes within one morphologic species of *Fusarium*. Deoxynivalenol (DON) levels and chemotype frequency in barley cultivars inoculated with two chemotypes of *Fusarium graminearum* were determined. Corn (maize) kernels colonized by *F. graminearum* were used as inoculum in an irrigated Fusarium head blight nursery at a 2:1 ratio of isolates producing 15-acetyldeoxynivalenol (15-ADON) to isolates producing 3-acetyldeoxynivalenol (3-ADON) in 2008, or 1:1 ratio in 2009 and 2010. Seed infection by *F. graminearum* ranged from 83.5% (2009) to 98.5% (2010) with no significant difference across the nursery. The chemotype distribution and DON levels in 2008 on infected kernels showed a several-fold difference from the eastern side (67.3% of 3-ADON isolates and 46 ppm DON) to the western end of the field (16% of 3-ADON isolates and 13 ppm DON). In all years, the recovery of isolates did not reflect the ratio of the initial inoculum, suggesting that other factors influenced the ultimate infection of seed. DON levels were much higher in the most susceptible cultivars in all three years, whereas infection levels were in lower in the most resistant cultivars (Clear et al. 2013).

Pathogen development and host responses to infection by *Fusarium graminearum* in wheat spikes of resistant (Sumai 3) and susceptible (Xiaoyan) cultivars were studied, using electron microscopy and immunogold labeling techniques. Pathogen spread was restricted in resistant cultivar, although the process of infection by *F. graminearum* was similar in both resistant and susceptible cultivars. In addition, the formation of thick-layered appositions and papillae was essentially more pronounced in infected tissues of resistant cultivar than in susceptible variety. The presence of β -1,3-glucan was detected in the appositions and papillae. Immunogold labeling technique revealed that labeling densities for lignin, thionins, and hydroxyproline-rice glycoproteins (HRGP) over the cell walls of infected tissues of susceptible cultivar increased only slightly. On the other hand, high accumulation of these compounds in cell walls of infected resistant cultivars was observed. A similar greater increase in the plant hydrolases, β -1,3 glucanase and chitinase were found in infected spikes of resistant cultivar. The mycotoxin DON concentrations produced by *F. graminearum* in the spikes of resistant cultivar was significantly less than in susceptible cultivar, as revealed by immunogold labeling technique (Kang et al. 2008).

Fusarium culmorum is also associated with wheat Fusarium head blight (FHB) disease. Ultrastructural and cytochemical alterations induced by the pathogen in wheat spikes were investigated using enzyme-gold and immunogold labeling techniques. The cell-wall components, namely cellulose, xylan, and pectin, in different tissues of healthy and *F. culmorum*-infected spikes were localized. The inter- and intracellular growth of the pathogen in the ovary, lemma, and rachis of infected spike caused pronounced alterations of cell walls and middle lamella matrices. Enzyme-gold and immunogold labeling in the infected hosts revealed that labeling densities for cellulose, xylan, and pectin were significantly reduced in the cell walls of infected ovary, lemma, and rachis when compared with healthy tissues. The host cells in contact with or close to hyphae of *F. culmorum* showed more pronounced changes in cell morphology and much greater reduction of labeling density than those at distance from the invading hyphae. The results indicated that *F. culmorum* could produce cell-wall degrading enzymes (CWDEs) such as cellulases, xylanases, and pectinases during infection and colonization of wheat spike tissues. As degradation of pectin was at a faster rate at the early stage of infection, pectinases might be secreted earlier at higher concentrations than cellulases and xylanases (Kang and Buchenauer 2000).

The distribution of β -1,3-glucanase and chitinase was traced in a later investigation using specific antisera in immunoassays. Uninoculated healthy and *Fusarium culmorum*-infected wheat spikes showed similar distribution of these enzymes. They were localized mainly in the cell walls of different tissues, including lemma, ovary, and rachis of the wheat spike, whereas the cytoplasm and organelles of cells in these tissues did not have a detectable label. Accumulation of β -1,3-glucanase and chitinase in infected spikes differed distinctly in resistant and susceptible wheat cultivars. Differential accumulation of β -1,3-glucanase and chitinase in resistant and susceptible wheat cultivars infected by *F. culmorum* might have a role in resistance to spread of the pathogen in wheat spike tissues (Kang and Buchenauer 2002).

Changes in cell-wall components in the tissues of wheat spikes infected by *F. avenaceum* were determined by using electron microscopy and cytochemical labeling techniques. Penetration of the host tissue occurred at 36 h after inoculation (hai). Dense mycelial networks were formed on the inner surfaces of the glume, lemma, and palea and ovary at 36–48 h after inoculation (hai). The pathogen penetrated the cuticle and hyphae extended subcuticularly or between the epidermal wall layers. Immunogold labeling procedures indicated that cell walls of spike tissues contained lower amounts of cellulose, xylan, and pectin near intracellular hyphae or infection pegs compared to healthy tissue cell walls. The results suggested that cell-wall degrading enzymes (CWDEs) produced by *F. avenaceum* facilitated rapid colonization of wheat spikes (Kang et al. 2005).

Enzymes secreted by fungal pathogens have been shown to play important roles in their pathogenicity. With a view to understanding the secretome of *Fusarium graminearum*, it was cultivated in liquid culture with barley and wheat flour as the sole nutrient source to mimic a host–pathogen interaction. A gel-based proteomics approach was employed to identify the proteins secreted into the culture medium. Sixty-nine unique fungal proteins were identified in 154 protein spots, including enzymes involved in degradation of cell walls, starch, and proteins. The majority of proteins (70%) were predicted to contain signal peptides. Proteins identified in the 72 spots showing a differential appearance between wheat and barley flour medium were primarily involved in fungal cell-wall remodeling and the degradation of plant cell walls, starch, and proteins.

The in planta expression of corresponding *F. graminearum* genes was confirmed by quantitative RT-PCR assay in barley and wheat spikelets, harvested at 2–6 days after inoculation. Furthermore, a significant difference in the accumulation of fungal biomass and the extent of pathogen-induced proteolysis of plant β -amylase was observed in barley and wheat (Yang et al. 2012).

Cladosporium variabile and *Stemphylium botryosum*, causing leaf spot diseases in spinach, are seedborne and outbreaks of the disease in fresh market crops of spinach were observed in the Salinas Valley, California and other areas in the USA. In order to determine the extent of infection by *C. variabile* and *S. botryosum*, component freeze-blotter seed assays were performed. *S. botryosum* was detected in the pericarps and embryos of all five seed lots with ranges of 13–91% and 5–76% of pericarps and embryos, respectively, showing infection. *C. variabile* and/or *C. macrocarpum* were present at a much lower incidence than *S. botryosum* in all five seed lots. Transmission electron microscopic (TEM) observations revealed that intercellular hyphae invaded the pericarps of all six seed from the lot 03-409. Penetration of the pericarp cell walls by intracellular hyphae could be clearly observed in some cells, while the intercellular hyphae and any other fungal structures were absent in all cells examined under TEM. On the other hand, scanning electron microscopic (SEM) examinations revealed the presence of conidia and conidiophores of *S. botryosum* and *C. variabile* or *C. macrocarpum* on the pericarps. The incidence and location of these fungi in spinach seed might be influenced by the maturity of the seed crop at the time of infection and the availability of favorable environmental factors for infection of seeds and disease development (Hernandez-Perez and du Toit 2006).

Fungal pathogens have to acquire iron from plant hosts for successful establishment and induction of disease in susceptible plants. Fungal pathogens have at least two pathways for iron uptake. Secretion and subsequent uptake of low-molecular-weight iron chelators known as siderophores form one pathway of iron uptake. Another pathway operating in some pathogens is based on the use of cell-surface reductases to solubilize ferric iron by reducing it to ferrous iron uptake. Five iron-related genes from *Fusarium graminearum* were identified and transcribed in response to iron limitation. In order to assess the relative contributions of the reductive and siderophore pathways of iron uptake, mutants disrupted at ferroxidase gene *FET3* ($\Delta fet3$) or the siderophore biosynthetic gene *SID1* ($\Delta sid1$) were generated. The $\Delta fet3$ mutants produced wild-type amounts of siderophores and grew at the same rate as the wild-type under iron limitation, but accumulated high levels of free intracellular iron. The $\Delta sid1$ mutants could infect single, inoculated spikelets, but were unable to spread from spikelet-to-spikelet through rachises of wheat spikes, while the $\Delta fet3$ mutant behaved as wild-type throughout the process of infection. The results suggested that siderophore-mediated iron uptake might be the major pathway of cellular iron uptake and might be required for full virulence in *F. graminearum* (Greenshields et al. 2007).

The virulence of fungal pathogens may be conditioned and controlled by different genes independently or in combination. Phosphatidylinositol-3-kinase signaling pathway was studied to determine its role in the virulence of *Fusarium graminearum* on wheat. *F. graminearum* locus FGSG 0482.3 (named *FgTEP1*) was identified as the functional homologue of *ScTEP1* in the sensitivity of *Saccharomyces cerevisiae* cells to wortmannin, the phosphatidylinositol-3-kinase inhibitor. Deletion of *FgTEP1* induced sensitivity of *F. graminearum* mycelial growth to lithium, in addition to reduced conidial production. Further, deletion of *FgTEP1* reduced the virulence of *F. graminearum* on

wheat. The results indicated that *FgTep1* was linked to the phosphatidylinositol-3-kinase signaling pathway in *F. graminearum* (Zhang et al. 2010). Mitogen-activated protein (MAP) kinases have been shown to be key components in signaling networks. Two MAP kinases involved in pathogenicity *Mgv1* and the *Gibberella* pathogenicity MAP kinase *Gpmk1* were identified. $\Delta gpmk1$ mutants with disrupted *GPMK1* gene were unable to infect wheat spikes. Among the extracellular enzymes secreted, the lipase *FGL1* was found to be a major virulence factor of *F. graminearum*. Expression of *FGL1* gene was reduced in $\Delta gpmk1$ strains during wheat head infection. $\Delta gpmk1$ strains that constitutively expressed *FGL1* showed that the level of extracellular lipolytic activity of these strains in culture was comparable to the wild-type strain of *F. graminearum*. These mutants exhibited complete restoration of conidiation and partial complementation of defects in development showed by the $\Delta gpmk1$ mutant. The results indicated that lipase *FGL1* might be necessary, but not sufficient, to restore complete pathogenicity to apathogenic $\Delta gpmk1$ mutant (Salomon et al. 2012).

The genetic basis of virulence of *Fusarium graminearum*, infecting wheat and maize (corn), was investigated. G-protein signaling has been implicated in pathogenesis in *F. graminearum*. Involvement of a putative phosducin-like gene (*BDM1*) in the growth, development, and pathogenesis in *F. graminearum* was studied. Targeted deletion of *BDM1* revealed roles in sexual and asexual sporulation, germ tube development, hyphal branching, and mycelial morphology. During disease development *BDM1* was shown to be required for wild-type levels of colonization of maize silk tissue and stalks, but it was not necessary for kernel colonization. Deletion of *BDM1* also reduced the virulence of *F. graminearum* during the infection of wheat seedlings and heads, resulting in a significant reduction in fungal biomass and delayed spread of visual symptom expression such as bleaching in heads. *BDM1* had another function in wild-type strains for the production of deoxynivalenol during infection of wheat heads and maize silks (Horevaji and Bluhm 2012).

Fusarium verticillioides, causal agent of maize ear rot and stalk rot diseases, produces carcinogenic mycotoxin fumonisins during colonization of corn kernels. *ZFR1*, predicted to encode a Zn(II)₂Cys₆ zinc finger transcription factor, was required for fumonisin B₁ (FB₁) production during the growth of the pathogen on kernels. The role of *ZFR1* in colonizing corn kernels and in inducing FB₁ biosynthesis by *F. verticillioides* was studied. The *ZFR1* deletion strains (*Dzfr1*) grew approximately 2.5-fold slowly compared to wild-type strain in endosperm tissue and a variety of other carbon sources, including glucose and amylopectin. However, the *Dzfr1* strain had a higher level of α -amylase activity and expression of genes involved in starch saccharification than the wild-type, indicating that the reduced mycelial growth of the mutant was not due to inhibition of amylolytic enzymes. The expression of six genes encoding putative sugar transporters was significantly greater on endosperm tissue than on germ tissue. The expression of at least three of the six genes was negatively affected by the disruption of *ZFR1*. However, disruption of *ZFR1* had no effect on growth, kernel colonization, or kernel pH, but decreased FB₁ production by approximately 82% on corn kernels (Bluhm et al. 2008).

Deletion mutants of two putative response regulator (RR) proteins, *FgRRG-1* and *FgRRG-2*, were generated from *Fusarium graminearum*. *FgRRG-1* deletion mutant ($\Delta FgRrg1-6$) showed increased sensitivity to osmotic stress mediated by NaCl, KCl, sorbitol, or glucose and metal cations Li^+ , Ca^{2+} , and Mg^{2+} . However, the mutant was

more resistant than the wild-type strain to dicarboximides and phenylpyrroles fungicides. Pathogenicity tests showed that the mutant was less virulent on wheat heads. Quantitative RT-PCR assays indicated that the expression of *FgOS-2*, the putative downstream gene of *FgRRG-1*, was reduced significantly in $\Delta FgRrg1-6$. All deficiencies were restored by genetic complementation of $\Delta FgRrg1-6$ with the wild-type *FgRRG-1* gene. The results indicated that *RRFgRrg-1* of *F. graminearum* was involved in osmotic stress response, pathogenicity, and sensitivity to fungicides and metal cation (Jiang et al. 2011). *F. graminearum* produces the red pigment aureofusarin, in addition to trichothecene mycotoxins. The velvet gene (*VeA*) is conserved in various genera of filamentous fungi. The velvet gene (*FgVe1*) was identified in *F. graminearum*. Disruption of *FgVe1* induced several phenotypic effects. However, complementation of the mutant with *FgVe1* gene restores the wild-type phenotypes. Variations in phenotypes of mutants exhibited hyperbranching of mycelium, suppression of aerial hyphae formation, reduced hydrophobicity of the mycelium, and highly reduced sporulation. *FgVe1* modulates the production of aureofusarin pigment. It was found to be essential for the expression of *Tri* genes and production of trichothecenes. Pathogenicity tests showed that *FgVe1* was a positive regulator of virulence in *F. graminearum* (Merhej et al. 2012).

Mycotoxin accumulation in cereal grains is influenced by several factors such as the pathogen strain, host resistance, duration of head wetness, and other environmental conditions. Trichothecene toxins produced by *Fusarium graminearum* (Fg complex) act as virulence factors on some hosts. Strain-specific difference in trichothecene metabolite profiles (chemotypes) were not found to be well correlated with the Fg complex progeny. A 19 kb region of trichothecene gene cluster was sequenced in 39 strains chosen to represent global genetic diversity of species in the Fg complex and four related species of *Fusarium*. Phylogenetic analysis of the sequences of the 19 kb fragment indicated that polymorphism within these virulence-associated genes persisted through multiple speciation events (Ward et al. 2002).

Restriction enzyme-mediated integration (REMI) mutagenesis was shown to be an efficient approach for the identification of pathogenicity genes. The REMI procedure was applied for generating mutants of *F. graminearum*. Eleven pathogenicity mutants defective in colonizing corn silks and flowering wheat heads were isolated. Defects in pathogenicity were tagged by transforming vector in six mutants. The *cbl-1* and *msy-1* deletion mutants had significantly reduced virulence on corn silks and wheat heads, indicating that methionine synthesis was critical for infection of plant tissues by *F. graminearum*. Furthermore, two genes, the putative b-z1 transcription factor gene and the transduction β -tubulin gene disrupted in mutants M7 and M75, respectively, appeared to be novel virulence factors in *F. graminearum* (Seong et al. 2005).

Fusarium culmorum, a component of Fusarium head blight (FHB) disease, infects the stem base of wheat (*Triticum aestivum*). In addition to ear infection, systemic translocation of deoxynivalenol (DON) may contribute to enhanced mycotoxin levels in grains after stem infection with toxigenic *Fusarium* spp. Two wheat cultivars differing in susceptibility to FHB were inoculated with *F. culmorum* in climate chamber conditions. The presence of fungal DNA was detected only in the stem base tissue, whereas DON and its derivative, DON-3-glucoside (D3G), were detected in upper plant parts. The infected stem base tissues contained $>10,000 \mu\text{g kg}^{-1}$ dry weight (DW) of DON and mean levels of DON in the ear and husks reached $1900 \mu\text{g kg}^{-1}$ DW. However, no DON or D3G was detected in mature grains. D3G quantification showed that DON

detoxification occurred primarily in the stem bases, where $\leq 50\%$ DON was metabolized into D3G. Expression of a gene putatively encoding a uridine diphosphate-glycosyltransferase was enhanced in the stem base after infection with *F. culmorum*. Histological observations revealed that vascular transport of DON labeled with fluorocein as a tracer from the peduncle to the grain was interrupted by a barrier zone at the interface between grain and rachilla, which was earlier designated “xylem discontinuity.” The results demonstrated that the effective control of influx of systemically translocated fungal mycotoxins into grains occurred at the rachilla–seed interface by the xylem discontinuity tissue in wheat ears (Winter et al. 2013).

The critical timing of infection, when the pathogen finds optimal conditions, has to be determined. Wheat plants exhibit high susceptibility to Fusarium head blight (FHB) disease during anthesis which is likely to be due to anther extrusion at this stage. The initial *Fusarium* infection is frequently seen on extruded anthers. Wheat has generally open-flowering type (chamogamous), whereas barley has both open-flowering and closed-flowering type (cleistogamous) in which the florets do not open and anthers are not extruded at anthesis. The effect of timing of *Fusarium graminearum* infection on FHB and mycotoxin accumulation in barley cultivars with different flowering types was investigated under greenhouse conditions. Barley cultivars (13) were spray-inoculated at two different developmental stages and the FHB severity was assessed. Closed-flowering cultivars were resistant at anthesis but they became susceptible at 10 days after anthesis (daa), whereas open-flowering cultivars were already susceptible at anthesis. The change in the response of Nishinochikara cultivar to inoculation between 0 and 10 daa actually occurred between 6 and 10 daa, because the severity of FHB and mycotoxin concentration with inoculation at 10 daa were significantly higher than with inoculation at 6 daa and earlier. At 6 daa, all anthers were still inside the closed florets. By 8–10 daa, many anthers were partially or completely extruded (Fig. 4.1). In the cultivars Nishinochikara and Minorimugi, accumulation of DON and NIV occurred at 9.0 and 10.1 $\mu\text{g g}^{-1}$, respectively, following inoculation at 20 daa, without displaying clear disease symptoms on the spikelet or kernels (Fig. 4.2). The results indicated that the most critical time for infection by *F. graminearum* and mycotoxin accumulation in barley differed with Japanese cultivars and that the flowering type of the cultivar might have a greater influence on these two biological activities of the pathogen (Yoshida et al. 2007).

In a further study, the influence of time of infection on mycotoxin accumulation in wheat grains was investigated. Under greenhouse conditions, four wheat cultivars were spray-inoculated at different stages with a mixture of DON and NIV chemotypes of *F. graminearum*. High levels of DON and NIV were produced by the chemotypes beyond 20 daa. Under field conditions, seven wheat cultivars were inoculated using corn kernels colonized by *F. graminearum* as inoculum. For all cultures, the levels of *Fusarium* damaged kernels (FDKs) (%) and toxin concentration at maturity were higher in 2008 than in 2007. In all cultivars except Sumai 3, both DON and NIV were detected at 20 daa and the concentrations largely increased after 20 daa to maturity (40 daa) in 2008. DON and NIV were not detected at 20 daa, but in mature grain, toxin (DON + NIV) attained a concentration of 1.6 $\mu\text{g kg}^{-1}$ (Table 4.2; Yoshida and Nakajima 2010).

F. graminearum secretes several extracellular enzymes considered to facilitate plant infection. The extracellular lipolytic activity of *F. graminearum* was strongly induced in culture by wheat germ oil. Based on this activity gene *FGL1*, encoding a secreted lipase, was isolated, cloned, and characterized. Expression analysis revealed that *FGL1* was

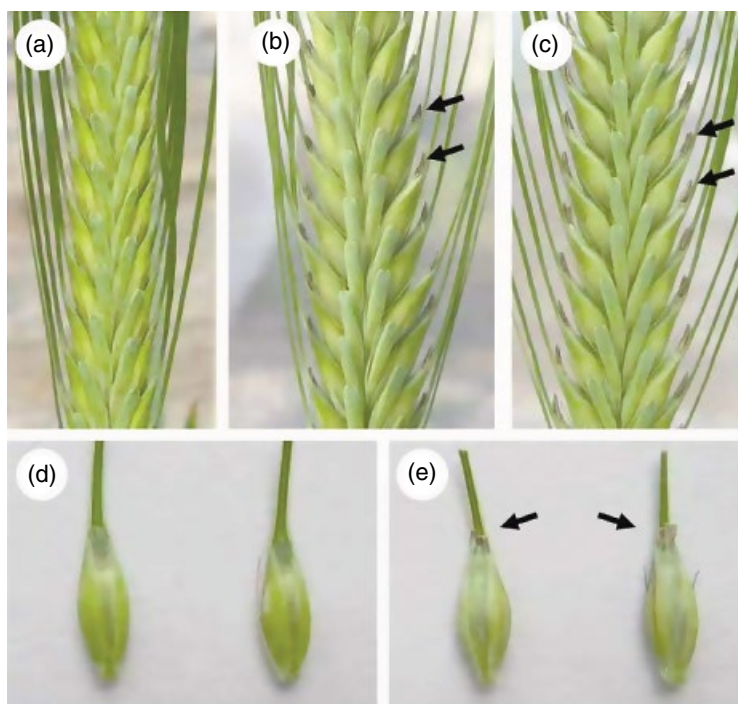


Figure 4.1 Response of chasmogamous (open-flowering) and cleistogamous (closed-flowering) barley to inoculation with *Fusarium graminearum* at different days after anthesis (daa). (a) Spikes at 2 daa; (b) spikes at 8 daa; (c) spikes at 10 daa; (d) view of ventral side of floret at 6 daa; and (e) 9 daa. Until 6 daa, anthers remained within closed florets; most anthers were partially extruded at 8 daa and anthers were entirely extruded at 10 daa. Arrows indicate extruded anthers. Source: Yoshida et al. (2007). Reproduced with permission of the American Phytopathological Society, MN, USA.

(1)



(2)

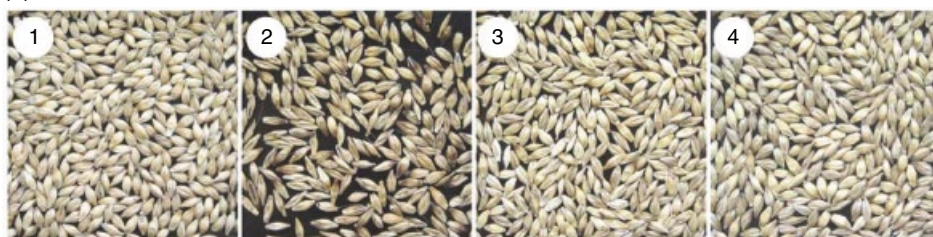


Figure 4.2 Effects of inoculation of two barley cultivars (1) Nishinochikara and (2) Minorimuge on the appearance of mature grains, following inoculation with *Fusarium graminearum* at different days after anthesis (daa). (a) Un inoculated; (b) inoculated at anthesis; (c) inoculated at 10 daa; and (d) inoculated at 20 daa. Mycotoxins contents (DON + NIV) ranged from 0.0 to $9.0 \mu\text{g g}^{-1}$ in Nishinochikara samples and from 0 to $10.3 \mu\text{g g}^{-1}$ in Minorimuge samples. Source: Yoshida et al. (2007). Reproduced with permission of the American Phytopathological Society, MN, USA.

Table 4.2 Effects of *Fusarium graminearum* on disease incidence and mycotoxin accumulation ($\mu\text{g g}^{-1}$) in grains of seven wheat cultivars (Yoshida and Nakajima 2010): A: Chikugoizumi; B: Norin 61; C: Shiroganekomugi; D: Minaminokaori; E: Saikai 165; and F: Sumai 3.

Pathogen effects	Year	Wheat cultivars						
		A	B	C	D	E	F	G
FHB disease	2007	7.3	16.0	10.8	13.9	11.6	8.1	5.0
FDK (%)	2007	4.3	4.6	2.2	5.0	3.7	3.7	0.6
DON*	2007	0.6	0.8	0.1	1.4	0.9	0.7	0.5
NIV*	2007	0.6	1.0	1.0	0.8	0.8	0.6	0.5
DON + NIV*	2007	1.3	1.9	1.1	2.2	1.7	1.2	1.0
FHB disease	2008	9.1	18.0	9.5	22.7	15.4	7.7	3.0
FDK (%)	2008	8.3	13.8	6.0	23.1	16.3	9.8	4.4
DON*	2008	1.9	2.9	1.7	5.6	3.1	2.3	5.5
DON + NIV*	2008	3.7	5.4	3.0	9.3	5.5	3.3	1.6

* Toxin at maturity

induced by lipid-containing substrates and repressed by glucose. *FGL1* transcription could be detected at 24 h postinfection of wheat spikes and the function of the *FGL1* gene product was verified by specifically demonstrating lipase activity after expression in a heterologous host. When wild-type conidia were supplemented with ebelactone B, a known lipase inhibitor, disease severity was appreciably reduced. The mutants generated by transformation-mediated disruption of *FGL1* of *F. graminearum* had reduced extracellular lipolytic activity in culture and virulence to both wheat and maize (Voigt et al. 2005).

Fusarium graminearum can produce several mycotoxins of which deoxynivalenol (DON) and zearalenone (ZEA) may accumulate in grains and feed materials to high levels. DON functions as a virulence factor, facilitating infection of wheat earheads by *F. graminearum*. However, disease severity in the field did not correlate with mycotoxin concentrations. Gene expression and mycotoxin production of lipase-deficient mutants (*Δfgl1*) with strongly reduced virulence and the respective wild-types were compared. During infection of earheads, *Δfgl1* mutants showed upregulated DON production. On individual wheat kernels, DON was detected only at lower concentrations compared to the wild-type isolates. In contrast, the wild-type or *Δfgl1* mutants were unable to produce ZEA, during head infection. But ZEA could be clearly detected on wheat kernels, *Δfgl1* mutants being more efficient in ZEA production. The altered amounts of DON and ZEA could be directly correlated with the expression of the toxin-related genes *Tri5* for DON and *PKS4* and *PKS13* for ZEA. Expression of *Tri5* and infection patterns of the wild-type and mutants suggested that the transition zone of rachilla and rachis appeared to be important in inducing biosynthesis of DON. Involvement of the lipase FGL1 in regulation of 8 PKS genes and production of ZEA was inferred from gene expression studies (Voigt et al. 2007).

Fusarium spp., infecting crops other than cereals, have been reported to produce mycotoxins in infected tissues. Asparagus spears are infected by *Fusarium* sp. in Germany and Poland. Spears of two cultivars Eposs and Gijnlim were tested for the

presence of mycotoxins. *Fusarium oxysporum* and *F. proliferatum* were detected at an average of 38.3% and 15.8%, respectively, in the spear sections sampled. Chemical analysis indicated that fumonisin (FB1) and moniliformin (MON) were present in some of the spears sampled in Poland. FB1 was not detected in the samples from Germany, but *F. proliferatum* could produce the mycotoxins in vitro in the range 101.4–205.8 $\mu\text{g kg}^{-1}$ of maize kernel substrate. However, asparagus samples from Poland contained FB1 up to 5.6 $\mu\text{g kg}^{-1}$ spear fresh weight. MON was found at a maximum concentration of 1350 $\mu\text{g kg}^{-1}$ in Eposs in Poland. Spears infected by both *F. oxysporum* and *F. proliferatum* contained both MON and FB1, with only the latter being the producer of both toxins (Weber et al. 2006).

Bipolaris sorokiniana causes black point disease of grains and shriveling of wheat spikelets of wheat, and can also infect leaves of barley plants. Interaction between the pathogen and barley plants was studied using the immunochemical method. Accumulation of pathogenesis-related (PR-) proteins as host defense response was revealed by localization of PR-1 protein, primarily in the intercellular spaces of mesophyll tissues in infected leaves. Light microscopy revealed labeling of PR-1 protein on mesophyll cell walls. Labeling in chloroplasts was observed in both infected and noninfected leaves. Subcellularly, PR-1 protein could be detected on the outer cell-wall layer and cytoplasm of primary hyphae. Intercellular electron dense material was observed at the junctions of host cells and in cell-wall appositions (Martilla et al. 2005).

The transfer of fungal pathogen from infected seeds to seedlings growing from them has been demonstrated to indicate the importance of seedborne inoculum as the source of inoculum for disease spread to near and/or distant locations. The effect of seedborne infection of *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*) in wheat and barley on the incidence of crown rot and root rot diseases in seven barley and three wheat cultivars was investigated. *B. sorokiniana* was consistently isolated from crown and roots of seedlings emerging from infected seeds. Furthermore, crown and root rot diseases of noninoculated barley cultivars correlated significantly with *B. sorokiniana* inoculum in seeds ($P=0.0019$). Pathogenicity tests with *B. sorokiniana* isolates revealed its role in inducing crown rot and root rot diseases, with two wheat cultivars being more resistant to crown rot and root rot than most barley cultivars. The results indicated the role of seedborne inoculum of *B. sorokiniana* in inducing crown and root rot diseases in wheat and barley (Al-Sadi and Deadman 2010).

The process of infection of wheat spikelets by *B. sorokiniana* was studied by light and electron microscopy. Germination of conidia inoculated on the spikelets commenced at 6–12 h on the surfaces of different spikelet tissues. Extracellular sheaths were present on germ tubes and appressoria attached to the surfaces of lemma, palea, and seeds, although their presence on the conidial surfaces was rarely seen. Appressoria, often noted over grooves, produced penetration hyphae invading the epidermal cell walls. Similar events occurred on the surface of the lemma, palea, and glume. Growth of the fungus in the epidermal and parenchyma cells was observed commonly in the cell walls and hyphal extension occurred both inter- and intracellularly in the infected tissues. Two pathways of pathogen invasion might lead to progress of the disease development: (i) direct infection of outer layers of pericarp cell walls; and (ii) entry through stigma into the pericarp cells. Hydrolytic enzymes and toxins of pathogen origin might facilitate the spread of the fungal pathogen and death of host cells that might provide required nutrients to the pathogen. The development of appositions between the cell wall and plasma membrane in cells adjacent to fungal cells and the presence of electron dense

material surrounding the pathogen hyphae were observed as the host responses to infection by *B. sorokiniana* (Han et al. 2010).

Barley seeds may be infected by *Rhynchosporium secalis*, causal agent of scald disease which generally overwinters in barley residues. Symptomless infection of barley seeds by *R. secalis* was revealed by using the isolation method and polymerase chain reaction (PCR) assay. Barley cultivar Harrington seeds without any visible scald lesions were plated on lima bean agar supplemented with Bengal rose and streptomycin. The pathogen was isolated from about 14% of seeds. The presence of *R. secalis* in barley seeds without symptom was detected by applying a species-specific primer set, based on the sequence analysis of the internal transcribed spacer regions of *R. secalis*. The PCR assay detected *R. secalis* in symptomless seeds of susceptible cultivar Harrington, but not in the seeds of resistant cultivar Seebe. The results indicated the usefulness of the assay methods for monitoring the pathogen spread through barley seeds (Lee et al. 2001).

Rice false smut disease caused by *Ustilaginoidea virens* has been reported to occur in epidemic proportions in some countries in Asia, America, Europe, and China. In the early stages of infection, ball-like colonies consisting of whitish hyphae are produced in spikelets and they turn into olive-black structures containing chlamydospores and sclerotia. Stigma, ovary, and young grain were considered to be primary sites of infection. The ability of the pathogen to infect roots at the seedling stage and to colonize the entire plant symptomlessly was revealed by histopathological and molecular techniques (Schroud and TeBeest 2005).

In a later investigation, an efficient method of inoculation of conidial suspension into the flag leaf sheaths of rice plants in the glasshouse was developed for performing cytological analysis of the infection process of *U. virens*. After removing lemmas and paleas, all fixed samples were stained and observed under light microscope and transmission electron microscope (TEM). All infection sites were found to be on the upper parts of filaments at 5 days after inoculation. The pathogen attached to the surfaces of the filaments and then “pressed” into the cell gaps. The primary infection sites were expanded along the filaments to the filament bases. The filaments of the three special stamens located between the lodicules and ovary near the lemma were infected (Fig. 4.3). The pathogen hyphae then extended invariably into the central vascular tissues along the cell walls. Some epidermal cells appeared to be digested completely and the spaces were occupied by the pathogen hyphae. No haustoria could be detected and the hyphae did not penetrate directly into the host cell wall. The primary infection sites were rice stamen filaments at the heading stage. Hyphal extension was limited to a few layers of lodicules cells and secondary hyphae were present only in a few styles of infected flowers (Fig. 4.4). The infection process was similar in both natural infection and artificial inoculation of spikelets. During the infection and development of rice false smut, no typical infective hyphae or haustoria which could penetrate host cell walls and enter the host cells through a hole were detected. The results suggested that *U. virens* has a distinct nutrient uptake mechanism and may possibly produce mycotoxins which make the infection mechanism unusual. The invasion paths of *U. virens* and the closely related *Claviceps* spp. show some similarities. *U. virens* infects a specific floral organ (filament) as *Claviceps* spp. (stigma). Both pathogens infect and invade in an intracellular mode without forming haustoria required for absorbing nutrients from the plant cells. Both are biotrophic pathogens and do not kill the infected organ during development. The results suggested that *U. virens* might produce mycotoxins as *Claviceps* spp. (Tang et al. 2013).

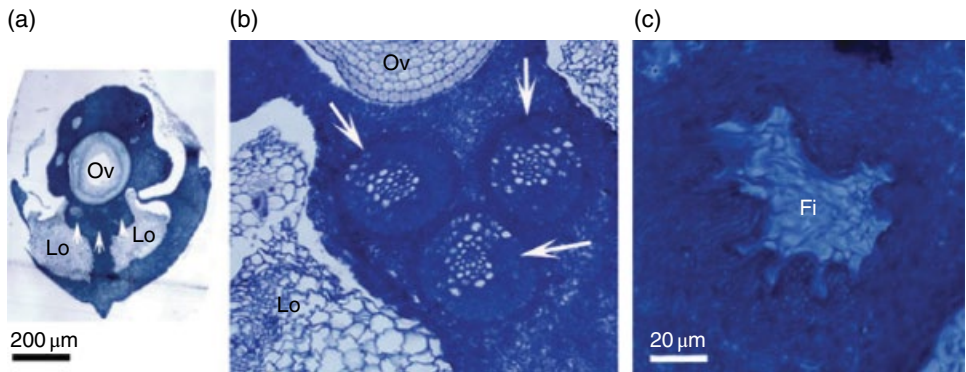


Figure 4.3 Visualization by light microscopy of pathogen invasion in floral parts of rice spikelets inoculated with *Ustilaginoidea virens*. Note infection of three special filaments located between ovary and lodicules near the lemma in semithin sections. (a, b) Floret filled with mass of dense pathogen hyphae; arrows show location of three infected filaments; and (c) shrunken uninfected filaments (F1) surrounded by dense hyphal mass. Source: Tang et al. (2013). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

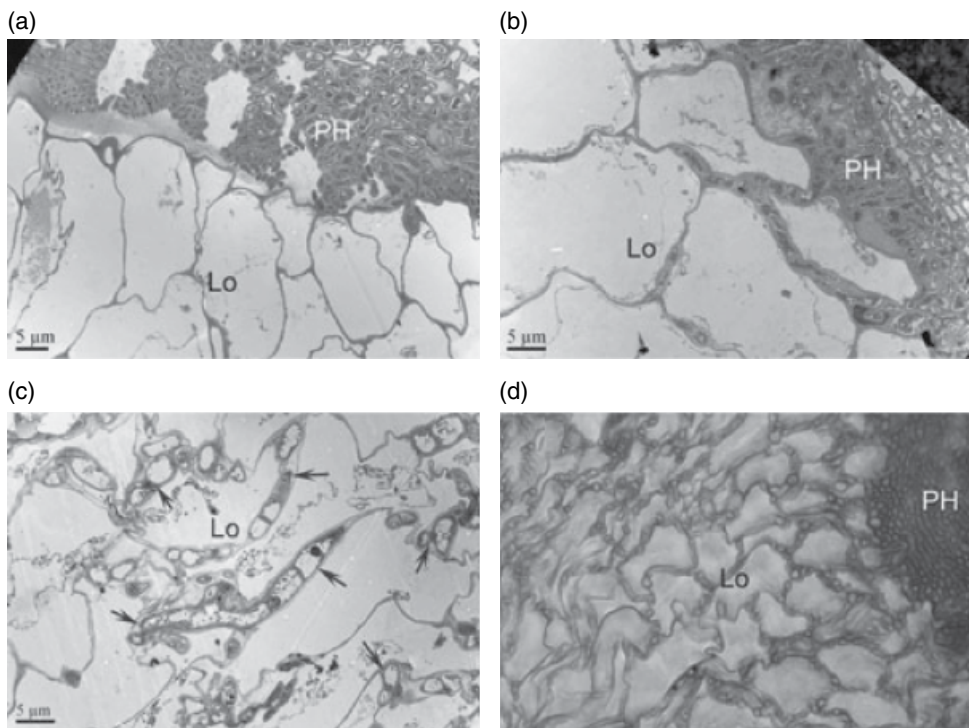


Figure 4.4 Transmission electron microscopic visualization of infection process of *Ustilaginoidea virens* in infected rice lodicules. Note the presence of (a): pathogen hyphae (PH) in firm contact with lodicules; (b) and (d): intracellular colonization in the outer layers of cells of lodicules and (c): light microscopic examination showing hyphae (arrowed) extending into intracellular space, but not reaching the vascular tissues. Source: Tang et al. (2013). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

4.1.2 Infection Through Inoculum in Soil/Plant Residues

Seedborne fungal pathogens produce different kinds of spores in the infected plant organs or tissues. These spores, which are resistant to environmental conditions, fall on the soil and remain viable for long periods. When they germinate under favorable conditions, they infect young seedlings and grow along with the host plants, reaching the reproductive structures as dormant mycelium. Alternatively, the spores (such as basidiospores) produced from teliospores of smut pathogens may be carried by wind and deposited on floral parts, facilitating initiation of infection and entry into the seed tissues. A vital staining procedure using fluorochrome acridine orange (AO) was employed to stain teliospores and primary sporidia (basidiospores) of *Tilletia indica*, causative agent of wheat Karnal bunt disease. This method allowed determination of teliospore viability in different soil types under different soil moisture levels. It was possible to qualitatively determine germination of teliospores in nonsterile soil taken directly from the field. This procedure was found to be simple and had the potential for use as a supplement to the quarantine inspection of seed lots for routine tests for the presence of *T. indica* extracted from seed consignments (Thinggaard and Leth 2003).

The process of infection in barley by the smut pathogen *Ustilago hordei* was studied using transmission electron microscopy (TEM). The interactions between compatible and incompatible barley cultivars and *U. hordei* were compared by the presence or absence of an avirulence gene (*VI*) on cultivar “Hannchen” harboring the cognate resistance gene (*Ruh1*). The pathogen penetrated the epidermal cells of the coleoptiles directly in both interactions within 2 days postinoculation (dpi). An electron-opaque interfacial matrix was observed around both inter- and intracellular hyphae in compatible interaction soon after penetration. Hyphae grew and extended into the host bundle sheath and invaded parenchyma cells. Cell-wall appositions surrounding the hyphae were recognized at 12 dpi. Tube-like structures of variable thickness and orientation were visible in the interfacial matrix mainly in the outer region bordering these appositions. On the other hand, in incompatible interactions cell-wall appositions could be visualized immediately after penetration of the host epidermal cells by the hyphae. Comparatively thicker and more granular material was deposited around the invading hyphae and extended onto the inner surface of invaded epidermal cells. In addition, host cell reactions of incompatible interaction included disorganization of cytoplasm and organelles, leading to necrosis and cell death. Very early hypersensitive response-like resistance representing gene-for-gene response was triggered; this response was extremely localized at the sites of primary infection, involving only a few plant cells (Hu et al. 2003).

Maize smut disease caused by *Ustilago maydis* is characterized by excessive tumor formation in infected plants. *U. maydis* differs distinctly in the initiation of infection and colonization of host plant tissues. The basidiospores produced from teliospores are disseminated by air currents or splashed by (rain) water to young corn seedlings. The basidiospores germinate on the host surface by producing a fine hypha which enters epidermal cells by direct penetration. Further development of the disease depends on its coming into contact and fusing with a haploid hypha produced by another basidiospore of compatible mating type. After fusion, the resultant hypha becomes either dikaryotic or multinucleate. The dikaryotic hypha grows intercellularly, stimulating the surrounding cells to enlarge (hypertrophy) and to divide repeatedly (hyperplasia), resulting in the formation of tumors (galls). The enlarged host plant cells

are invaded by the fungal hyphae and the invaded cells collapse and die soon. *U. maydis* displays dimorphic growth alternating between a budding haploid and a filamentous dikaryon formed by mating of two haploid cells. This morphological change in the asexual phase of the pathogen plays a critical role in pathogenicity, since only the filamentous dikaryon is able to infect maize. A role for the cAMP signaling pathway in dimorphism and pathogenicity was indicated. Further, the involvement of upregulated genes in filamentous cells in pathogen morphogenesis was later inferred (Andrews et al. 2004).

Ustilago maydis is capable of producing indole-3-acetic acid (IAA) efficiently from tryptophan. The pathways leading to fungal IAA biosynthesis in maize plants were investigated. Two indole-3-acetaldehyde dehydrogenase genes *iad1* and *iad2* were identified. The mutants $\Delta iad1$ and $\Delta iad2$ were blocked in the conversion of both indole-3-acetaldehyde and tryptomine to IAA. Deletion of the aromatic amino acid aminotransferase genes *tam1* and *tam2* in $\Delta iad2$ mutant resulted in a further reduction in IAA levels by 5- and 10-fold in mutants harboring deletions. The results indicated that fungal IAA production might be a critical factor contributing to the IAA levels in infected tissues (Reineke et al. 2008).

In a later investigation, candidate pathogenesis genes were identified based on their representation in the dikaryon library only or in the dikaryon and diploid cDNA libraries, followed by comparative analysis with the genome sequences of other plant pathogens. Six genes conserved only among pathogenic fungi and six genes unique to *U. maydis* were confirmed to be expressed in planta, using RT-PCR assay. The transcript levels of three of these genes varied during the transition from filamentous dikaryotic growth to formation of the smut teliospores. Many of the newly identified *U. maydis* noncoding RNAs were differentially represented among cDNA libraries, suggesting potential functional roles in different *U. maydis* cell types (Morrison et al. 2012).

Ustilaginoidea virens (teleomorph *Claviceps virens*), causing false smut disease of rice, produces conidia, pseudosclerotia, and true sclerotia in the fructifications in place of rice grains. Ascospores, produced following germination of sclerotia, cause primary infection of spikelets, while conidia play an important role in the secondary infection. Inoculation of leaf sheath, just prior to ear emergence, resulted in successful infection. In order to study the mode of infection of spikelets during booting stage, a strain of *U. virens* expressing green fluorescent gene (*gfp*) was employed. Conidial suspension of the transformed strain was injected into rice leaf sheaths. The presence of several conidia on the spikelet surfaces was recorded at 4 h postinoculation. The hyphae after growing sufficiently invaded the spikelets at 144 h postinoculation through their apices, via the small gap between the lemma and palea. All floral organs were subsequently invaded with lapse of time after inoculation (Ashizawa et al. 2012).

Fusarium verticillioides, infecting maize (corn) plants, induces root rot, stalk rot, ear rot, and seedling blight at different stages of plant growth. In order to study the path of invasion of maize tissues by *F. verticillioides*, a strain of the pathogen was transformed with a gene (*gfp*) for green fluorescent protein (GFP). Seeds of a commercial maize hybrid were inoculated with the transformed strain, planted in a greenhouse potting mix, and incubated in growth chambers. The plants were incubated at three temperature regimes (low, average, and high), designed to simulate the range of temperature conditions that occurred in Iowa during different periods after planting. Transformation did not affect the growth of pathogen strain in liquid cultures. The transformant and the wild-type strain colonized maize stalk tissue at similar rates. The GFP-expressing

strain infected >90% of root and mesophyll tissues at all three temperature regimes. Symptomless systemic infection of below-ground and aboveground internodes was also detected at all three temperature regimes. Seed-to-kernel transmission involves several stages and seed-to-seedling is the first stage. GFP was found to be a suitable marker for *F. verticillioides* transformant. This technique allowed differentiation of the transformant from other naturally occurring *Fusarium* strains in the environment. The results indicated that seed transmission of *F. verticillioides* was found to be common and symptomless systemic infection could be initiated under a broad range of temperature conditions (Wilke et al. 2007).

Ustilago maydis, incitant of maize (corn) smut disease, systemically colonizes infected plants and induces symptoms exclusively in the inflorescences. The pathogen infects primordia of all aerial organs, resulting in the formation of large plant tumors. *U. maydis*-induced organ-specific transcriptional changes in the pathogen and seedling leaves, adult leaves, and tassels were observed. *U. maydis* genes encoding secreted proteins were differentially expressed, depending on the colonized maize organ. The pathogen might secrete virulence-related proteins (effectors) that act in an organ-specific manner. In support of this presumption, identification and functional characterization of 20 presumptive organ-specific *U. maydis* effector genes were accomplished. Deletion mutants of *U. maydis* were generated and their infectivity on maize leaves and tassels was determined. Eleven effector genes were required for the full virulence of *U. maydis*. Virulence was affected in one of the tested plant organs in nine deletion strains. The results indicated that individual effector proteins had a role in an organ-specific manner (Schilling et al. 2014.)

Pyricularia grisea, incitant of one of the important diseases of rice, causes leaf-, node-, and neck-blast phases and also infects the seeds. In the tropics, airborne inoculum is available throughout the year. On the other hand, in regions such as Japan where overwintering is necessary for the pathogen, infected seeds may play an important role in the disease cycle (Lee 1994). The location of *P. oryzae* in the infected germinating and nongerminating rice seeds was determined. Sporulation of *P. oryzae* was most often confined to the embryonal end of germinating seed. In contrast, sporulation of the pathogen was observed all over the surface of the nongerminating seed. The embryonal end of the living seed was found to be the most susceptible to infection by *P. oryzae*. It is likely that the embryonal end of rice seeds has high protein content which provides the nutrition for the invading pathogen. The extent of transmission of *P. oryzae* from seed to plant was very low. However, the latent infections by *P. oryzae* result in lesion development on 2-week-old rice seedlings. The results indicated that such infected seedlings could be a potential source of blast disease under field conditions (Manandhar et al. 1998).

The possibility of rice seeds infested with *Pyricularia grisea* present on the soil surface, causing primary infections and sustaining rice blast epidemic during the growing season by inducing symptoms on leaf, collar, and neck of rice plants, was investigated. A marked strain of *P. oryzae*, a sulfate nonutilizing (*sul*) mutant was used to infest rice grains that were applied on the soil surface at the time of seedling emergence at densities of 0, 0.5, 5, 25, and 50 grains/0.1 m² in plots planted to susceptible cultivar M201. Leaf blast symptoms were observed and the *sul* mutant was isolated from more than 90% of lesions sampled from rice seedlings at 35–45 days after plant emergence. At the end of seasons, the *sul* mutant was recovered from over 90% of the leaf, collar, and neck blast lesions. Sporulation on artificially infested grains occurred up to two weeks after

application of infested rice grains to the soil under field conditions and was similar in overall appearance to that of naturally infested seed. Conidiophores and conidia formation were predominantly associated with the embryonic end of rice seed for both artificially and naturally infested seed. The *sul* mutant of *P. grisea* could be easily differentiated from wild-type isolates recovered from rice blast lesions by a differential medium containing sodium selenate. The *sul* mutant predominated in all leaf, collar, and neck blast lesions sampled in two years (1996 and 1997). The development of blast disease caused by the wild-type strain and *sul* mutant progressed in a similar pattern, indicating the usefulness of the marked strain of *P. oryzae* to follow the process of blast disease development (Long et al. 2001).

Magnaporthe oryzae (*grisea*), incitant of rice blast disease, occurs in widely different ecosystems. The main overwintering and primary inoculum sources were found to be infected plant residues and seeds. To follow the different stages of the infection process, microscopic observations were made from seed germination to seedling stage under aerobic conditions and water seeding. Green fluorescent protein (*gfp*) gene-transformed *M. oryzae* strains were employed to track the pathogen invasion. Conidia produced from contaminated seed infected emerging seedling tissues (coleoptiles and primary root) and produced mycelium that colonized the newly formed primary leaf and secondary roots. When the rice plants were inoculated either at ripening stage, heading stage, or flag leaf stage, *M. oryzae* was able to infect the seeds. The seedlings growing from infected seeds were generally killed and such seedlings would serve as inoculum source for the healthy rice plants (Faivre-Rampant et al. 2013).

Verticillium dahliae, a soilborne pathogen causing spinach Verticillium wilt disease, is seedborne. It became a major concern because of the pathogen dispersal to areas where fresh and seed processing crops may be grown in rotation with other susceptible crops. The spinach seedlings were inoculated with conidial suspension of *V. dahliae* for studying colonization events, using confocal laser-scanning microscopy. Conidia germinated at 24–96 h postinoculation (PI), followed by production of hyphal colonies on root tips and root elongation zones. Colonization of root cortical tissues occurred both inter- and intracellularly within two weeks. The pathogen colonized the taproot xylem with abundant mycelium and conidia, resulting in vascular discoloration coincident with foliar symptom expression by eight weeks PI. The xylem of the upper stem, inflorescence, and spinach seed parts, including the pericarp, seed coat, cotyledons, and radical, were colonized by the pathogen which did not invade the perisperm (diploid maternal tissue). The seed coat, the outermost layer of the vasculature, had the maximum fungal population. The results indicated that infection of *V. dahliae* in spinach was systemic and could be transmitted to seedlings emerging from infected seeds (Maruthachalam et al. 2013).

Botrytis cinerea, causal agent of gray mold diseases affecting several vegetable and fruit crops, induced systemic, endophytic infection of lettuce plants which frequently remained symptomless. Endophytic infection may commence from seeds. The test plants were grown in a conventional glasshouse and in a spore-free air-flow provided by an isolation propagator. *B. cinerea* was detected by plating surface-sterilized sections of roots, stem pieces, and leaf discs from symptomless plants on selective media. The presence of *B. cinerea* in different tissues of symptomless plants was confirmed by immuno-labeling of the tissues with the *Botrytis*-specific monoclonal antibody (MAb) BC-12-CA4. Infection spread from the roots to stem and subsequently to leaves with an increase in interval after inoculation with *B. cinerea*. Seed surface-sterilization led to a

reduction in the number of symptomless lettuce plants. Genetically fingerprinted selected isolates, using microsatellite loci, were employed to confirm the ability of *B. cinerea* isolates to spread systemically in inoculated plants in which other isolates were also detected. This indicated that single lettuce plants could host multiple isolates of *B. cinerea*. The results revealed the potential of *B. cinerea* to develop as endophyte in lettuce plants and the endophytic phase might be an important component of species perpetuation, as the aggressive necrotrophic phase leads to development of symptoms characteristic of gray mold disease (Sowley et al. 2010).

Ergot disease is caused by *Claviceps purpurea*, *C. sorghi*, and *C. africana* in small grain crops. Rye inflorescence infected by *C. purpurea* produces ergot sclerotia in place of grains. The pathogen overwinters as sclerotia on or in the soil surface or mixed with seed. At the heading stage, sclerotia germinate by producing flesh-colored stalks at the tip of which spherical heads are formed at the periphery. Perithecia-bearing asci containing ascospores are produced. Ascospores released from the asci are carried by wind currents or insects to freshly opened flowers. The ascospores infect the ovaries directly or through stigma. Detection of the *cpnox1* gene from *C. purpurea*, probably encoding a membrane-associated NADPH oxidase, had an impact on the germination of conidia and pathogenicity. $\Delta cpnox1$ mutants could penetrate the host epidermis, but they were impaired in colonizing the floral ovarian tissues. When macroscopic signs of infection (honeydew-containing spores) were observed, the initial appearance of the symptoms was extremely delayed and fully developed sclerotia were never formed. The results suggested that *C. purpurea* Nox1 might be important for the interaction with its host, probably directly affecting pathogenic differentiation of the fungal isolates (Giesbert et al. 2008).

Phaeosphaeria nodorum (anamorph *Stagonospora nodorum*), infecting wheat, is seedborne and the infected seed may be a major source of primary inoculum for foliar epidemics. Seed infection in commercial wheat seed lots is common, the range of and mean infection being 1–71% and 23% (Shah and Bergstrom 1993). *P. nodorum* is transmitted effectively from infected seed to aerial portions of seedlings over a broad range of soil temperatures. The pathogen forms on the seedling surfaces of pycnidiospores that are dispersed by splashing rain (Shah and Bergstrom 2000). The relative contribution of seed-transmitted *P. nodorum* in winter wheat versus all other primary inocula to foliar and grain populations was assessed by releasing identifiable genetically marked isolates in infected seed and using greenhouse-generated clean seed for control field plots. Seeds infected by two distinct groups of marked isolates of *P. nodorum*, containing rare alleles that were identified by amplified fragment length polymorphisms (AFLPs) and balanced for mating type, were planted in experimental field plots at two locations in New York and Georgia states, USA. Both infected seed and other sources of the pathogen significantly contributed primary inocula to populations recovered from leaves and harvested grain. Seed-transmitted genotypes accounted for a total of 57% of all isolates recovered from inoculated plots, with a range of 15–90% of the populations of *P. nodorum* over the season in individual, inoculated plots at four locations. Isolates of *P. nodorum* sampled from the upper leaves and mature seed were derived from seed-transmitted, primary inoculum as well as from other primary inocula. Isolates with genotypes identical to those inoculated on seed were generally only recovered from plots where they were released. Plants in the noninoculated control plots were also infected and 95% or more of the isolates recovered from these plots were nonreleased genotypes which might be derived from immigrant ascospores, potentially from sources at a considerable distance from the experimental plots (Bennett et al. 2007).

Leptosphaeria maculans (anamorph *Phoma lingam*) causes Phoma stem canker, one of the most destructive diseases of canola (oilseed rape-*Brassica napus*). The role of infected stubble pieces containing pseudothecia and/or pycnidia and airborne ascospores in the development of disease at different stages of canola was investigated. Mean disease severity was significantly higher ($P < 0.001$) on plants infected at cotyledonary stage than at 1- and 3-leaf stage. Plants at these stages were more severely infected than the plants infected at later stages (6-leaf). The results suggested that pycnidiospores might be the primary source of inoculum in western Canada under field conditions (Ghanbarnia et al. 2011). The process of infection was studied using the cultivar Surpass 400 containing single dominant gene-based resistance (SDGBR). Two strains of *L. maculans*, UWA192 and UWAP11, were inoculated on cultivars Surpass 400 and Westar. The prepenetration and penetration behavior of both strains were found to be similar. However, significant differences in the further stages of infection were observed. When UWAP11 infected Surpass through stomata, guard cells were killed rapidly within a few hours and necrosis set in in the surrounding mesophyll cells, resulting in a hypersensitive reaction (HR). Hyphal growth continued slowly through the intercellular palisade, mesophyll, and spongy mesophyll spaces, but hyphae rarely spread on the HR region and did not sporulate. Accumulation of phenolic compounds occurred in the area bordering the HR. In contrast, when UWA192 infected through stomata, symptoms became visible at 10–12 days postinoculation. Pale to tan/gray circular lesions with abundant pycnidia were observed. Hyphae extensively spread beyond the lesion border, reaching the veins and progressing down the petiole towards the stem. HR reaction to UWAP11 strain restricted its spread to a few palisade cells, although the presence of hyphae in the lower tissue layers of cotyledons could be noted. In the absence of SDGBR, as in UWA192 strain infection, extensive death of epidermal and upper and lower palisade cells occurred throughout infected areas of the cotyledon abundantly. Accumulation of phenolic compounds characteristic of HR did not occur in the interaction with UWA192 strain (Li et al. 2007).

In a later investigation, the cytological responses in the hypersensitive reaction (HR) induced in the cotyledon and stem tissues of *B. napus* inoculated with an avirulent strain of *L. maculans* were studied. The cotyledon or the sixth true-leaf stage plants were inoculated with pycnidiospores of *L. maculans* strain UWAP11. Condensation of cytoplasm, shrinkage in cell size and nuclear DNA fragmentation in the cotyledon and stem tissues, shrinkage and condensation of cytoplasm, chromatin fragmentation, and lobing of the nucleus were the cytological responses induced by the avirulent strain of *L. maculans* in Surpass 400 canola (Li et al. 2008).

Pyrenophora graminea, causative agent of barley leaf stripe disease, is seedborne; the possibility of long- and short-distance spread of the disease through infected seed lots is therefore of concern. In order to study the genetic interaction of the pathogen strains and host cultivars, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was employed to determine hordein pattern (groups D, C, and B) of five barley cultivars inoculated with selected isolates of *P. graminea*. The absence of particular hordein subunits and degradation of others in most of the cultivars inoculated with virulent isolates was observed. Identical numbers of polymorphic bands were observed through the different assays for each pathogen isolate–cultivar combination. The changes in the hordein profiles of infected seeds were considered to be due to infection with *P. graminea* (Arabi et al. 2005).

Bipolaris zeicola (teleomorph *Cochliobolus carbonum*) causes ear rot disease of maize (corn) plants and is carried in the seeds. Maize seed was reported to contain a chitinase ChitA, an antifungal protein. ChitA was converted to a modified form by a protein secreted by *B. zeicola*. In denaturing chitinase zymograms, ChitA could be detected in protein extracts from healthy seeds but not in the infected seeds from ears inoculated with *B. zeicola*. Further, addition of proteins extracted from diseased seeds to those from healthy seeds led to the loss of detectable ChitA activity, indicating the presence of a molecule in diseased seeds that was able to target ChitA. This molecule was identified as a secreted protein which was designated *B. zeicola* chitinase-modifying protein (Bz-cmp). Biochemical investigations showed that Bz-cmp isolated from pure cultures of *B. zeicola* catalytically converted ChitA into a modified form with reduced molecular mass in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Bz-cmp was impaired in its chitin-binding ability. The results suggested that ChitA was an important component of defense in developing maize seeds and Bz-cmp might be a potential contributor to pathogenicity of *B. zeicola* (Naumann et al. 2009).

Pectolytic enzymes, namely polygalacturonase (PG), pectin methylesterase (PME), and pectate lyase (PL), are produced by fungal pathogens during disease development. These enzymes degrade the pectin in plant tissues, resulting in their maceration, and enzymatic activities are enhanced under conditions for rapid development of disease. *Phytophthora capsici* infects pepper and other vegetable crops, causing blight diseases. *P. capsici* produced high levels of PG, PME, and PL in planta. A high correlation between enzymatic activity and pathogenicity of isolates of *P. capsici* was observed (Jia et al. 2009). Visualization of the host–pathogen interaction was accomplished by employing *P. capsici* transformed with green (*pgfp*) or red (*tdTomato*) fluorescent protein. All transformants emitted fluorescence at all stages of the life cycle, but the intensity of fluorescence differed. They contained one, two, or five copies of *pgfp* or *tdTomato*, as determined by Southern analyses. One of the transformants expressing *pgfp* had reduced growth on artificial medium, produced smaller lesions on detached pepper fruit, and showed reduced virulence on pepper seedlings compared with wild-type isolate. However, four transformants with similar growth rate and virulence as the wild-type isolate could be useful for tracking the pathogen spread in the pepper tissues at different intervals after inoculation (Dunn et al. 2013).

Soil is a reservoir for *Aspergillus flavus*, which causes peanut aflaroot disease. Densities of *A. flavus* and *A. parasiticus* in soil varied widely and this factor might influence the severity of peanut infection. The relationship between soil density of the pathogens and the incidence of peanut seed colonizing under laboratory conditions was therefore investigated. Viable peanut seeds were wounded and inoculated with the pathogens from 20 soils differing in composition and densities of *Aspergillus* spp. The percentages of seeds colonized were well below 100%, despite high densities of *Aspergillus* spp. in some soils. Competition among section Flavi species might be the possible reason for reduced incidences of seed colonization. An average of two or more propagules of each *Aspergillus* spp. in the soil at the wound site was required for colonization of 20% peanut pods (Horn 2006).

The role of proteolytic enzymes produced by *A. flavus* and *A. parasiticus* in peanut seed infection and aflatoxin contamination was examined. *A. parasiticus* produced protease before infection of peanut seed. The pathogen was inoculated on seeds of resistant and susceptible peanut cultivars. *A. parasiticus* grown on casein medium (inductive medium) induced greater internal and external infection and the seeds had higher

fungal protease content than those induced by the pathogen grown on PDA or sucrose media (noninductive media). Higher fungal colonization and aflatoxin contamination in seeds of resistant cultivar preincubated with *Aspergillus* extracellular proteases were observed than in those incubated without proteases. *A. flavus* and *A. parasiticus* produced metallo and serine proteases, the greatest activity being associated with the latter enzyme (Asis et al. 2009). In another study, the presence of aflatoxins was detected using high-performance liquid-chromatography (HPLC) technique in husks and kernels of peanut infected by *A. flavus* and *A. parasiticus*. However, the results revealed that the presence of these pathogens did not necessarily indicate the presence of aflatoxins in the substrate. Isolation of *A. flavus* and *A. parasiticus* from soil samples confirmed that the main route of peanut infection and aflatoxin contamination was through soilborne inoculum (Atayde et al. 2012).

Aspergillus flavus, an opportunistic fungal pathogen, infects maize kernels at the pre-harvest stage, becoming a major human health concern in addition to being responsible for substantial grain loss. In order to understand the pathways of invasion of kernels by *A. flavus*, localization, morphology, and transcriptional profile of the pathogen were investigated during internal seed colonization. *A. flavus* was found to be capable of infecting all tissues of the immature kernel by 96 h after inoculation. The presence of mycelia in and around the point of inoculation in the endosperm was observed and the hyphae were advancing down the germ. At the endosperm–germ interface, hyphae seemed to differentiate and form a biofilm-like structure that surrounded the germ. A custom-designed Afymetrix GeneChip® microarray system was used to monitor genome-wide transcription during pathogenicity. A total of 5061 genes were differentially expressed. Genes encoding secreted enzymes, transcription factors, and secondary metabolite gene clusters were upregulated and considered to be potential effector molecules responsible for disease in the kernel. The results indicated the possibility of preventing or slowing down the colonization of *A. flavus*, using suitable disease management strategies (Dolezal et al. 2013).

4.1.3 Infection Through Inoculum Present in Seed-Bearing Organs

Seeds may be invaded by the fungal pathogens that do not have direct access to the seeds which are enclosed in fruits or pods. Infection of pods of leguminous crops such as bean, pea, peanut, and grain legumes and fruits of tomato and cucurbits may result in seed infection in due course. The pathogens develop on the lesions initiated on the seed-bearing organs and the fungal structures, such as acervuli and pycnidia that produce asexual spores or chlamydospores, and other overwintering spores are formed. Infection of seeds may occur in the field in the same season or after harvest during transit and storage.

Ascochyta rabiei (teleomorph *Didymella rabiei*), a necrotrophic fungal pathogen, directly penetrates and infects all aerial plant parts of chickpea (*Cicer arietinum*). During germination of conidia and infection, germ tubes secrete a mucilaginous substance to facilitate attachment to the host surface and the pathogen produces cell-wall-lytic enzymes to penetrate the host plant cells. In addition, the pathogen produces several phytotoxins, solanapyrones A, B, and C, cytochalasin D, and a proteinaceous toxin which may be involved in cell necrosis and subsequent death. Further, the pathogen may be able to degrade the preformed antimicrobial compounds present in the host tissues and/or suppress their production in chickpea. Two pathotypes of *A. rabiei*, pathotype I (less aggressive) and pathotype II (aggressive), have been identified. A high

degree of variation in resistance among chickpea cultivars has been reported and resistance to the Ascochyta blight disease declines with plant age. The symptoms of infection and disease severity showed a quantitative continuum based on virulence and cultivar resistance to the pathogen (Jayakumar et al. 2005).

Colletotrichum lindemuthianum, the incitant of anthracnose diseases of leguminous crops including common bean (*Phaseolus vulgaris*), exists in the form of several races, differing in their virulence assessed by inoculating a set of differential varieties that respond to the pathogen races differently. Isolates (85) of *C. lindemuthianum* from kidney bean were differentiated into 12 races on the basis of their reaction types (Sharma et al. 1999). The conidia surrounded by a fibrillar spore coat, comprising many major glycoproteins, were formed from acervuli present on plant residues left after harvest on the soil. The conidia of *C. lindemuthianum* adhered to the aerial parts of bean plants to initiate the infection process. The extent of seedborne infection (0, 1, 2, 5, 10, and 20%) influencing the seedling emergence, seedling infection, and the buildup of anthracnose disease inoculum in the crop, were investigated. The incidence of seedling infection was significantly higher in the cultivar Acole than in Navigator. A linear increase ($P < 0.01$) in anthracnose severity within the crop canopy and on the pods with higher rates of seedborne infection was observed in both cultivars. The percentage of seedborne infection was positively correlated with loss in yield and seed mass (Conner et al. 2009).

A monoclonal antibody (UB20) that was specific to the glycoproteins inhibited the adhesion of conidia to hydrophobic surfaces. Conidia germinated on glass, polystyrene, and water agar, but appressoria were produced only on hard surface. Removal of the spore coat with protease inhibited adhesion of spores at 30 min, but before the secretion of extracellular matrix (ECM) glycoproteins. Protease treatment inhibited development of appressorial formation or pathogenicity on inoculated plant leaves. The intact spore coat seemed to have an important role in conidial adhesion and for the formation of appressoria required for initiation of infection process by *C. lindemuthianum* in common bean (Rawlings et al. 2007).

Mutants of *Colletotrichum lindemuthianum*, blocked at different stages of appressorial development – namely differentiation, maturation, function, and signal exchange between the pathogen and host – were employed to study the functions of genes involved in pathogenesis. Strain H18 was blocked at the appressorium differential stage and this strain did not form any genuine appressoria. Strain H191, blocked at appressorium maturation stage, exhibited a pigment defect and was only able to develop partial internal turgor pressure required for mechanical penetration of host cell surface. Strain H290, defective in appressorium function, could not penetrate into bean tissues. Strain H290 produced nonexpanding lesions, whereas mutant H101 was unable to induce lesions even on wounded plant tissues and mutant H18 could not colonize even wounded plant surfaces. The results indicated that for successful infection, genes governing all stages of appressorial development were required (Veneault-Fourrey et al. 2005).

In a later investigation, the role of STE12-like gene-encoding (CLSTE12) transcription factor essential for appressorium-mediated host penetration by *C. lindemuthianum* was studied. Expression of a spliced variant of *C. lindemuthianum* was negatively regulated during the early stages of pathogenesis, whereas the correctly spliced mRNA expressed up to the penetration step, suggesting distinct roles for these two transcripts. Comparison of the cell surface protein patterns generated from the wild-type strain and the *Clste 12Δ* mutant revealed the distinct presence of a major protein (Clsp1p9) of 16kDa in the wild-type strain, but not in the mutant strain. The Clsp1p9 protein

belonged to a new family of wall-associated proteins found only in the Euscomycetes. The results suggested that the activity of CLSTE12 could be molded by a regulated alternative splicing mechanism, and this factor may be involved in the production of cell surface proteins and host cell-wall-degrading enzymes (Hoi et al. 2007).

Colletotrichum lagenarium causes anthracnose diseases of cucurbits. *C. lagenarium* produced melanized appressoria from which infection hyphae, essential for penetration of the host surface, are formed. During the final step of melanin biosynthesis, the enzyme laccase has been shown to be involved in the oxidation of 1,8-dihydroxynaphthalene (1,8-DHN). The role of a laccase gene *LAC1* from *Colletotrichum lagenarium* in the melanin biosynthesis was investigated. *LAC1* was cloned from *C. lagenarium* that could synthesize 1,8-DHN melanin and characterized. The genomic DNA of *C. lagenarium* was subject to PCR with degenerate oligonucleotide primers for cloning the *LAC1* sequences. The *LAC1* gene contained an open reading frame (ORF) composed of 589 codons and three introns of 51, 49, and 59 nucleotides. Under melanin-induced culture of *C. lagenarium*, laccase activity significantly increased and *LAC1* expression was also detected. However, the *lac1Δ* mutants were also able to retain laccase activity and had no significant phenotypic differences in melanin production or pathogenicity from the wild-type strain. The results indicated that laccase gene might not be essential for melanin biosynthesis by *C. lagenarium* and for its pathogenicity (Tsuji et al. 2001).

C. lagenarium exhibited synchronous cellular differentiation from conidia. Two types of cellular differentiation could be controlled by the incubation temperature. At 24°C, conidia produced darkly melanized appressoria. On the other hand, at 32°C they produced elongated germ tubes and lacked the ability to produce appressoria. The presence of melanin was detected only in the appressoria, but not in conidia or germ tubes. RNA gel blot analysis revealed that melanin biosynthesis structural genes were expressed during the early stage of conidial germination, regardless of appressorium formation, suggesting the involvement of posttranscriptional regulation of appressorium melanization. Temporal translational patterns of melanin biosynthetic enzymes during the development of germinating conidia at 24°C and 32°C were examined. Western blot analysis revealed that two melanin biosynthetic enzymes, namely scytalone dehydratase (Scd1p) and 1,3,8-trihydroxynaphthalene reductases (Thr1p), were expressed only when appressorial differentiation occurred at 24°C but not at 32°C (Tsuji et al. 2003).

The role of cyclic-AMP (cAMP) signaling in the process of development of anthracnose disease caused by *Colletotrichum lagenarium* in cucurbits was investigated. The *cpk1* and *cac1* mutants, knockout mutants of *CPK1* and adenylate cyclase gene *CAC1*, were nonpathogenic on cucumber. Both mutants had low level of conidial germination, suggesting the involvement of cAMP signaling in conidial germination. Although conidial germination occurred in the mutant, the appressoria were nonfunctional resulting in the failure of infection. The results indicated that cmk1 mitogen-activated kinase (MAPK) may regulate germination, appressorium formation, and growth of the pathogen. The cAMP signaling appeared to control multiple steps of fungal infection in cooperative regulation with cmk1MAPK in *C. lagenarium* (Yamaguchi et al. 2004).

The gene *ClapEX6* (PEX6) encoding a protein involved in peroxisome biosynthesis was identified through analysis of nonpathogenic mutants of *Colletotrichum lagenarium* (Kimura et al. 2001). The knockout analysis of this gene revealed the requirement of *PEX6* gene for pathogenicity of *C. lagenarium*. The *pex6* mutants formed only small appressoria and severely reduced melanization, resulting in failure of production of penetration (infection) hyphae. Metabolic functions of peroxisome seemed to be essential

for appressorium functionality, especially for melanization of appressoria. The role of peroxisome-associated metabolism in pathogenicity of *C. lagenarium* was investigated. *ICL1* was isolated from the pathogen and characterized. *ICL1*-encoded isocitrate lyase was involved in the glyoxylate cycle in peroxisomes. The *icl1* mutants were unable to utilize fatty acids and acetate for mycelial growth. These mutants formed highly pigmented appressoria, suggesting that glyoxylate cycle was required for melanin biosynthesis in appressoria. However, *icl1* mutants were poorly virulent and penetration hyphae failed to develop in the inoculated plants, suggesting that *ICL1* was involved in invasion of host tissues. Exogenous supply of glucose partially restored the virulence of the mutants. The results indicated that *PEX6* was required for melanization of appressoria, a vital requirement of successful host tissue penetration (Asakura et al. 2006).

Colletotrichum coccodes, causing tomato anthracnose, is a devastating disease of ripe fruits. The pathogen can infect tomato roots, stem, and leaves. The disease may be transmitted to the emerging tomato seedlings when contaminated seeds are sown. Seed infection by *C. coccodes* may therefore lead to anthracnose disease incidence on tomato fruits in the subsequent generation. The proportion of seeds infected by *C. coccodes* ranged over 20–63% for all isolates of the pathogen tested and correlated with the aggressiveness (virulence) of the isolates to tomato fruits. An isolate of *C. coccodes* was transformed with green fluorescent protein (*gfp*) gene, employed to visualize the progress of disease development in tomato fruits. The mycelium was detected both on the surfaces of seed coat and within 1% of the embryos in infected tomato fruits (Ben-Daniel et al. 2010). Several factors including pH are involved in the regulation expression of genes encoding proteins. The structural and functional characterization of the *pacCl* gene, which encodes the transcription factor PacC of *Colletotrichum lindemuthianum*, were performed. The *pacCl* gene showed reduced expression in acid pH and its transcription was activated by elevated extracellular pH. A *pacCl* disruption mutant line of *C. lindemuthianum* was used to study the importance of the *pacCl* gene. The mutant line was able to penetrate the host tissue through differentiation of primary hyphae. However, it could not cause maceration of the infected plant tissue. The results suggested that PacCl was a regulator of a gene activation and its expression is required for fungal growth in alkaline conditions, as well as for the transcription of genes required for the passage from the biotrophic to the necrotrophic phase (Soares et al. 2014).

Macrophomina phaseolina causes charcoal rot and premature death of plants following infection at postflowering stages of mungbean plants. The modes of infection of seed by *M. phaseolina* were investigated. The pathogen was isolated from serial portions of ten mungbean plants infected naturally. There were discrete portions of infected tissue separated by apparently healthy tissue in most of the plants. In addition, molecular analysis of isolates collected from infected tissue on two of the plants was carried out. The results suggested that aerial infection of aboveground parts by different isolates was common. Inoculations of roots and aboveground parts of mungbean plants at nine temperature–soil-moisture-incubation combinations and of detached green pods strongly supported the view that seed infection resulted from infection of pods by microsclerotia, rather than from hyphae growing systemically through the plant root or stem infection. However, when inoculum of *M. phaseolina* was placed within 130 mm of green developing pod and the herbicide containing paraquat and liquat was sprayed on the inoculated plants, *M. phaseolina* was able to grow systemically from vegetative tissue into the pods and seeds, indicating the possibility of seed becoming infected through movement of the pathogen from other plant parts (Fuhlbohmer et al. 2013).

4.1.4 Role of Enzymes and Toxins in Pathogenesis

The enzymes and toxins elaborated by fungal pathogens have important roles in the different stages of pathogenesis (disease development). Various kinds of primary cell-wall components are present in higher plants. Pectin forms a major structural component of the middle lamella of primary cell walls, and consists of rhamnogalacturonan and homogalacturonan. The enzymes produced by the fungal pathogens degrade the pectin by depolymerization (as in the case of polygalacturonases, pectin, and pectate lyases) or altering its structure (as done by pectin acetylsterases and pectin methylsterases) (Kars and van Ken 2004). In general, optimal activity of fungal pectin methylsterases (PMEs) occurs between pH 4 and pH 6, whereas plant and bacterial PMEs peak between pH 6 and pH 8 (Alebeek et al. 2003).

Fusarium graminearum, the primary pathogen of Fusarium head blight (FHB) disease affecting wheat and barley and other small grain crops, infects ears during anthesis. *F. graminearum* penetrated the spikelets through ovary and colonizes inner surfaces of the lemma and palea. After penetration, pathogen hyphae invaded both intra- and intercellularly in the host tissues. The pathogens spread through the vascular bundles in the rachis and rachilla, between florets inside the spikelet, and from spikelet to spikelet. Investigations based on gold labeling of cellulose, xylan, and pectin revealed that *F. graminearum* degraded all three cell-wall polymers during penetration and wheat spike tissues (Ribichich et al. 2000; Wanyoike et al. 2002; Miller et al. 2004).

In a later study, the role of two endo-polygalacturonases (end-PGs) produced by *F. graminearum* during pathogenesis in wheat spikelets was investigated. Experiments on expression and characterization of two PGs during infection of wheat spikelets were performed. The endo-PGs were found to be products of two unique endo-PG genes annotated in the genome database of the pathogen. The genome of *F. graminearum* had two genes encoding two different endo-PGs and, as indicated by N-terminal Edman sequencing, the PG1 and PG2 from in vitro culture corresponded to the products of the *fg1101* and *fg03194* genes, respectively. Both genes (*Fgpg1* and *Fgpg2*) were expressed in vitro and during spike infection. PG1 had a greater specific activity (at pH 5–7), was largely secreted in liquid culture, and could be detected frequently in the infected ovary. On the other hand, PG2 was more active at a higher pH range (7–7.8), was poorly secreted in liquid culture, and faintly detectable in infected ovaries. Expression analysis during host–pathogen interaction showed that transcription of both *pg* genes occurred within 12 h. Both PG-encoding genes were maximally expressed at 24 h after spikelet infection, paralleling the expression of a pectin lyase (*Fgpnl1*) gene. The *pg1* transcript was strongly induced, whereas the *pg2* transcripts appeared to be slightly anticipated. The timing of expression of *pg* genes appeared to be consistent with the characteristics of the tissue initially infected after inoculation. Expression analysis of xylanases showed that *FgxylA* transcript could be detected later than the *pg* and *pnl* transcripts and reached a maximum at 4 days after infection. Xylanases gene expression appeared to be more related to hyphal colonization of the floral bracts (Tomassini et al. 2009). Degradation of pectin preceded the growth of *Botrytis cinerea*, and endo-polygalacturonase (endo-PG) genes (*Bcpg*) were expressed during pathogen invasion of different plant tissues. A purified glycoprotein from strain T14 of *B. cinerea* was identified as endo-PG1 (T4BcPG1) and this glycoprotein was shown to be both a virulence and avirulence factor (Poinssot et al. 2003).

A phytotoxic metabolite botrydial, another virulence factor of *B. cinerea*, acted in a strain-specific manner. All pathogenic strains of *B. cinerea* secrete a nonspecific phytotoxin, the sesquiterpene botrydial. The functional characterization of *bcbot1*, encoding a P450 monooxygenase, indicated its involvement in pathogenesis. The botrydial biosynthetic gene *bcbot1* was expressed in planta. Deletion of *bcbot1* resulted in reduced virulence only in strain T4 among three strains tested, indicating that the effect on virulence of *B. cinerea* was strain-specific. *B. cinerea*, being a versatile pathogen capable of infecting a wide range of plant species, may possess multiple infection mechanisms with functional redundancy (Siewers et al. 2005).

Plant cell walls contain polygalacturonase-inhibiting proteins (PGIPs). The interactions between PGIPs and endo-PG enzymes secreted by fungal pathogens have been studied in some pathosystems. *Sclerotinia sclerotiorum* produced oxalic acid, endo-PGa, and endo-PGb with acidic and basic activities. The activity of PGb3 was enhanced by oxalic acid (oxalates) and PGa activity was maintained at pH 3.6. The activity of PGa was not affected by soybean PGIP, possibly because of inactivity of the PGIP at this pH. Reverse-transcription polymerase chain reaction (RT-PCR) assay indicated that, during infection of soybean by *S. sclerotiorum*, the expression of the putative *pga* gene was delayed when compared to the basic one (Favaron et al. 2004). The involvement of other enzymes, in addition to pectinases and cellulases, in the degradation of host plant cell walls during pathogenesis has been demonstrated. The immunogold labeling technique was applied to demonstrate the presence of homogalacturonan (pectin) in the cell walls of rye ovaries. Deposition of labels increased at the rye–*Claviceps purpurea* (causing ergot disease) interface. Two xylanases genes *cpxyl1* and *cpxyl2* were isolated from a genomic library of *C. purpurea*, using the genes of *Cochliobolus carbonum* (*xyl1*) and *Magnaporthe grisea* (*xyn33*) as heterologous probes. Evidence of PME, PG, xylanases, and cellulase activity was obtained in the rye–*C. purpurea* pathosystem. The genes *cpxyl1* and *cpxyl2* were single-copy genes and were expressed both in vitro and in planta during all stages of infection (Giesbert et al. 1998; Tenberge 1999).

The role of enzymes, other than cell-wall-degrading enzymes of pathogens in disease development, was investigated in wheat–*Stagonospora* (*Septoria*) *nodorum* pathosystem. Ornithine decarboxylase (ODC) was suggested to be essential for the virulence (aggressiveness) of *S. nodorum*. A knockout strain lacking the ODC allele was obtained through targeted gene replacement technique. The mutants were unable to obtain the supply of ODC required for utilizing polyamines from host plants because of the disruption of the gene concerned (Bailey et al. 2000). Seed transmission of *Mycosphaerella pinodes* in field pea was demonstrated under growth chamber and field conditions. The percentage of seeds infected by *M. pinodes* was negatively correlated with seedling emergence (Xue 2000).

In a later study, the host–pathogen interaction was investigated by determining the role of pathogenicity factors in the breakdown of host plant defense responses. *M. pinodes* secreted two well-characterized fungal signal molecules, a glycoprotein elicitor (c. 70 kDa), and polypeptide suppressor (<5 kDa) in the conidial germination fluids. The suppressors inhibited defense signaling induced by the pathogen elicitors, including activation of the cell wall and plasma membrane, ATPases, and protein kinases (Shiraishi et al. 1997). The involvement of a MAPK pathway in *M. pinodes* elicitor-induced defense response in pea and the interruption of the elicitor-induced MAPK cascade(s) in pea by the suppressor have been elucidated. A polypeptide fungal suppressor from *M. pinodes* inhibited the polysaccharide elicitor-induced (from the pathogen) activation of only

p44 kinase, a myelin basic protein (MBP-) dependent kinase, but not p48 kinase. The results suggested that the suppressor from *M. pinodes* might act through inhibition of a MAPK (p44), facilitating infection of pea by *M. pinodes* (Uppalapati et al. 2004).

During the development of diseases caused by fungal pathogens, in addition to enzymatic degradation of preformed antimicrobial compounds present in host plants, the production of toxins (host specific or nonspecific) considered as virulence or pathogenicity factors assist in the pathogen establishment in susceptible (compatible) plant species. *Alternaria alternata* infects several host plant species, including carrot. The pathogen produces different host-selective toxins (HSTs) that cause necrosis on specific host plant species and are designated "pathotypes" of *A. alternata* (Kohmoto et al. 1995). Carrot seeds are infected by *A. alternata* and *A. radicina*. A profile of toxin production by these pathogens was determined. All isolates of *A. alternata* (10) produced alternariol (AOH), alternariol methylether (AME), altenuene (ALT), and tenuazonic acid (TeA). An additional toxin altertoxin1 (ATX1) was produced by eight of ten isolates. The amount of toxin produced varied with the isolate concerned. Tenuazonic acid (TeA) was found to be the most dominant toxin among the toxins tested. On the other hand, isolates of *A. radicina* produced radicinin (RAD) only. Treatment with the solutions of extracts containing *Alternaria* toxins induced necrosis of seedlings growing from seeds, in addition to reducing germination of seeds (Tylkowska et al. 2003).

Pyrenophora tritici-repentis, causing tan spot in wheat, produces a host-selective (specific) toxin encoded by *ToxA* gene. ToxA was shown to be a protein and product of a single gene (Ciuffeti et al. 1999). The transformation of a nonpathogenic isolate of *P. tritici-repentis* with *ToxA* gene rendered it pathogenic on ToxA-sensitive wheat lines. The *ToxA* gene encoded a preprotein that contained a single sequence to target the protein and a prosequence (N-domain) that was necessary for proper folding that might be removed prior to secretion (Tuori et al. 2000). A related *toxB* gene present in nonpathogenic race 4 exhibited high similarity (86%) to *ToxB* and appeared to be a single-copy gene. This high level of similarity between pathogenic and nonpathogenic isolates (races) in a gene(s) encoding HSTs has not been reported in other fungal pathogens producing HST (Martinez et al. 2004).

The introduction of HST of *P. tritici-repentis* into the apoplastic space of a sensitive plant induced a necrotic response similar to the disease symptoms produced by ToxA-producing isolates, indicating the ability of ToxA protein to induce cell death in the absence of the pathogen. Sensitivity to ToxA and ToxB in the susceptible host plant was conferred by a single gene for each toxin. Toxin-sensitive and -insensitive wheat cultivars were treated with ToxA followed by proteinase K (PK) to study the site of ToxA (PtrToxA) action. ToxA was resistant to PK but only in sensitive leaves, suggesting internalization of ToxA into toxin-sensitive cells. The results revealed that ToxA protein was able to cross plant plasma membrane from apoplastic space to the interior of the plant cell in the absence of the pathogen (Manning and Ciuffetti 2005). Acetolactate synthase (ALS) catalyzes the first common step in leucine, isoleucine, and valine biosynthesis and is the target of several classes of inhibitors. In order to characterize MoILV2 and MoILV6 (subunits of ALS enzyme) functions in *Magnaporthe grisea*, incitant of rice blast disease, deletion mutants $\Delta Moilv2$ and $\Delta Moilv6$ were generated. Phenotypic analysis showed that both mutants were auxotrophic for leucine, isoleucine, and valine and were defective in conidial morphogenesis, appressorial penetration, and pathogenicity. The results suggested that Moilv2 and Moilv6 may play a critical role in

maintaining the balance of intracellular amino acid levels. *Moilv2* and *Moilv6* were both localized to the mitochondria and the signal peptide of *Moilv6* was found to be critical for its localization. The genes *MoILV2* and *MoILV6* had a crucial role in the biosynthesis of isoleucine and valine and isoleucine and leucine, respectively. Both genes are required for the pathogenicity of *M. oryzae* (Du et al. 2013).

Mycotoxins produced by *Aspergillus* spp. in infected seeds crucially affect trade because of the poor quality and contamination of seeds that adversely affect human and animal health. *Aspergillus flavus* and *A. parasiticus* infect a wide variety of crops, including peanut (groundnut). A histological investigation was conducted to study the interaction between peanut and *A. parasiticus*. Peanuts attached to plants were inoculated with the pathogen without wounding. A genetically tagged strain of *A. parasiticus* G5 was engineered to harbor the β -glucuronidase (GUS) reporter gene under control of the *nor-1* promoter from the aflatoxin biosynthetic pathway. The transformed strain was used to follow infection and aflatoxin production during colonization of undamaged, drought-stressed peanuts. The fungus colonized all tissues of peanut pod and appeared to gain ingress through the corky layer of the pericarp. Both intra- and inter-cellular colonization were visualized. Fungal colonization of the cotyledons resulted in visible depletion of storage bodies within cells. The pod tissues showed the presence of two morphologically distinct types of hyphae: wider and narrower hyphae. The narrower hyphae were significantly more likely to produce GUS activity than wider hyphae. GUS activity was detected in hyphae infecting the pericarp, embryo, and cotyledons, indicating expression of aflatoxin biosynthetic genes in these tissues. However, GUS activity was not seen in the hyphae colonizing the testa (Xu et al. 2000).

Aspergillus flavus, incitant of maize (corn) ear and kernel rot disease, produces aflatoxins, structurally related metabolites that are products of the polyketide pathway. Maize genotypes with sugary kernels supported more pathogen growth and higher aflatoxin production than the genotypes with starchy kernels (Widstrom et al. 1984). The importance of α -amylase in aflatoxin production was investigated by disrupting the α -amylase gene in an aflatoxigenic strain of *A. flavus* by gene disruption. The mutant strain 101 contained disrupted *Amy1* and strain 33 had the wild-type *Amy1*. The mutant had slower mycelial growth rate (45% of wild-type strain) and produced aflatoxin in mediums containing glucose but not in mediums containing starch. However, both strains produced similar aflatoxin in the embryo-wounded kernels over the range 300–400 μg of aflatoxin g^{-1} kernels. When the endosperm of kernels was wounded aflatoxin was occasionally detected in those inoculated with strain 101, whereas strain 33 produced aflatoxin in nearly all kernels. Aflatoxin was detected in both embryo and endosperm tissues of kernels inoculated with strain 33. After removal of the embryo, maize kernels were inoculated with strains 33 and 101. Both strains grew and sporulated on kernels, but only strain 33 was able to produce aflatoxin. The results suggested that α -amylase might facilitate aflatoxin production and growth of *A. flavus* from a wound in the endosperm to the embryo (Fakhoury and Woloshuk 1999).

Maize (corn) kernels are infected by *Aspergillus niger* and *A. flavus*. Ears were inoculated using the silk-channel, side-needle and spray inoculation methods at 7 days after mid silk (50% of the plants with silk emergence). Ears were also inoculated with a modified-pinbar procedure at 21 days after mid silk. Resistant and susceptible hybrids showed 2–11% and 2–45% infection, respectively. The silk-channel, side-needle and modified-pinbar techniques resulted in significantly higher levels of kernel infection in the susceptible hybrid in 2008 and 2009 than the spray inoculation. The level of *A. niger*

pathogenicity and virulence increased when conidia were placed inside the husks of developing ears by wounding (modified-pinbar and side-needle methods) or non-wounding (silk-channel procedure) inoculation. *A. niger* infection levels were greater than that of *A. flavus* in both susceptible and resistant corn hybrids (Windham and Williams 2012).

Mycotoxins are produced by fungal pathogens in infected seeds at pre- and postharvest stages. Some may have a role in pathogenesis, but they are more important as health hazards because of their carcinogenic properties resulting in mycotoxicoses in human beings and animals following consumption of contaminated grains and feedstuffs. *Fusarium graminearum* (teleomorph-*Gibberella zeae*), the primary pathogen causing Fusarium head blight (FHB) disease in wheat, barley, and other small grain crops, produces several mycotoxins, the trichothecenes, deoxynivalenol (DON) and its acetylated derivatives (3-ADON and 15-ADON), nivalenol (NIV), and estrogenic polyketide zearalenone (Desjardins 2006). Production of DON was detected during plant infection, although its production could be induced in vitro under specific conditions. Experiments were performed to determine the presence of DON and 15-ADON in the macroconidia of *F. graminearum* and also in spikelets inoculated with the macroconidia of the pathogen. Both toxins were detected in spikelets at 48 h postinoculation (PI). Differences in the quantities of toxin accumulation at different periods after inoculation were significant ($P < 0.0001$) when averaged over two barley cultivars and three isolates of pathogen. The range of variability in toxin accumulation in spikelets of germplasm genotypes was also revealed in this investigation (Evans et al. 2000).

The qualitative and quantitative effects of environmental factors on initial infection, pathogen development, and production of mycotoxins by *Fusarium* spp. (causing Fusarium head blight disease) in inoculated wheat earheads were assessed. A single isolate each of *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae* was inoculated on wheat ears separately. In addition, combinations of two or three isolates were also used to inoculate the ears simultaneously. The inoculated plants were exposed to various combinations of duration of wetness (6–48 h) and temperatures (10–30°C). FHB infection and concentration of mycotoxins generally increased with increasing periods of wetness and temperature. Mycotoxin accumulation increased with increasing temperatures ($\geq 20^\circ\text{C}$) during initial stages of infection. Mycotoxin production dramatically increased in the coinoculation by as much as a factor of 1000, whereas the fungal biomass of weaker isolates was greatly reduced due to competitive interactions between isolates of different *Fusarium* spp. The results of both diagnostic and competitive PCR assays provided evidence for competitive interactions between isolates of *Fusarium* spp. Almost all grain samples contained quantifiable amounts of fungal DNA. In combinations with other isolates *F. graminearum* DNA was detected and quantified in almost all samples, whereas only a few samples had sufficient population of other isolates for quantification. *F. graminearum* isolate was the most competitive, whereas isolates of other *Fusarium* spp. had similar levels of competitiveness (Xu et al. 2007).

Fusarium graminearum produces different trichothecene mycotoxins, deoxynivalenol (DON) and its acetylated derivatives (3-ADON and 15-ADON), nivalenol (NIV), and the estrogenic polyketide zearalenone. The biosynthetic pathway of DON production is well characterized and DON and NIV function as virulence factors. DON was shown to be essential for the spread of *F. graminearum* from florets to rachis in wheat by preventing host cell-wall reinforcement. However, DON had a limited role in the spread of *F. graminearum* in barley, indicating the variable importance of DON in invasion of

floral tissues by this pathogen. The expression of the core DON biosynthetic gene *Tri5* during wheat head infection of susceptible and resistant cultivars was studied. Transcription of *Tri5* gene was most active at the infection front before symptom expression, consistent with the role of DON in establishing infection and colonization. As infection progressed, earliest-infected kernels exhibited diminished relative *Tri5* expression which did not cease during the entire period (21 days) of observation. *Tri5* and fungal GAPDH (glyceraldehydes 3-phosphate dehydrogenase) and translation elongation factor 1 α (EF1A) transcripts remained detectable, although reduced, in infected tissues of susceptible cultivar after the plant tissue had completely senesced. The remarkable increase in expression of several *Tri* genes (including *Tri5*) at 72 h after inoculation of barley corresponded to the initial appearance of lesions, when the pathogen development was rapid in the plant. The peak expression of *Tri5* in wheat appeared consistently at the infection front. Infection of spikes spread out from inoculation point (IP) both upward and downward. By the time infection reached the bottom of the head (at 12 dpi), the upper florets were severely shriveled due to plugging of the vascular system. The results suggested that the highest DON content might be associated with early infection. The genes for DON synthesis could be relatively highly expressed in a very small quantity of mycelium before symptom expression. Transcripts for DON biosynthetic genes persisted even after senescence of host tissue, suggesting the possibility of the pathogen to resume toxin biosynthesis rapidly in dried infected grain under favorable environmental conditions. This might be the possible mechanism for high DON levels in asymptomatic seeds of wheat infected by *F. graminearum* (Hallen-Adams et al. 2011).

Fusarium moniliforme (= *F. verticillioides*; teleomorph *Gibberella fujikuroi*) causes root rot and ear rot of maize and has a worldwide distribution. *F. moniliforme*-producing fumonisins could be recovered from maize kernels of earheads in plants growing from maize kernels inoculated with the pathogen at the time of planting. Strains of *F. moniliforme* (malting population A) with a different capacity to produce fumonisins were studied. One strain producing high levels of fumonisin and two strains producing very low levels of fumonisins were applied to maize kernels at planting. The distribution of fumonisins in symptomatic and symptomless kernels was determined by high-performance liquid-chromatography (HPLC) technique. Both symptomatic and symptomless kernels were extensively colonized by *F. moniliforme* and kernels with symptoms had highest concentrations of the mycotoxin. All three strains applied to the kernels at planting could be recovered from kernels. Low-fumonisin producers under laboratory conditions might be high-fumonisin producers under field conditions, since both strains caused symptomatic and asymptomatic kernel infections (Desjardins et al. 1998).

The comparative efficacy of four inoculation methods – namely (i) injection of inoculum through ear husk leaves at R2 (blister) growth stage; (ii) silks sprayed with inoculum at R2 and covered with a shoot bag; (iii) reinoculated one week later and covered with shoot bag until harvest; and (iv) insertion of six *F. moniliforme*-colonized toothpicks into the silk channel at R2 – was assessed to select the most efficient method of inoculation for maize (corn) breeding program for resistance to infection by *Fusarium moniliforme* and fumonisin production. Injection of inoculum through ear husk leaves was the most effective procedure, resulting in the highest concentration of fumonisin and severity of *Fusarium* ear rot disease compared with uninoculated control. The concentrations of fumonisin in grain and disease severity were not affected by the interaction of hybrids with years. The enzyme-linked immunosorbent assay (ELISA) technique using a rabbit polyclonal antibody (PAb) was applied to quantify fumonisin in the maize grains.

The detection limit of ELISA was $\geq 1 \mu\text{g}$ of intact fumonisins B1, B2, and B3 per gram of ground maize (equivalent to 20 ng g^{-1} in the diluted test solution). The ELISA technique was as efficient as the HPLC procedure for quantifying fumonisins (Clements et al. 2003).

Fusarium proliferatum infestation of cowpea seeds is common in tropical and subtropical countries. Fumonisin B1 (FB1) produced by *F. proliferatum* was detected in cowpea seeds. All concentrations of FB1 significantly reduced seed germination at $50\text{--}100 \mu\text{g mL}^{-1}$, and the toxin also inhibited root and shoot elongation. FB1-treated embryonic tissues showed compaction of the protoplasm and separation of the plasma-lemma from the cell wall. Lipid bodies accumulated, resulting in the apparent lining of the cell wall (Kritzinger et al. 2006).

Fusarium verticillioides (= *F. moniliforme*) produces fumonisins that contaminate maize grains, making them unfit for consumption by human beings and animals as the concentration of fumonisin exceeded the permissible limit of 5 mg kg^{-1} of grains in some areas of the USA (Marasas 1996). Investigations were therefore conducted to determine both the initial onset of infection and appearance of fumonisin, and the progression of infection and accumulation of fumonisin in the maize kernels grown in the field. Maize ears were harvested at two locations in North Carolina, beginning 2 weeks after pollination and continuing for 14 weeks. Kernel infection by *F. verticillioides* and fumonisin contamination appeared as kernels tended to reach physiological maturity and increased up to the average harvest date for maize. Fumonisin B1 was detected in kernels at 4 weeks after pollination and steadily increased until the sixth week. A second peak in the concentration of fumonisin was observed in kernels harvested nine weeks after pollination. All three hybrids showed a similar pattern of infection in two locations. The susceptible hybrid showed a greater infection rate than either the resistant or intermediate hybrids. Further, these hybrids showed a similar pattern for fumonisin accumulation during the first 9 weeks and differences in fumonisin concentration in these hybrids were recorded later. The results suggested that the suitable time to screen hybrids for kernel infection and fumonisin production appeared to be between 7 and 10 weeks (Bush et al. 2004).

Fusarium graminearum has attracted the attention of plant pathologists as the primary incitant of Fusarium head blight (FHB) disease of wheat and other small grain crops. Further, it is known to have extended its host range to noncereal crops, including soybean on which it induces pod blight, seed, and root rot diseases. *F. graminearum* causes seed infection which may either show symptoms or remain asymptomatic. In order to determine the optimum conditions for disease development, individual soybean seed was inoculated with $100 \mu\text{L}$ suspension with differing concentrations of macroconidia of *F. graminearum* in a rolled-towel assay at temperatures of 18, 22, and 25°C . Optimal concentration of macroconidia was 2.5×10^4 macroconidia mL^{-1} or more to obtain successful infection of soybean seed at all temperatures tested. The rolled-towel inoculation procedure developed in this investigation was found to be effective to provide required infection levels suitable for assessing the efficacy of various fungicides for the management of soybean seed rot disease (Ellis et al. 2011).

4.2 Invasion Paths of Propagule-Borne Fungal Pathogens

Asexually propagated seed materials are invaded by many fungal pathogens, as in the case of true seeds. Infected plant propagules like tubers form the primary sources of infection, when they are planted in the same location or carried to other faraway locations.

Potato seed tubers are infected by *Phytophthora infestans*, causing late blight disease. The potato–*P. infestans* pathosystem was investigated to understand the role of genes induced during interaction between susceptible cultivar and the pathogen by applying suppression subtractive hybridization (SSH) procedure. Potato leaves were treated with benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methylester (BTH), a known synthetic inducer of resistance in plant against several plant pathogens and used as control. A low-redundancy library with a relatively low frequency of pathogenesis-related (PR) genes was generated. Of the 288 clones screened for induced sequences, 75 clones were detected using inverse Northern analysis (hybridizing the arrayed clones with radiolabeled cDNA populations) and 43 clones appeared to be induced. Eleven clones were selected for total RNA blot analysis, and the elevation of transcript levels during *P. infestans* infection was confirmed for 10 of them. Some of the cDNAs analyzed by RNA blot analysis had homology to genes induced during infection, such as β -1,3-glucanase. In addition, cDNAs having homology to enzymes involved in detoxification, such as gamma glutamylcysteine synthetase and cytochrome P450, were also identified. Further, infection-induced putative proteins encoded by cDNAs, such as the ER-located chaperone Bip, also made their appearance at a detectable concentration. The protocol used in this investigation provides the possibility of analyzing the metabolic changes occurring during infection of potato by *P. infestans* (Beyer et al. 2001).

The infection process of *Phytophthora infestans* was studied in several potato cultivars and somatic hybrid genotypes and their parents, using fluorescence microscopy and electron microscopy techniques. The cultivar–pathogen interactions did not affect the germination of zoospores of *P. infestans* and the frequency of invasion by infection hyphae. However, the extension of hyphae after penetration of host cells differed markedly among the genotypes examined. In susceptible genotypes, hyphae of *P. infestans* grew rapidly both inter- and intracellularly at 15 h after inoculation (hai). Digital (finger-like) haustoria were formed and disorganization of the cytoplasm of invaded host cells soon followed. On the other hand, pathogen development was restricted to the site of initial penetration in the resistant genotypes. Host cells in resistant genotypes produced resistance responses, although some hyphae were able to penetrate epidermal cells. Cell-wall appositions were produced at points of contact with pathogen hyphae and no haustoria-like structures could be seen in the cells of resistant genotypes. In the somatic hybrid genotypes, the host response was dependent on their parents. In the hybrid genotype 1508/2, as in the wild species *Solanum bulbocastanum*, no hyphae could be seen in the host cells. In other genotypes, hyphae of *P. infestans* spread intracellularly and formed haustoria, but the cytoplasm of hyphae and haustoria was disorganized. Other resistance responses such as host cell disorganization and production of cell-wall apposition were observed (Han et al. 2013).

Patterns of potato tuber tissue colonization by *Phytophthora infestans* and other tuber decay pathogens were investigated. Potato tubers were artificially inoculated with *P. infestans* and other fungal pathogens such as *Pythium ultimum*, *Fusarium sambucinum*, *Verticillium dahlia*, or *V. albo-atrum*. Polymerase chain reaction (PCR) assays were applied to obtain an early and accurate diagnosis of pathogen infection and to follow precisely the progress of development of each pathogen in tuber tissues. Samples were collected over time from both the surface (peel, 0–2 mm depth) and internal tissues (flesh, depth >2 mm) of tubers at various distances from the point of inoculation at different time intervals (3–19 days) after inoculation. By using specific PCR primers, the fungal pathogens were detected several centimeters ahead of visible lesions on tubers.

It was possible to track and determine the extent of colonization both on the surface of the tubers and in internal tissues by different pathogens, either after single or multiple infections involving the primary pathogen. The development of *P. ultimum* was reduced in the presence of *P. infestans*, whereas tuber colonization by *F. sambucinum*, *V. dahlia*, or *V. albo-atrum* was not perceptibly affected by infection. The PCR procedure was found to be helpful in monitoring the sanitary status of stored tubers (El-Hadrami et al. 2007).

Transmission of *Phytophthora infestans* from potato seed tubers to aboveground shoots was assessed by inoculating the tubers with isolates BF05 (US8 genotype), WA10.1 (US24), and 110B (US11). After inoculation, plant stems were incubated in a humidity chamber at 10–21.5°C for 7–28 days. The number of aboveground stems with sporulation was significantly less when stems were inoculated with isolate 110B than with isolates BF05 and WA10.1, but length of latent period did not differ among the three isolates tested. The incidence of aboveground stems with sporangia was significantly greater ($P < 0.05$) for Russet Norkotah than for Umatilla Russet when inoculated near the point of seed piece attachment. The incidence of aboveground stems with sporulation did not differ among incubation temperatures 10, 15, and 21.5°C. Latent period significantly increased with decrease in temperature. The age of stem did not appear to affect transmission of *P. infestans* (Johnson and Cummings 2013).

Potato seed tubers are infected by *Colletotrichum coccodes* causing black dot disease and *Verticillium dahliae* causing wilt disease. The effect of incidence of seed lot infection by *C. coccodes* and *V. dahliae* on disease severity and yield was investigated using infested lots and fields with and without potato cultivation histories. A higher level of seed tuber infection by *C. coccodes* led to more root colonization in one year (2008) but not in the previous year (2007). However, the yield of tubers was not affected. The incidence of infection by *V. dahliae* was not related to canopy necrosis, infection of progeny tubers, or yield. However, seed lots showed more *V. dahliae* aboveground vascular colonization, canopy necrosis, infested plant debris, and infected progeny tubers in 5-year potato rotation fields compared with long potato rotation fields. Foliar disease symptoms and yield loss did not have any relationship with levels of either *C. coccodes* or *V. dahliae* in seed lots. The importance of soilborne inoculum was indicated in fields with recent cropping histories where yields were significantly reduced (Dung et al. 2012).

Potato powdery scab disease is caused by *Spongospora subterranea* f.sp. *subterranea* (Sss), which is soilborne. The relationship between initial soil inoculum level of the pathogen and incidence and severity of the disease at harvest was investigated. The soil inoculum level of Sss (number of spore balls g⁻¹ soil) was determined using a quantitative real-time PCR assay. Soil inoculum ranging over 0–148 Sss spore balls g⁻¹ soil in soil samples was detected in 75% of the 113 commercial potato fields across the UK. The number of progeny crops developing powdery scab increased with increase in the level of soil inoculum estimated in the field soil prior to planting progeny crop. The link between the amount of inoculum added to the soil in four field trials and disease incidence was investigated. Disease incidence and severity on progeny tubers was significantly greater in plots with increased levels of inoculum incorporated. Disease incidence and severity scores were greater in some cultivars than in others, indicating the cultivar effect, possibly due to variation in the resistance of potato cultivars to powdery scab disease (Brierley et al. 2013).

Molecular biology of development of Fusarium wilt disease of banana caused by *Fusarium oxysporum* f.sp. *cubense* race 4 (Foc 4) was studied. Foatf 1, a basic leucine

zipper (bZIP) transcription factor in Foc 4, was characterized; a deletion mutant could cause an oxidative burst of banana seedlings in early stages of infection, and this mutant exhibits higher sensitivity to hydrogen peroxide (H_2O_2) than the wild-type strain Foc 4. Foatf 1 showed significantly reduced virulence on Cavendish banana (*Musa* spp.). The results indicated that Foatf1 might be required to induce the full virulence of Foc 4 by regulating the transcription of catalase to impair plant defenses mediated by reactive oxygen species (ROS) (Qi et al. 2013). Differences in the virulence of *Fusarium sulpherum* and *F. sambucinum* and changes in the reactive oxygen species (ROS) production and metabolism in inoculated slices of potato tubers were compared. *Fusarium* infection induced significant production of ROS, lipid peroxidation, and loss of cell membrane integrity, but low activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX). *F. sulpherum* induced larger lesion diameters on potato tubers and slices, resulting in more superoxide anions (O_2^-) and an earlier peak of hydrogen peroxide (H_2O_2), but lower catalase activity (CAT) and APX. This was accompanied by higher malondialdehyde (MDA) content and lower cell membrane activity compared with the effects induced by *F. sambucinum*. The results suggested that overproduction of ROS was primarily involved in the pathogenicity of *Fusarium* spp. in potato tubers (Bao et al. 2014).

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5

Detection and Identification of Bacterial and Phytoplasmal Pathogens

Bacteria and phytoplasmas pathogenic to plants are classified as prokaryotes. They are much simpler in structure and smaller in size. Comparatively they have less complex structural features than the fungal pathogens. As they have insufficient variations in their morphological characteristics for classification, other biological, biochemical, physiological, immunological, and genomic properties are considered for reliable identification and meaningful classification of bacterial pathogens. On the other hand, phytoplasmal pathogens pose formidable obstacles for their identification, primarily because of their nonculturability in cell-free media. It has not been possible to determine their nutritional requirements due to their obligate parasitic relationship with plants. Further, very little is known about the process of infection of plants by phytoplasmas, resulting in a large vacuum in the knowledge of their life cycle in plant and insect hosts that are the natural vectors involved in their dispersal over short/long distances. Since it has not been possible to culture the phytoplasmas in a cell-free medium, the conventional Koch's postulates have not been satisfied. However, spiroplasmas have been grown in artificial culture medium and their pathogenicity to the natural plant hosts has been demonstrated in some cases (Narayanasamy 2011).

5.1 Detection and Identification of Bacterial Pathogens

Bacterial plant pathogens are carried by seeds and propagules which form the primary sources of inoculum, introducing the bacterial pathogens to new locations where they may be absent, and also carrying the inoculum to subsequent generations of crops. As the majority of bacterial diseases are transmitted through contaminated seed or propagative materials, early detection and precise identification of the bacterial pathogen(s) is of paramount importance, especially to international trade.

5.1.1 Detection of Bacterial Pathogens in Seed

Bacterial pathogens present on or inside the seeds can be detected and identified by applying various techniques that are based on the biological, biochemical, physiological, immunological, and genetic characteristics of the bacterial species. The taxonomic characteristics and guidelines for naming and describing bacteria are presented in *Bergey's Manual of Systematic Bacteriology* (Kreig and Holt 1984). The names proposed for newly discovered bacterial species should be in accordance with the International

Code of Nomenclature of Bacteria. As the bacteria have insufficient variations in morphological characteristics required for differentiating different species, several physiological and biochemical properties of bacteria are also considered as the basis for classifying them into class, family, and genus. Naming a new bacterial species requires the results of polyphasic tests which include nucleic acid analyses, such as DNA-DNA and DNA-rDNA hybridization, chemotaxonomic comparison, such as cell-wall composition, lipid composition, isoprenoid quinines, soluble and total proteins, fatty acid profiles, and enzyme characterizations, in addition to biochemical and nutritional tests, so that determinative keys may be formulated (Young et al. 1992; Young 2000). The International Society of Plant Pathology established the criteria for use of the term “pathovar” for those plant pathogenic bacteria that did not satisfy the criteria for species designation. The nomenclature of “pathovar” is applied at the infra-subspecific level of bacteria, distinguished chiefly based on the differences in pathogenicity on a set of plant species/cultivars used as differentials. Plant pathogenicity occurs because some populations of bacteria have particular gene complexes that give them specific capabilities to infect some of the differentials severely, but others to different levels of intensity (severity). The bacteria do not have distinctive morphological characteristics. The taxonomic systems of nomenclature are therefore less stable than that of fungi. Bacterial isolates/strains that share certain phenotypic and genotypic similarities are included in one species. The predominantly occurring strain is designated “type strain,” whereas other strains of the same species may vary from the type strain to different magnitude. Variations in morphological, cultural, physiological, biochemical, or type of effects induced in plants may be exhibited by strains of the same bacterial species.

Kingdom: Procaryotae: bacteria with distinct cell membrane and cell wall

Division: Gracilicutes: enclosing Gram-negative bacteria

Class: Proteobacteria: enclosing mostly single-celled bacterial species

Family: Enterobacteriaceae

Genus and species: *Erwinia* (= *Pectobacterium*) *carotovora*, causing soft rot of potato

Family: Pseudomonadaceae

Genus and species: *Acidovorax avenae*, infecting barley; *A. avenae* subsp. *citrulli*, causing watermelon bacterial fruit blotch; *Ralstonia solanacearum*, causing potato brown rot; *Xanthomonas oryzae* pv. *oryzae*, causing rice bacterial leaf blight

Family: Not named yet

Genus and species: *Xylella fastidiosa*, infecting grapevine; *Candidatus Liberibacter*, causing citrus huanglongbing (greening)

Division: Firmicutes enclosing Gram-positive bacterial species

Class: Thallobacteria, producing filamentous, branched threads with chain of bacterial cells

Genus and species: *Clavibacter michiganensis* subsp. *michiganensis*, causing tomato bacterial canker; *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, infecting cucurbits; *Streptomyces scabies*, causing potato common scab

Division: Tenericutes

Class: Mollicutes: lacking cell wall, with triple-layered cell membrane

Family: Spiroplasmataceae

Genus and species: *Spiroplasma citri*, causing corn stunt and citrus stubborn diseases

Family: Not defined

Genus: *Phytoplasma*, causing yellows type of diseases of several plant species

The relationships of the phylogenetically misnamed phytopathogenic taxa, *Pseudomonas avenae* and others belonging to the family Comamonadaceae, were compared based on DNA-rRNA hybridizations, DNA-DNA hybridizations, PAGE of whole cell proteins, and a numerical analysis of carbon assimilation tests. Most of the taxa clustered closely around the type strain *P. avenae*. When they were compared phenotypically, all forming a rRNA branch could be differentiated from each other and were found to be most closely related to the genus *Acidovorax*. DNA-DNA hybridization results indicated that these bacteria constituted two genotypic groups. The generically misnamed phytopathogenic *Pseudomonas* species were therefore transferred to the genus *Acidovorax* as *A. avenae* within which three species – *A. avenae* subsp. *avenae*, *A. avenae* ssp. *citrulli*, and *A. avenae* ssp. *cattleyae* – were distinguished (Willem et al. 1992). Bacteria belonging to *Pseudomonas* rRNA group II were separated from members of *Pseudomonas sensu stricto* (*P. fluorescens* rRNA group) on the basis of plant association and chemotaxonomic characteristics, DNA-DNA hybridization data, rRNA-DNA hybridization data, and sequences of 5S and 16S rRNA. *P. solanacearum*, *P. plantarii*, and *P. glumae* were transferred to the genus *Burkholderia* based on the abovementioned characteristics (Urakami et al. 1994). The phytopathogenic coryneform bacteria with 2,4-diaminobutyric acid in the peptidoglycans of their cell walls were classified in the genus *Corynebacterium*. Later studies indicated that these phytopathogens should be reclassified as *Clavibacter michiganensis* ssp. *michiganensis*, *C. michiganensis* ssp. *sepedonicus*, and *C. michiganensis* ssp. *nebraskensis*, however (Davis et al. 1984). The name *Streptomyces scabies*, causative agent of potato common scab disease, was revived. *S. scabies* could be differentiated by a phenotypic analysis of 42 criteria from six atypical pathogen strains, three saprophytic streptomycetes isolated from scab lesions and reference strains of *Streptomyces griseus* and *Streptomyces tendae*. *S. scabies* strains (12) formed a homogeneous group characterized by smooth gray spores borne in spiral chains, melanin production and usage of all International Streptomyces Project sugars (Lambert and Loria 1989).

5.1.2 Conventional/Isolation-Dependent Methods

These methods are generally based on the isolation of bacteria from seed or extracts by culturing on semiselective or selective media, followed by colony identification by morphological and biochemical characteristics and pathogenicity tests. These tests need a long time (one to several weeks) before the identity of the pathogen(s) can be confirmed.

5.1.2.1 Isolation of Bacterial Pathogens

Detection of bacterial pathogens involves a three-step procedure: (i) extraction of bacteria from seeds; (ii) isolation using appropriate medium; and (iii) identification using one or more methods. The target bacterial pathogen has to be recovered from the seed sample(s) and this depends on the optimum release of the bacterial pathogen. Release of the bacteria from seeds in turn depends on the presence of saprophytic microorganisms and inhibitory compounds in the seeds. Washing the seeds or surface sterilization may result in reduction in saprophytes associated with seeds and in turn enhance the efficiency of extracting the target pathogens. However, this procedure will not be useful for the isolation of deep-seated bacterial pathogens for which grinding or milling the seeds into flour before extraction may be required. Bacteria may be extracted from seed

flour or seeds using a liquid medium, sterile water, or buffers. *Pseudomonas syringae* pv. *phaseolicola* was isolated from a water suspension of bean seed flour plated onto King's medium B (KB) on which the pathogen produced fluorescence pigments (King et al. 1954). The media, generally used for isolation of bacterial pathogens, may be sufficient if the target pathogen is in large numbers and fast-growing and the bacterial saprophytes are at a low concentration. Semiselective medium may be required for isolating the target pathogen if it is in low population levels. The medium KBC is a modification of KB medium which is amended with boric acid, cycloheximide, and cephalixin. Seed-associated saprophytes were inhibited to a great extent (90%). KBC medium was shown to be highly effective for the isolation of *Pseudomonas syringae* pv. *syringae* (Pss) from contaminated bean seeds (Mohan and Schaad 1987). Sterile buffered saline is frequently used for bacterial extraction (Roth 1989).

Xanthomonas sp., causal agent of onion bacterial blight disease, occurs in several tropical and subtropical areas. A semiselective medium was developed for the isolation of the pathogen from seeds on which other bacterial microflora were also present. The medium NCTM1 medium containing 7 g yeast extract, 7 g peptone, 7 g glucose, 15 g agar, 10 mg neomycin, 30 mg cephalixin, 3 mg trimethoprim, 100 mg pivmecillinam, 20 mg propiconazole, and 1000 mL distilled water was used for the isolation of the pathogen. All onion isolates of *Xanthomonas* sp. from several countries grew on NCTM1 medium well. The pathogen could be isolated consistently, using the medium from seed samples containing approximately 10^6 saprophytic bacteria g^{-1} of seed and also from asymptomatic plant materials. *Xanthomonas* sp. was detected only in seeds originating from one seed product site. The medium NCTM1 could be a valuable tool for isolating *Xanthomonas* spp. from seeds and asymptomatic plant tissues (Roumagnac et al. 2000). Crude extracts of *Brassica* sp. seed were used for the detection of *Xanthomonas campestris* pv. *campestris*, causing black rot disease of crucifers. The seed extracts were plated on nutrient medium for the development of the pathogen colonies (Chitarra et al. 2002). Four semiselective media were compared for their effectiveness for isolation of the black rot pathogen *Xanthomonas campestris* pv. *campestris* (Xcc). Eight seed lots were distributed among thirteen laboratories in the USA, France, and the Netherlands. Xcc was extracted from seed lots by shaking for 5 min or 2.5 h, followed by dilution plating onto mCS20ABN, mFS, NSCA, and NSCAA semiselective media. Suspected Xcc colonies were subcultured to YDC medium and tested for pathogenicity on cabbage plant or excised cotyledon. Based on the proportion of positive subsamples, mCS20ABN and mFS media were equally efficient and were more effective than NSCAA and NSCA media. The population of Xcc extracted was greater after 2.5 h of extraction than after 5 min of extraction. The semiselective media mCS20ABN and mFS were recommended for the detection of Xcc based on the results of experiments organized by the International Seed Health Initiative (ISHI). The identity of the suspected colonies was confirmed reliably by employing the cabbage plant pathogenicity assay (Koenraad et al. 2005).

Xanthomonas oryzae pv. *oryzicola* causes rice bacterial leaf streak (BLS) transmitted through seeds. Seed washings containing the pathogen were inoculated on rice leaf segments which were placed on 1% agar amended with the fungicide benzimidazole (75–100 ppm) to maintain freshness of inoculated leaf segments. The agar plates were incubated in a moist chamber. Typical bacterial leaf streak lesions developed on inoculated leaf segments followed by the appearance of bacterial ooze on the leaf surface, indicating the seed infection by *X. oryzae* pv. *oryzicola* (Xie and Mew 1998). An efficient

seed extraction buffer (SEB) is critical for the isolation of *Acidovorax avenae* subsp. *citrulli* (= *A. citrulli*) (*Aac*) from seeds and other plant tissues. The addition of an antibiotic could reduce the numbers of saprophytes. Most of the saprophytic bacteria present in seed washes were eliminated by combining SEB containing vancomycin (SEB-V) with plating on isolation media (Zhao et al. 2009).

The pathogen in the extraction solution is transferred to the general, semiselective, or selective media that favor the development of the target bacterial pathogen. A semiselective medium (XTS agar) was found to be useful for the isolation of *Xanthomonas campestris* pv. *translucens* (*Xct*), causal agent of wheat black chaff disease from the seeds of wheat (*Triticum aestivum*) and barley naturally infected under field conditions. The XTS agar medium containing Difco nutrient agar, glucose, cycloheximide, gentamycin, and cephalixin inhibited more than 91% of the seed-associated saprophytic bacteria and facilitated rapid development of *Xct* present in the wheat seeds, since the competition by the saprophytic for nutrients was kept at minimal levels. This procedure was found to be simple and the levels of seed contamination by *Xct* could be assessed effectively. Contamination levels of approximately 1000 CFU of *Xct* mL⁻¹ of seed washings might be required for the incidence of black chaff disease (Schaad and Forster 1985; Section 5.4.1). In a later investigation, barley seed samples (100 g) were placed in Erlenmeyer flasks containing cold sterile buffered saline (0.1 M PO₄ buffer, pH 7.0 in 0.85% saline and three drops of Tween 20 per liter) and shaken in a rotary shaker for 5–10 min. Solutions from each flask were serially diluted from 10 to 10⁻³ on XTS medium. Cultures were incubated for 3–5 days at 28°C and then transferred to yeast extract, dextrose, and calcium carbonate (YDC) medium in plates. Pure cultures of *Xct* were maintained on YDC medium (Olsen et al. 2002).

A new selective medium designated CCNT medium was developed for the isolation of *Burkholderia glumae* from rice seeds. *B. glumae* produced a yellowish-white colony with a diffusible yellow pigment on this selective medium and the pathogen could be differentiated from other bacteria when incubated at 41°C for 2–4 days. *B. glumae* was detected at the same rate as the S-PG medium used earlier (Kawaradani et al. 2000; Section 5.4.1). The population of *Xanthomonas axonopodis* pv. *vignicola* (*Xcv*) and *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), infecting cowpea and tomato respectively, were determined by counting the numbers of viable colony-forming units (CFUs). The seeds were soaked individually in phosphate-buffered saline, followed by dilution-plating onto MXP and CNS semiselective agar medium for *Xcv* and *Cmm* respectively (Dutta et al. 2013). Symptomatic bean seeds were washed thoroughly and then placed in a Stomacher bag, covered with SPS (2 mL/seed) and soaked overnight at 4°C. The seeds were crushed with a hammer, filtered to eliminate seed fragments, and then centrifuged at 6000 g for 20 min at 4°C. The pellet was resuspended in 1 mL of SPS to have a final concentrate (FC). Yeast peptone glucose agar (YPGA), nutrient broth yeast (NBY) extract agar medium, and the semiselective medium were employed for isolating *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* from infected bean seeds (EPPO 2011). The possible transmission of *Erwinia aphidicola* through bean seeds was verified by testing 120 commercial bean seeds and 120 seeds from the fruiting plants. Extracts from seed coats, cotyledons, and leaves of plants originating from those seeds were analyzed. The test plants inoculated with extracts of different tissues developed interveinal chlorosis, necrotic pits, and rough roots and these symptoms were similar to those in plants naturally infected by *E. aphidicola*. The isolates from the plants growing

from the seeds of infected plants were analyzed by biochemical and molecular techniques. These assays confirmed the presence of *E. aphidicola* in seeds and plants that originated from infected seeds (Marin et al. 2011).

Pantoea stewartii subsp. *stewartii*, causing Stewart's wilt disease of sweet corn, was isolated by a kernel plating method. Kernels were surface sterilized for 2 min in 0.5% sodium hypochlorite solution and the tips of kernels were embedded in soft NBY agar (1.2% agar). After 48 h of enrichment at 27°C, the kernel tip was touched onto a plate of NBY-CRN agar medium which was highly selective for the rif-9A isolate of the pathogen. NBY-CRN agar was prepared by amending NBY agar with cycloheximide (50 mg), rifampicin (50 mg), nalidixic acid (25 mg), and water (1000 mL). Characteristic *P. stewartii* subsp. *stewartii* colonies were formed in this selective medium (Block et al. 1998). *Pantoea ananatis*, causing onion center rot disease, was isolated from infected seed using the semiselective medium PA-20. Colonies resembling those of *P. ananatis* were isolated from four seed lots. Pathogenicity tests showed that the South African strains induced symptoms similar to those caused by American strains on onion (Goszczyńska et al. 2006). Tomato bacterial canker pathogen *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) transmitted through seeds could not be isolated effectively by using the semiselective media developed earlier. A new bacterial canker of tomato (BCT) medium was therefore developed and proved to be superior to other available media because of its higher plating efficiency, amounting to $\leq 89\%$ within 7 days for all 30 strains tested. This medium was highly selective, as other bacterial species associated with tomato plants/seed were inhibited to the extent of 98–100%. Further, this medium was highly sensitive, since as small as 8 CFU of *Cmm* in field plant homogenates containing 12,750 CFU of accompanying saprophytes could be detected on the BCT medium. The selective medium developed in this investigation can be used for effective isolation and detection of *C. michiganensis* subsp. *michiganensis* in the tomato seed or plant tissues (Ftayeh et al. 2011).

5.1.2.2 Bacteriophage Typing

Bacteriophages are viruses infecting bacteria, and the specificity of pathogenicity to bacterial pathogens has been exploited for the detection and establishment of the identity of some phyto-bacterial pathogens. In addition, the possibility of utilizing the bacteriophages for the control of certain bacterial diseases of crops such as apple, cotton, potato, and tomato has been indicated (Narayanasamy 2013). The bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was detected and differentiated by employing OP₁ phage. Isolates of *Xoo* were grouped into 15 lysotypes based on their sensitivity to phage (Ou 1985). Two races of *Xanthomonas axonopodis* pv. *malvacearum* showed distinct differences in their sensitivity to phage, providing a reliable basis for their detection and differentiation (Freigoun et al. 1994). The genome of OP₂ phage has 464 bp and the average G + C content is 60.9%. This bacteriophage shows similarity to the phage Bcep1 which lyzes *Burkholderia cepacia*, infecting onion. As an exception, dUTPase gene had 94% identity with the host bacterium *Xoo* and no insertions or deletions were detected in it (Inoue et al. 2006). *Burkholderia glumae* and *B. plantarii* cause rice seedling rot and seedling blight disease, respectively. Two phages could lyse both bacterial pathogens and suppress disease development. Seed treatment with phage was effective in reducing seedborne infection by *B. glumae* and *B. plantarii*. The phages could be employed for detection of the pathogens and also for the management of the diseases caused by them (Adachi et al. 2012).

5.1.3 Isolation-Independent Methods

5.1.3.1 Seedling Grow-Out Assay

Symptoms of infection by bacterial pathogens may or may not be observed on seeds. Isolation of the suspected bacterial species from the seed lot may not indicate the extent of transmission of the pathogen to the seedlings when the infected seeds are sown in the next season. The potential risk of utilizing the contaminated seed can be assessed by performing seedling grow-out assays. A stem-printing technique was used on greenhouse grow-outs and field grow-outs to evaluate seed transmission of *Pantoea stewartii* subsp. *stewartii* from corn kernels to the emerging seedlings (Section 5.4.2). Seed transmission of the pathogen was evaluated by assays of more than 76,000 plants in greenhouse and field grow-out trials. The percentages of infected kernels ranged over 0.8–72%, as determined by agar-plating or by enzyme-linked immunosorbent assay (ELISA) test. Plants grown from these seeds were assayed by stem-printing technique, involving cutting and pressing a cross-section of each stem into agar media. Prints were examined at 24 and 48 h for the development of pathogen colonies. Seed transmission rate from seed produced from inoculated plants was 0.066% (28 of 42,206 plants), while the transmission rate of the pathogen from seed produced on naturally infected plants was 0.0029% (1 of 34,924 plants), based on all seedlings assayed and 0.022% from infected kernels. Seed transmission of *P. stewartii* subsp. *stewartii* was very low and seeds from heavily infected plants were rejected because of poor seed germination. Seed transmission in good-quality seeds might therefore be a remote possibility (Block et al. 1998).

In a later investigation, the seeds of corn collected from infected Jubilee sweet corn plants were planted and the seedlings at two-to-three leaf-stage were examined for symptoms of Stewart's wilt. Leaf tissue was sampled from any seedlings with symptoms that remotely resembled Stewart's wilt disease. Leaf samples were examined, using light microscope, for the presence of bacterial ooze. All samples positive for ooze were assayed by ELISA to confirm the infection by Stewart's wilt pathogen. Samples that did not show ooze were also tested by ELISA. Rates of transmission of *P. stewartii* subsp. *stewartii* were estimated by dividing the number of infected seedlings (bacterial ooze + ELISA-positive) grown from infected seed by the total number of seed that were infected with *P. stewartii* subsp. *stewartii*, as estimated by ELISA tests. Combined data for all three trials showed that 59 of approximately 58,300 seedlings grown from infected seeds were infected with the pathogen, based on symptoms of Stewart's wilt and ELISA-positive samples. These 59 seedlings were probably infected from seed-to-seedling transmission of the pathogen and due to natural transmission of the disease by flea beetles. Seed-to-seedling transmission was estimated to be 0.038%. This level of seed transmission was substantially lower than the seed transmission rates reported by earlier workers (Michener et al. 2002).

Acidovorax avenae subsp. *citrulli* (*Aac*) causes bacterial fruit blotch (BFB) disease in watermelon, melon, and other cucurbitaceous crops. *Aac* is seedborne and the extent of seed transmission of the pathogen was assessed by grow-out test. *Aac* was multiplied on nutrient agar (NA) medium amended with ampicillin ($100\text{ }\mu\text{g mL}^{-1}$) for 48 h at 28°C. The bacterial growth was suspended in sterile water and the concentration was adjusted to about 10^8 CFU mL^{-1} , using a spectrophotometer. Seeds of watermelon cultivar Malali, melon cultivar 6404 and cucumber cultivar Olympus were incubated in a suspension of *Aac* (10^8 CFU mL^{-1}) with gentle shaking (100 rpm) for 1 h at room temperature.

The seeds were then separated and washed gently with sterile water, and air-dried for 4 h at room temperature. The inoculated seeds and control seeds were planted in plastic pots in sand and maintained in the greenhouse at a temperature of 25–28°C. The percentages of symptomatic seedlings and seedling mortality were determined at 18 days after inoculation (dai). The M isolates (from melon) of *Aac* were more aggressive than the W isolates (watermelon) in two experiments conducted. The percentages of both symptomatic plants and seedling mortality were significantly higher for M isolates than that of W isolates. Further, W isolates developed more slowly to a lesser extent than M isolates (Burdman et al. 2005).

A modified method, better than the conventional grow-out method, was developed to detect *Acidovorax avenae* subsp. *citrulli* (*Aac*) in cucurbit seeds. The seeds collected from field-grown plants were placed in test tubes for 2 weeks and the bacteria proliferated semiselectively on seeds and developing seedlings. The plants were shaken vigorously in water to suspend bacterial cells. The resultant suspension was spread on a selective medium and the bacterial colonies were identified by colony characteristics as *A. avenae* subsp. *citrulli*. This method was found to be suitable for evaluating the percentages of seeds infested with *Aac*, but it might not be useful for detection of the pathogen in commercial seed lots (Kubota et al. 2011). The USDA National Seed Health System approved a seedling grow-out test to detect *Aac* in seeds in the greenhouse. For each seed lot 10,000–30,000 seeds are planted in the sanitized greenhouse trays and maintained at 21–35°C, 55–90% relative humidity. In addition, seedlings are exposed to light for 12 h and watered daily to hasten bacterial fruit blotch (BFB) symptom development. Negative and positive controls (uninfected and artificially inoculated) seed samples have to be maintained. Visual examination of the seedlings is continued up to 21 days after planting. Development of water-soaked areas along the veins of cotyledons and leaves is recorded. Isolation and confirmation tests are performed for suspect seedlings (Feng et al. 2013).

5.1.3.2 Biochemical Methods

Physiological tests involving the assessment of different enzymatic activities or fatty acid analysis have been performed to identify the bacterial pathogens tentatively. The API System depending on the visualization of growth of target bacterium was applied for the identification of *Xanthomonas* spp., and this test required 1 week for results. Pathovars of *Pseudomonas syringae* could be identified and the pathotypes of *X. campestris* were also differentiated by the API System (Gardan et al. 1984; Verniere et al. 1991). Another system of identifying Gram-negative bacteria on the basis of metabolic fingerprints was developed by Biolog Inc., Hayward, California. The Biolog automated identification system is based on the differential utilization of 95 carbon sources by different bacterial species/isolates/strains. A redox dye, tetrazolium violet, is used to visualize the increased respiration of bacteria while utilizing a carbon source. Each bacterial species produces a metabolic fingerprint that can be compared to those of known bacterial species whose profiles are entered into a database. The Biolog GP microplates with wells are inoculated with predetermined aliquots (150 µL) of target pathogen (Klinger et al. 1992).

Pseudomonas syringae pv. *coriandricola* is the causal agent of coriander bacterial umbel blight and seed decay disease. On Biolog plates the coriander strains showed a uniform metabolic pattern and could be clearly distinguished from other *P. syringae* pathovars. Typical hosts of *P. syringae* pv. *syringae* were not susceptible to the coriander isolates.

Further, most of the tested members of Umberlliferae were not infected by the coriander isolates. Based on the results of the investigation, the pathogen infecting coriander was proposed as a new pathovar as *P. syringae* pv. *coriandricola* (Toben and Rudolph 1996). Isolates (12) of *Acidovorax avenae* subsp. *citrulli* (*Aac*) infecting watermelon and melon were examined to determine the substrate-utilization profiles by employing Biolog System. Forty-four percent (42 of 95) of the substrates included in the Biolog procedure were utilized by at least one isolate. All W isolates (watermelon), but not M isolates (melon), grow on L-leucine, propionic acid, and α -hydroxybutyric acid as the sole carbon source. All W isolates with the exception of W5 grew on D-glucose, whereas none of the M isolates could develop on this carbon source. The results indicated the existence of two groups of *A. avenae* subsp. *citrulli*, one group being associated with watermelon and other group associated with melon plants (Burdman et al. 2005). In a later investigation, the Biolog microstation (version 4.20.05, Biolog Inc., Hayward, CA) indication system, based on the ability of the target bacterial strain(s) to oxidize 95 different carbon sources, was employed to identify the strains of *A. avenae* subsp. *citrulli* (*Aac*). The Biolog System could identify the strains of *Aac*, but it required 2–3 days to provide the results. In addition, the Biolog database had only one strain of *Aac* which did not represent the complete range of substrate utilization profiles displayed by natural populations of *A. avenae* subsp. *citrulli* (Feng et al. 2013).

The Biolog System was applied to determine carbon source utilization patterns of 140 plant pathogenic corynebacterial strains. This system was effective for identifying all *Curtobacterium* strains up to the genus level. However, variations in the identification of *Clavibacter* strains ranged over 27–77%, depending on subspecies (Harris-Baldwin and Gudmestad 1996). For the identification of *Xanthomoas axonopodis* pv. *vesicatoria* (*Xav*) strains, the utilization patterns of 95 carbon sources were analyzed using the Biolog GN Microplate as per the manufacturer's instructions. Positive and negative growth reactions of bacterial isolates were recorded after 28 h. The results indicated that all strains present in Macedonia belonged to *X. axonopodis* pv. *vesicatoria* group A (Mitrev and Kovačević 2006).

5.1.3.3 Physical Techniques

The direct colony thin-layer chromatography (TLC) method was found to be useful for identifying several bacterial plant pathogens, including *Xanthomonas* spp., *Clavibacter* spp., and *Pseudomonas* spp. The usefulness of the direct colony TLC method for rapid identification of different *Pseudomonas* spp. in ribosomal RNA (rRNA) homology group II was demonstrated. The high-performance liquid-chromatography (HPLC) technique was applied to detect and differentiate *Clavibacter* and *Erwinia*. Distinct differences were recorded at species level in *Erwinia* (Matsuyama 1995). By employing HPLC procedure in conjunction with the direct colony thin-layer chromatography technique, *Burkholderia gladioli* isolates could be identified by distinct peak at Rt 6.2 min and they could be differentiated from isolates of *B. glumae* and *B. plantarii* which did not exhibit such a peak (Matsuyama et al. 1998). Phytopathogenic bacteria may be identified and differentiated by TLC profiles using chloroform-methanol-NaCl or 2-propanol solvent system. Comparison of TLC of aminolipids extracted from 315 phytopathogenic and nonpathogenic bacteria showed that TLC profiles were characteristic of the target isolates at the genus or species level and the results were highly reproducible. The profiles of pathovars of *Pseudomonas syringae* were identical and simple, but distinct diversity was revealed in the profiles of *Xanthomonas campestris* and *X. oryzae*. The TLC profiles

of aminolipids could be prepared easily and they might be employed for presumptive identification and differentiation of bacterial plant pathogens (Matsuyama et al. 2009).

Electrophoresis technique is employed to separate complex mixtures of DNA into different fragments of varying sizes. The DNAs isolated from target organisms are treated with restriction enzymes that cleave at specific sites in the DNA to generate DNA fragments small enough to resolve by electrophoresis in agarose or acrylamide. The DNA migrates in an electrical field to a distance that is inversely proportional to the logarithm of its length. Polyacrylamide gel electrophoresis (PAGE) has been commonly used to resolve the DNA fragments amplified by polymerase chain reaction (PCR). Two major phenotypic groups could be identified by employing the PAGE procedure and silver staining of sodium dodecylsulfate (SDS-) lysed cells of *Xanthomonas campestris* pv. *vesicatoria*. The α -band (32.35 kDa) was detected in 192 of 197 tomato race 1 strains, whereas the β -band (25.57 kDa) was present in all 55 strains of tomato race 2. Silver staining of profiles of proteins and testing for amylolytic activity may be useful for differentiating strains of *X. campestris* pv. *vesicatoria* (Bozar et al. 1994). The limitation of PAGE technique could be overcome by applying the pulsed-field gel electrophoresis (PFGE) which allows the electrophoretic separation of large molecules in agarose gels. *Acidovorax avenae* subsp. *citrulli* (*Aac*), incitant of cucurbit bacterial fruit blotch (BFB), exists in the form of different strains with varying degrees of virulence (aggressiveness) as revealed by seed, seedling, and fruit inoculation with the isolates of *Aac*. The PFGE analyses of isolates of *Aac* indicated that 23 unique DNA bands and five different profiles could be recognized. Each profile contained 9–13 bands. The isolates of *Aac* were classified into two groups. One group included strains that were more associated with watermelon (group II) and the other group enclosed strains that were usually associated with nonwatermelon cucurbits (group I). Further, based on the PFGE analysis, the 12 analyzed isolates could be divided into five different haplotypes of which four were considered to be new. The PFGE technique provided an advantage over extensively employed rep-PCR assay. PFGE offered a greater level of reproducibility of results (Burdman et al. 2005).

The genetic diversity of *A. avenae* subsp. *citrulli* strains (11) occurring in China and other countries was assessed, using PFGE and multilocus sequence typing (MLST) techniques. Analysis by PFGE method resulted in nine genotypes which could be typed into two groups based on host, namely watermelon and melon. Group I included strains mainly from melon and group II contained strains largely from watermelon, as indicated by earlier investigation. The results obtained through PFGE analysis were corroborated by MLST, clustering the strains from melon and watermelon. The difference between these two techniques was that 10 watermelon strains from Beijing clustered with melon strains group based on the MLST procedure, suggesting that these 10 watermelon strains had a close relationship with melon strains placed in group I (Yan et al. 2013).

5.1.4 Immunoassays

Bacterial plant pathogens do not form different kinds of spore forms as fungal pathogens do. A variety of antigenic determinants (epitopes) such as proteins, polysaccharides, lipopolysaccharides (LPS), O and K antigens, and murein lipoproteins has been detected on the cell surface of bacterial species pathogenic to humans, but much less is known about membrane protein envelopes of plant pathogenic bacteria. However, polyclonal antibodies (PABs) have been raised against some bacterial species infecting plants, and

they are mixtures of antibodies with multiple specificities (Schaad et al. 1978). Cross-reactions with taxonomically unrelated species are therefore common. The development of hybridoma technology enhanced the specificity of immunological reactions between the antigen and antibody. Hybridomas are somatic cell hybrids formed by the fusion of β -lymphocytes (antibody-producing cells) with myeloma cells (capable of indefinite multiplication). The hybridomas derive the ability to produce a specific antibody from the β -lymphocyte and be cultured indefinitely in vitro from the myeloma cells. Each hybridoma clone produces identical antibodies that are specific for a single epitope present in the bacterial immunogen. The generation of monoclonal antibodies with characterized clones of hybridomas has singularly improved the specificity and sensitivity of immunoassays. Panels of MAbs, after characterization of MAbs, may be combined to form a reactive group that can be employed to detect and identify up to genus, species, subspecies, and pathovars of a bacterial pathogen (Alvarez et al. 1996). The MAbs raised against two German isolates of *Xanthomonas campestris* pv. *campestris* (Xcc) were employed to subdivide Xcc isolates into three serogroups S1, S2, and S3 (Rabenstein et al. 1999).

Immunoassays have been found to be efficient for detecting the bacterial pathogens in infected seeds and asexually propagated planting materials, especially when symptoms of infection are not clearly visible as in the case of potato tubers latently infected by brown rot pathogen *Ralstonia solanacearum* (Priou et al. 1999). Two types of symptoms – black rot and blight – induced by *X. campestris* pv. *campestris* (Xcc) in cabbage seedlings were strain-dependent. In seed beds, most of the ELISA-positive seedlings were asymptomatic and infection by Xcc of these seedlings could be detected, indicating the usefulness of the immunoassay for detecting the latent infection by *X. campestris* pv. *campestris* (Shigaki et al. 2000). Immunoassays differ in their specificity, sensitivity, rapidity, and reliability, depending on the nature and volume of antigen and antibodies employed.

5.1.4.1 Agglutination-/Diffusion-Based Tests

Among the immunoassays, agglutination or precipitation in microscope glass slides or test tubes is the simplest. This test depends on the formation of visible clumps or precipitates, following interaction between the bacterial antigen(s) and the antibodies generated against them. *Pseudomonas syringae* pv. *Phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* were detected in bean and pea by agglutination tests (Trujillo and Saettler 1979; Van Vuurde and Van den Bovenkamp 1981). The agglutination test needed to be improved because of its low level of sensitivity and requirement of relatively high titer antiserum in large quantity. An improvement was made by sensitizing the cells of *Staphylococcus aureus*, which have a high concentration of protein A on the cell surface. PABs specific to the bacterial species to be detected were conjugated with the cells of *S. aureus*. Rapid slide detection of *Pseudomonas syringae* pv. *phaseolicola* in bean seed and *P. syringae* pv. *pisi* in pea seed (Lyons and Taylor 1990) and *X. campestris* pv. *campestris* was achieved. The bacterial pathogens were effectively detected in washings of *Brassica* seeds by employing the improved slide agglutination test (Mguni et al. 1999).

Ouchterlony double diffusion test performed in petriplates depends on the diffusion of the antibodies and antigen towards each other and the formation of a visible white precipitin line where the reactants meet at optimal concentrations. This test was effective for the detection and differentiation of pathovars of *Pseudomonas syringae*.

P. syringae pv. *pisi* in infected pea seeds, using antibodies generated against glutaraldehyde-fixed pathogen cells (Marzarei and Kerr 1990). In a later study, the antisera raised against glutaraldehyde-fixed flagella of two strains UGM 551 of *P. syringae* pv. *pisi* (*Psp*) and strain L of *P. syringae* pv. *syringae* (*Pss*) had high levels of specificity for the detection of respective strains. In addition, the antisera specific to heat-killed cells of *P. syringae* could be used for distinguishing *P. syringae* from all other bacterial species and genera tested (Marzarei et al. 1992).

5.1.4.2 Immunofluorescence Microscopy

Xanthomonas campestris pv. *campestris* (*Xcc*), the incitant of cabbage black rot disease, is transmitted through seed. Use of disease-free seed is an important strategy for the management of cabbage black rot disease. A specific and sensitive method was therefore developed for the detection of *Xcc* in cabbage seed. Both polyclonal and monoclonal antibodies were evaluated for the efficacy in detecting *Xcc* in cabbage seeds, using immunofluorescence (IF) microscopy. PAb 94, MAbs 20H6, 2F4, 18G12, and a mixture of MAbs 20H6, 18G12, 2F4, and 16B5 were employed for the detection of *Xcc* in seed extracts with 5 min and 2.5 h shaking of seeds for extraction of the pathogen. Some of the virulent *Xcc* strains extracted from the seed lots did not react with MAbs 2F4, 18G12, 17C12, and 16B5. The saprophytic isolates from one seed lot cross-reacted with MAb 17C12 and to a lesser extent with MAbs 2F4, 18G12, and PCA94, indicating the absence of relationship between positive IF reactions and pathogenicity of the isolates of *Xcc* (Franken 1992). Immunofluorescence colony-staining (IFC) method is based on sample pour-plating in combination with immunofluorescence staining for recognition of the target colony. An indirect immunofluorescence colony-staining method was developed for the detection of seedborne bacterial pathogens carried in tomato seeds. A specific antiserum for initial binding by visualization of positive colonies with commercially available secondary antiserum conjugated with fluorescent isothiocyanate (FITC) stain and observation under the fluorescence microscope revealed the presence of the pathogen. The sensitivity of the assay could be enhanced by using monoclonal antibodies specific to the target bacterial species. This assay was found to be easy to perform and the results could be easily compared by visual scoring or image analyzer (Veena and van Vuurde 2002).

Immunofluorescence colony-staining (IFC) was optimized to detect *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) and *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in tomato seed lots. Optimum incubation periods for colony growth were 2 and 3 days for *Xcv* and *Cmm*, respectively. Positive correlations were observed between fluorescence intensity of target colonies and incubation temperatures in the range tested and period of colony growth of both pathogens. Generally IFC preparations of pure cultures showed lower levels of brilliance compared with those of seed extracts. The maximum density of bacterial colonies in the pour plates for both target pathogens was about 1000 times higher per square centimeter as for surface plating. The sensitivity of IFC was ten times greater than that of immunofluorescence cell staining and isolation methods. In samples with a target to saprophyte ratio higher than 1/100, 10–100 times more positive colonies of the target were detected in IFC using 16 mm diameter wells in miniaturized routing system than by dilution plating on trypticase soy agar (TSA) and selective media. The recovery of *Xcv* in pour plates at various densities of the target was still 46–68% for samples with a high microbial background (10^5 or more colonies/cm). In the case of slow-growing *C. michiganensis* subsp. *michiganensis*, recovery was

reduced to 12–17% for samples with high microbial contamination. Of the 23 commercial seed lots tested, IF detected the presence of *Xcv* in seven samples. In contrast, no typical fluorescent positive colonies of *Xcv* could be observed when IFC procedure was applied. The weak cross-reacting colonies grown from the viable cells were found to be other species of bacteria (Németh and van Vuurde 2006).

Xanthomonas campestris pv. *campestris* (*Xcc*), causing cabbage black rot disease, is seedborne. Infection of cabbage seedlings by *Xcc* could not be visually recognized since the seedlings had latent infection by the pathogen. Application of ELISA test was successful in detecting latent infection of cabbage seedlings before production of any visible symptoms induced by different strains of *Xcc* (Shigaki et al. 2000). *Xcc* was chromosomally tagged with *gfp* gene encoding green fluorescent protein (GFP). The presence of *gfp* gene in the chromosome in the transformant was detected by Southern hybridization technique. The virulence-related gene of the transformant was not affected by the insertion of *gfp* gene. Cabbage plants were inoculated with the transformed strain and movement of the pathogen was monitored by using fluorescence microscope. The migration and distribution of *gfp*-labeled strain could be visualized in real time. Visualization of the presence of the pathogen easily enabled the infection process to be monitored from the early stages. A delay of 2 days was required between the distribution of *Xanthomonas* cells in cabbage plant and appearance of visible necrosis (So et al. 2002).

5.1.4.3 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was developed for the detection of plant viruses, using plastic microplates with wells to allow reaction between antigen and antibodies (Clark and Adams 1977). These tests have been employed for the detection, differentiation, and quantification of plant bacterial pathogen and fungal pathogens less frequently. ELISA and its variants are well-established procedures for testing large number of samples in quality assurance programs worldwide. The protocol readily allows automation and produces relatively reproducible results for large-scale testing of seeds and especially propagules. Both polyclonal and monoclonal antibodies (PABs and MABs) have been produced commercially for important bacterial plant pathogens. The MABs generated against bacterial pathogens are initially screened using appropriate ELISA format. The ELISA tests with sensitivity levels of 10^3 – 10^5 CFU mL⁻¹ are sufficient for the detection of most bacterial pathogens. The sensitivity of ELISA may be increased ten-fold by using an extraction buffer containing EDTA and lysozyme which liberate LPS present in the bacterial cell surface, resulting in the significant enhancement of antigen-antibody reaction. In addition, by boiling the bacterial samples to destroy proteins or using commercial extraction buffers, it may be possible to increase the sensitivity of ELISA tests. Commercial detection kits have been developed by Agadia Inc. and other commercial agencies for the detection of single or multiple pathogens in plant samples (Alvarez 2004).

Monoclonal antibodies produced against *Pseudomonas syringae* pv. *pisi* (*Pss*) were used in indirect and competitive ELISA formats for efficient detection of this pathogen in pea seeds. Strains of *Pss* were also differentiated by applying the ELISA tests (Candlish et al. 1988). The presence of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) could be detected in leaves and seeds of 60 rice accessions (Zhu et al. 1988). The monoclonal antibodies (*Xoo*-1) generated specifically against *X. oryzae* pv. *oryzae* reacted positively with all 178 strains collected from diverse geographical locations. The MAb did not react with

X. oryzae pv. *oryzicola* (causing rice bacterial leaf streak disease) or other xanthomonads in modified ELISA tests. Four groups of *Xoo* isolates, namely groups I, II, III, and IV, were formed based on their reactions with four MABs. However, two of these MABs reacted positively with *X. oryzae* pv. *oryzicola*, although there was no reciprocal reaction between the MAB specific to the bacterial leaf streak (BLS) pathogen with any of the four groups of *Xoo* isolates (Benedict et al. 1989). In a later study, 63 strains of *Xoo* were classified into nine reaction types consisting of four serovars and seven subserovars, based on their immunological activity with PABs and MABs (Huang et al. 1993). In another investigation, pathovar-specific MAB was employed to detect *X. oryzae* pv. *oryzae* in contaminated rice seed by using ELISA test (Gnanamanickam et al. 1994).

Carrot bacterial leaf blight disease caused by different strains of *X. campestris* pv. *carotae* was detected by using DAS-ELISA format. Positive reactions, as reflected by color development, required contamination levels $>10^5$ CFU g⁻¹ of seeds present in the seed-wash suspensions. The absorbance values were not significantly different from control samples (uncontaminated seeds) if seed-wash suspensions from seed lots had contamination levels $<10^4$ CFU g⁻¹ of seeds. However, DAS-ELISA test was less sensitive than the seed-wash dilution plating assay (Meng et al. 2004).

X. campestris pv. *vesicatoria* (*Xcv*), incitant of bacterial spot disease, infects tomato and pepper. Three specific MABs – 7AH10, 5HB3, and 4AD2 – reacted with lipopolysaccharides present on bacterial cell surface. Three formats of ELISA were employed to assess the effectiveness of the MABs in detecting *Xcv*. Competitive-ELISA test was the most sensitive, as it could detect populations of *Xcv* as low as 10^3 – 10^4 CFU mL⁻¹. Bacterial growth in artificially inoculated tomato and pepper plants could be successfully monitored. Assays on field-collected samples reconfirmed the diagnostic efficiency of the MABs. Assessment of the accessibility of the MABs to their lipopolysaccharide epitopes confirmed that sample preparation could be improved to enhance the sensitivity of the assay to a significant extent. Immunological properties of *Xcv* were studied by using other immunoassays. Immunoblotting of different extracts of bacterial cells and immunoelectron microscopy demonstrated that the epitopes were distributed throughout the bacterial cell wall and involved in complexes that might be loosened through the action of proteinase K or lysozyme (Tsuchiya and d'Ursel 2004). In a later investigation, PABs specific to *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) were used in indirect ELISA format in 96-well microtitre plates. Clear positive reactions were observed for all eight strains from Macedonia and two reference strains of *Xcv* from California. *X. campestris* pv. *malavacearum* strain did not react positively with the antibodies for *Xcv*, indicating their specificity of reaction. If the bacterial suspension was heated at 60°C for 30 min prior to performing ELISA test, the rapidity and unambiguity of the test could be enhanced (Mitrev and Kovačević 2006).

Double-antibody sandwich (DAS-) ELISA was shown to be more efficient in detecting Stewart's wilt pathogen *Pantoea* (= *Erwinia*) *stewartii* subsp. *stewartii* in corn (maize) seeds than four other procedures tested. DAS-ELISA was the most suitable method, using PABs for capture and MABs for detection of the pathogen. This ELISA format could detect the pathogen antigen in corn seeds from plants inoculated with an antibiotic-tolerant strain of *P. stewartii* subsp. *stewartii*. The presence of viable *P. stewartii* subsp. *stewartii* was confirmed by culturing the pathogen from infected corn seeds. A highly positive relationship could be discerned between the recovery of the pathogen from seeds and ELISA reactivity (Lamka et al. 1991). The rate of transmission of *P. stewartii* subsp. *stewartii* from seeds to seedlings was determined. Sweet corn

hybrid Jubilee seedlings were repeatedly inoculated with pinprick inoculators or suspensions of the pathogen were injected into the shanks of the primary ears of each adult plant. Seedlings growing from the seeds collected from inoculated plants were examined at two-to-three-leaf stage for development of Stewart's wilt symptoms. Infected plants were confirmed by microscopic observations of bacterial ooze and also by the ELISA test. Of the 61,800 seedlings, 115 showed symptoms of infection and leaf tissue samples were taken from the symptomatic plants. The bacterial ooze test showed the presence of *P. stewartii* subsp. *stewartii* in 41 samples which were ELISA-positive. Symptomatic leaf tissues that did not have bacterial ooze were also negative for the pathogen by ELISA test. The results indicated that the rate of seed to seedling transmission of *P. stewartii* subsp. *stewartii* was low (0.038%) (Michener et al. 2002). Efficiency of detection of *P. stewartii* subsp. *stewartii* by ELISA tests was not affected even after the treatment of corn seeds with the fungicides or insecticides, either singly or in combination. There was no consistent evidence from the experiments that fungicide or insecticide treatments substantially affected the ability of ELISA-based seed health test to detect the pathogen in corn seed. Assays of treated seed appeared to be no different than assays of nontreated seed. ELISA tests therefore have the potential for use in estimating the effectiveness of seed treatments with chemicals for elimination of the pathogen from corn seeds (Pataky et al. 2004). In a later study, *Pantoea stewartii* subsp. *stewartii* populations were estimated by comparing ELISA absorbance values from naturally infested corn seed to standard curves prepared from a serial dilution of *P. stewartii* subsp. *stewartii*. The bacterial populations ranged from 1.6×10^5 to 8.5×10^8 CFU per seed (Dutta et al. 2013).

A double-antibody sandwich (DAS-) ELISA format was developed for the detection of *Xanthomonas campestris* pathovars *undulosa*, *cerealis*, *translucens*, and *phleipratensis* in pure cultures and wheat seeds contaminated with *X. campestris* pv. *undulosa* (*Xcu*). The pathovars *undulosa*, *cerealis*, and *translucens* are known to be pathogenic to wheat crops under natural conditions. Antiserum prepared against somatic antigens of *Xcu* reacted positively with cells of pv. *undulosa*, *cerealis*, *translucens*, and *phleipratensis*, but not other bacterial species belonging to *Xanthomonas*. The lower limit of detection of pure cultures was 5×10^3 CFU mL⁻¹. A semiselective enrichment broth (SSEB) improved the recovery of *Xcu* in cultures mixed with contaminating bacteria commonly present on wheat seeds. The enriched samples provided two-to-three-fold increases in A₄₀₅ nm readings when viable cells of *Xcu* were present. Following enrichment, *X. campestris* pv. *undulosa*, *cerealis*, *translucens*, and *phleipratensis* could be detected in samples that originally had less than 5×10^2 CFU mL⁻¹.

Semiselective enrichment combined with ELISA (SSEB-ELISA) allowed for determination of the percentages of infestation of wheat seed lots. Potential seedling infection (PSI) of naturally infested wheat seed lots was determined by growing seed samples in the greenhouse under optimal conditions for disease development. Percentages of seed infestation determined by SSEB-ELISA procedure were significantly correlated with PSI ($r = 0.87$; $P \leq 0.05$). On the other hand, assessments made by ELISA or direct plating onto semiselective medium XTS agar did not correlate with PSI determined in the greenhouse. The SSEB-ELISA procedure provided a highly sensitive and reliable detection and enumeration method for wheat seeds infested with <100 CFU mL⁻¹ (Table 5.1). Further, this assay could offer reliable results within 24 h, forming a useful tool for rapid initial screening of wheat seed lots in certification programs. Furthermore, the SSEB-ELISA procedure was able to efficiently detect three bacterial pathovars belonging to

Table 5.1 Comparative efficiency of three different laboratory methods in detecting *X. campestris* pv. *undulosa* in wheat seeds and influence of seed infestation on seedling infection (Frommel and Pazos 1994).

Seed lot no.	Seedling infection (%) ^a	Infected seeds (%) ^b		
		XTS medium ^c	ELISA-ST ^d	SSEB-ELISA ^e
1	12.0	5.2	2.72	17.93
2	14.0	6.5	2.73	16.30
3	1.0	0.1	7.07	3.26
4	8.75	8.0	15.78	10.30
5	2.50	6.6	14.72	5.00
6	14.0	5.8	1.10	18.29
7	6.50	3.6	2.10	8.54
8	8.75	4.5	2.45	10.97
9	6.50	8.0	2.48	6.10
10	12.0	3.6	1.22	7.60
11	9.0	4.1	2.28	12.17
12	6.5	2.6	4.88	8.50
r ^f		0.54	-0.56	0.87
t-value ^g		2.033	-2.169	5.68 ^h

^a Seed germination in all seed lots was 95–99%; ^b Four replicates of 1200 each; ^c Blotting of seeds in XTS medium; ^d Seed washings in saline and Tween solution tested by DAS-ELISA; ^e Seed washings in semiselective enrichment broth tested by DAS-ELISA; ^f Coefficient of correlation; ^g Student's *t*-test at 5% level; ^h Uninfected control seeds had no infestation and no disease on seedlings.

the *translucens* group, thus providing another advantage of using this method for the detection of bacterial pathogens associated with wheat seeds (Frommel and Pazos 1994). *Xanthomonas translucens*, causing barley bacterial blight disease, is seed-borne. The pathogen was detected by dilution plating method, using XTS medium. The indirect ELISA format was found to be more sensitive, rapid, and reliable. However, the ELISA test could identify the bacteria isolated from infested seeds only up to genus (*Xanthomonas*) level, but not the species (*translucens*) specifically. Since all *Xanthomonas* spp. are considered to be pathogenic, any *Xanthomonas* spp. isolated from barley seed were presumed to be *X. translucens*. Indirect ELISA was deemed to be desirable for seed testing using the isolates from dilution plate assays (Olsen et al. 2002).

For immunoassays, the specificity and sensitivity of reactions depend on characteristics of antibodies. In the case of *Acidovorax avenae* subsp. *citrulli* (*Aac*), causative agent of cucurbit bacterial fruit blotch (BFB) disease, the available PABs gave false-positive results because they also reacted with closely related bacterial species. A novel MAb specific to the seedborne bacterial pathogen *Aac*, MAb 11E5, was therefore generated. The MAb 11E5 reacted specifically with 19 strains of *Aac*, but not with three closely related bacteria in the family Comamonadaceae. The MAB

Table 5.2 Comparative efficacy of MC-sELISA and ELISA formats in detecting *Acidovorax avenae* pv. *citrulli* in seeds of watermelon and cucumber from plants infected by the pathogen (Himananto et al. 2011).

Seed samples	Seedling grow-out test		Reactivity (A_{405})	
	Diseased seedlings ^a	Percentage %	MC-sELISA ^b	ELISA ^c
Watermelon Khonkaen 1	14/100	16	2.00 ± 0.12 (+)	2.54 ± 0.51 (+)
Watermelon Khonkaen 2	3/100	4	0.79 ± 0.07 (+)	0.97 ± 0.03 (+)
Watermelon Khonkaen 3	0/100	0	0.12 ± 0.03 (–)	0.09 ± 0.04 (–)
Cucumber 40680101	0/32	0	0.17 ± 0.07 (–)	0.19 ± 0.02 (–)
Cucumber 40682001	0/32	0	0.21 ± 0.048 (–)	0.12 ± 0.02(–)
Healthy watermelon seed	0/100	0	0.16 ± 0.01 (–)	0.12 ± 0.02 (–)
Healthy cucumber seed	0/100	0	0.13 ± 0.01 (–)	0.19 ± 0.02 (–)
Seed from infected watermelon	23/200	13	0.85 ± 0.04 (+)	1.26 ± 0.12 (+)
Seed from infected cucumber	6/32	20	0.58 ± 0.01 (+)	0.58 ± 0.06 (+)

^a Number of diseased seedlings/total; ^b MC-sELISA: MAb capture-sandwich ELISA; ^c Commercial ELISA kit marketed by Agadia Inc.; data are average of absorbance values for three replicates; absorbance values greater than twice of those from healthy seed were considered positive reaction.

11E5 detected a strain of *Aac* that was not detected by a commercial ELISA-based kit and a commercial immunochromatographic strip test. In Western blot analysis, MAb 11E5 reacted with an *Aac* protein of a molecular mass >170 kDa. The MAb 11E5 was used in two sandwich ELISA systems – MAb-captured-sandwich ELISA (MC-sELISA) and PAb-captured-sandwich ELISA (PC-sELISA – and a commercial ELISA kit (Agadia) for detection of *Aac* in watermelon seed lots with different percentages of artificially infested seed. Seed macerate was more suited for detection of *Aac* by both MC-sELISA and commercial ELISA kit as detection of *Aac* in seed wash gave lower sensitivity and higher background. The MC-sELISA procedure was 10 times more sensitive than PC-sELISA for the detection of *Aac* in cucurbit leaf and seed extracts. Ten commercial seed lots were tested using MC-sELISA and the commercial ELISA kit. The results obtained from both methods were similar and agree with those of standard seedling grow-out (SGO) method. The detection limit of MC-sELISA method was 5×10^4 CFU mL⁻¹ of *Aac* suspension (Table 5.2). MAb 11E5 could detect *Aac* strains isolated from imported squash and bottle gourd seed, whereas the commercial ELISA kit and immunochromatographic strip test failed to detect squash type B strain. The MC-sELISA procedure developed in this investigation had the potential for effective detection of *A. avenae* subsp. *citrulli* directly in leaf, fruit, and seed samples without the need for isolation of the pathogen in pure culture. Furthermore, this procedure is simple, suitable, and inexpensive for application for routine field inspection to assess the disease incidence (Himananto et al. 2011; Section 5.4.3).

Tomato seeds carry the seedborne bacterial pathogen *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), as well as several saprophytes. Specificity of the monoclonal antibody (MAb) Cmm1 to geographically diverse strains of *Cmm* was assessed.

The MAb was also tested for its usefulness as a tool to separate *Cmm* from other saprophytes associated with tomato seed. The MAb Cmm1 reacted positively with 99% of the 236 international *Cmm* strains tested. Further, MAb Cmm1 strongly reacted with an additional 32 strains isolated from tomato seed that could be identified as *Cmm*, using the Biolog MicroLogTM microbial identification system (Biolog Inc., CA, USA) and 16S rDNA analysis. The specificity of MAb Cmm1 to the pathogen and the near universality of the MAb Cmm1-reactive antigen among diverse *Cmm* strains were considered as a reliable feature of this antibody and a useful diagnostic and identification tool. A large proportion of the *Cmm* strains associated with naturally infested tomato seed were found to be putatively hypovirulent or nonvirulent. The role of such populations therefore must be investigated to determine their significance in *Cmm*–tomato interactions (Kaneshiro et al. 2000).

5.1.4.4 Immunomagnetic Separation of Bacterial Pathogens

Immunomagnetic separation (IMS), a variant of immunoisolation technique, is a process by which immunomagnetic beads (IMBs) are coated with antibodies specific to the target pathogen, allowing selective trapping of target pathogen cells from the samples. They are resuspended in a smaller volume of solution processed for polymerase chain reaction (PCR) assay. The IMS method combines the advantages of serologic recognition, target bacteria concentration, and growth characteristics of the colonies developing on the culture medium, facilitating the process of obtaining axenic culture of the target pathogen for further investigations. Utilization of IMS prior to plating on agar media improves the sensitivity of detection and isolation of pathogenic bacteria found along with fast-growing saprophytic bacterial species. Immunomagnetic separation (IMS) procedure was employed for the detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*), causative agent of watermelon bacterial fruit blotch (BFB) in watermelon seed. IMS yielded a 10-fold increase in recovery of the pathogen over direct spread-plating on King's medium B (Walcott and Gitaitis 2000).

IMS technique was applied for selective isolation of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causing tomato bacterial canker disease, from tomato seeds to improve the specificity and sensitivity over the standard isolation procedure. Two commercially available PABs for *Cmm* were evaluated for their efficacy in binding magnetic beads. The PAB-PRI, recommended by the European Plant Protection Organization (EPPO), was better than PAB-BR. IMS with 10^6 IMB mL⁻¹ coated with polyclonal antiserum at 1/3200 dilution recovered more than 50% of the target cells from the initial inocula of 10^3 – 10^4 CFU mL⁻¹. The threshold detection was lower than 10 CFU mL⁻¹, even in seed extracts containing seed debris and high populations of nontarget bacteria. The detection and isolation of *C. michiganensis* subsp. *michiganensis* from naturally infected tomato seeds with a higher level of sensitivity was possible by employing IMS procedure, when compared with direct isolation on the semiselective medium used earlier (Fig. 5.1). IMS has the potential for large-scale applications for routine testing of tomato seeds by phytosanitary agencies (de León et al. 2006; Section 5.4.4).

The efficacy of IMS-plating method, DAS-ELISA, immunofluorescence (IF), and direct PCR assays was assessed for detection of *Cmm* in tomato seed lots. The IMS-plating method allowed the detection of less than 10 CFU mL⁻¹ of *Cmm* in all samples

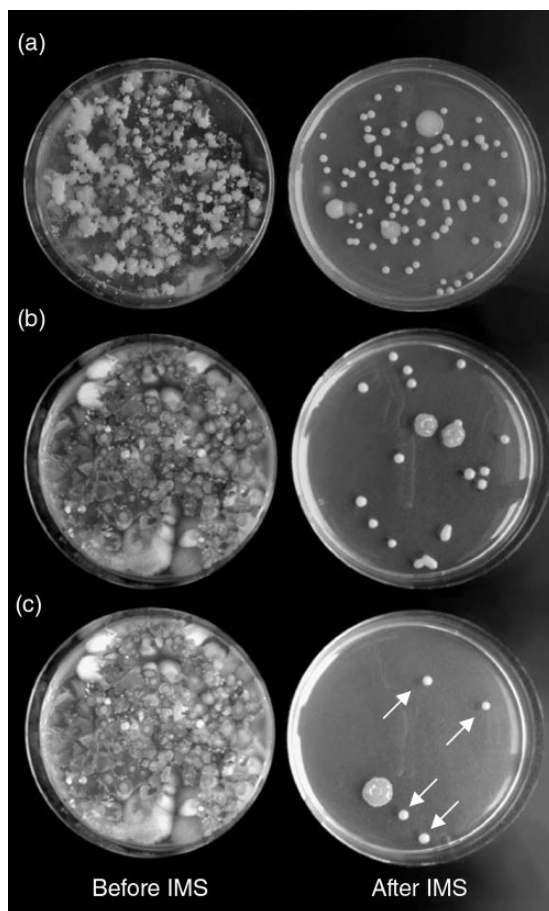


Figure 5.1 Isolation of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) from tomato seed extracts on YPGA medium at different concentrations before and after immunomagnetic separation (IMS). Left: saprophytic bacterial populations at high levels and right: effective reduction of saprophytic bacterial population by IMS, favoring the development of *Cmm*. Source: de León et al. (2006). Reproduced with permission of Elsevier Publishers BV, USA.

assayed. On the other hand, DAS-ELISA and IF tests could detect *Cmm* at concentrations of 10^4 and 10^3 CFU mL⁻¹, respectively. Direct PCR assay needed DNA extraction from seeds and positive results were obtained if the pathogen population was 10^3 CFU mL⁻¹ or more. IMS-plating procedure was the most sensitive and specific for *Cmm* detection, allowing the recovery of viable bacteria from seed extracts. When combined with standard procedures, IMS-plating method could be the preferred procedure for the detection of *Cmm* in tomato seed lots (de León et al. 2008).

5.1.4.5 Immunolabeling and Electron Microscopy

Immunolabeling and electron microscopy techniques have been applied to visualize immunological reactions on electron microscope grids and to monitor the distribution and localization of various proteins of pathogen/host origin in certain host–pathogen interactions. Immunosorbent electron microscopy (ISEM) and immunofluorescence

(IF) techniques were employed for characterization of MABs for the detection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), causative agent of rice bacterial leaf blight (BLB) disease (Benedict et al. 1989). Indirect and direct immunofluorescence staining involves observations under a microscope fitted with ultraviolet light source. The target antigen was stained with homologous antibodies conjugated with fluorescent dyes such as fluorescein isothiocyanate (FITC). Seed leachates from bean seeds were mixed with agar in petridishes and the colonies were produced after 48 h. The agar was incubated with FITC-conjugated antiserum. After several washing steps, observations under ultraviolet microscope revealed the presence of fluorescing colonies of bacterial pathogen. The intensity of fluorescence was proportional to the population size of the bacteria in the seed preparation (Franken and van Vuurde 1990).

The effectiveness of MABs and PABs generated against *Xanthomonas campestris* pv. *campestris* (*Xcc*) in detecting the pathogen in cabbage seeds was assessed using immunofluorescence (IF) microscopy technique. The PAB 94 and MABs 20H6, 18G12, 2F4, and 16B5 efficiently detected *Xcc* in seed extracts when 5 min or 2.5 h shaking of seeds were used as extraction procedures. The reliability of detection of *Xcc* in cabbage seeds depended on the seed lot and the antibody employed. Some of the virulent strains of *Xcc* derived from seed lots could not be detected using some MABs, while saprophytic isolates cross-reacted with MAB 17C12. The results indicated that the MAB capable of reacting with all strains of the pathogen should be employed for detecting *Xcc* in cabbage seeds (Franken 1992). By employing immunoelectron microscopy technique, the presence of epitopes specific to *Xanthomonas campestris* pv. *vesicatoria*, causing bacterial spot of tomato and pepper, was detected on the bacterial cells. The epitopes were well distributed throughout the cell wall and involved in complexes that might be loosened through the action of proteinase K or lysozyme (Fig. 5.2) (Tsuchiya and d'Ursel 2004).

"*Candidatus* Liberibacter asiaticus," the incitant of an economically damaging disease of citrus, huanglongbing (HLB) (also known earlier as citrus greening disease), was suspected to be vertically transmitted from seed to seedlings. Real-time polymerase chain reaction (PCR) assays indicated the presence of abundant bacterial 16S rRNA

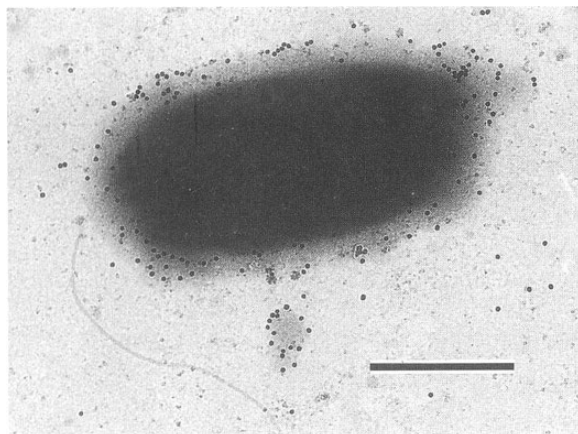


Figure 5.2 Detection of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) by immunogold electron microscopy using the monoclonal antibody MAB 4AD2 specific to the pathogen. Note the uniform distribution of immunospecific epitopes on the cell wall of *Xcv*. Source: Tsuchiya and d'Ursel (2004). Reproduced with permission of Springer Science, Heidelberg, Germany.

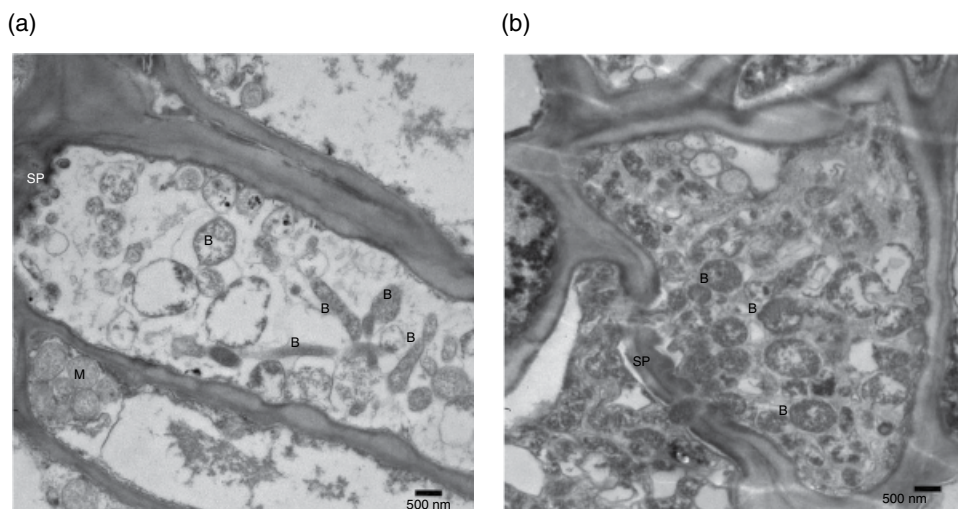


Figure 5.3 (a) Visualization of “*Candidatus Liberibacter asiaticus*” in the transverse sections of phloem sieve element in seed coat of grapefruit cultivar Conners using transmission electron microscopy (TEM). B: bacterial cells; SP: sieve plate, and M: mitochondria (b) Cross-section of phloem sieve element showing large number of bacterial cells. Source: Hilf et al. (2013). Reproduced with permission of the American Phytopathological Society, MN, USA.

sequences in seed coats of citrus seed, but the bacterial cells were not detected. Transmission electron microscopy (TEM) was used to verify the presence of intact bacterial cells in citrus seed coats. Thin sections of Conners seed coats collected from immature, nonharvestable fruit were examined under TEM. The presence of bacteria in phloem sieve elements of seed coat vascular bundle was detected and they showed pleomorphic round and elongated bacilliform-like cells characteristic of “*Ca. L. asiaticus*” (Fig. 5.3a). These bacterial cells were similar to those present in additional seed coat samples with dimensions of 0.14–0.26 μm , values within the ranges reported earlier for this pathogen. In addition there were many round bacterial cells, sometime in large numbers (Fig. 5.3b), which had a size range of 0.30–0.99 μm . During the study on seed transmission, the seed coat was the seed tissue in which the bacterial DNA was most frequently detected. The electron micrographs indicated that intact “*Ca. L. asiaticus*” cells were also present in the sieve elements in the seed coat vascular bundle. Because the bacteria in these seed coat tissues appeared to be near homogeneity, the seed coats could be used as a source of relatively pure pathogen cells for culturing and other analyses (Hilf et al. 2013).

5.1.4.6 Flow Cytometry

Rapid identification and quantification of cells or particulate substances as they move through a sensor in a liquid system may be accomplished by flow cytometry (FCM) technique. Fluorescent antibodies conjugated to specific antibodies are useful for the identification of bacteria. Multiple cellular parameters are determined simultaneously based on the cell's fluorescence and its ability to scatter light. The results may be confirmed by further confirmatory tests, after sorting out the cells electronically or culturing the subpopulations of selected cells. Vital stains are used as fluorescent markers to

indicate cell viability. Carboxy fluorescein diacetate (cFDA) and calcein acetoxy methyl ester (Calcein AM) are used for green fluorescent staining to indicate cell viability, whereas propidium and hexidium iodide are employed for red fluorescent staining to show that cells are dead (Alvarez 2001; van der Wolf and Schoen 2004). Fluorescein diacetate (FDA), when absorbed into the cells, is cleaved by nonspecific esterases to release fluorescein, a polar fluorescent compound which is retained inside the cells. The cell viability is therefore related to the ability of the cells to accumulate fluorescein due to esterase activity. In contrast, propidium iodide (PI) may pass through damaged cell membranes only and form complexes with ds-DNA and RNA. PI-stained cells are considered to be nonviable (Chitaara et al. 2006).

The viability of cells of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causing bacterial canker of tomato, was assessed by using fluorescent probes calcein AM, cFDA, and PI in combination with flow cytometry (FCM) technique. The ratio of viable/nonviable cells in *Cmm* populations formed the basis of assessment. Heat-treated and viable (untreated) *Cmm* cells labeled with calcein AM, cFDA, and PI could be distinguished based on their fluorescence intensity in FCM analysis. Untreated *Cmm* cells exhibited relatively high green fluorescence levels due to staining with either calcein AM or cFDA. In contrast, *Cmm* cells damaged by heat treatment showed high red fluorescence levels due to staining with PI. The number of labeled cells detected by flow cytometry analyses was always higher than the number of CFU mL⁻¹ determined by the plating procedure, implying that a large proportion of *Cmm* cells were enzymatically active, but only about 24–55% of stained cells were able to form colonies. A linear relation ($r^2 > 0.80$) was observed between the percentage of heat-treated cells in samples and the FCM counts of *Cmm* cells labeled with PI (Table 5.3). Both plate count and FCM results showed a linear relationship with the percentage of viable *Cmm* cells in pathogen population. FCM analysis of calcein AM stained cells could be used as a cell viability indicator and this procedure was found to be superior to the conventional plate count, as the FCM required a short assay time (Chitaara et al. 2006).

Table 5.3 Colony formation of sorted *Cmm* cells labeled with Calcein AM, cFDA, PI or combinations of Calcein AM, cFDA with PI, after plating on GNA medium (Chitaara et al. 2006).

Label	<i>Cmm</i> population % heat-treated cells	Concentration of sorted cells (cells mL ⁻¹)	Plate counts of sorted cells (CFU mL ⁻¹)
Calcein AM	0	2.4×10^3	9.8×10^2 (42%)
PI	100	4.8×10^2	0
Calcein AM+	50	9.7×10^{2a}	6.3×10^2 (65%)
PI		4.2×10^{2b}	0
cFDA	0	3.9×10^3	2.0×10^2 (5%)
PI	100	2.6×10^3	0
cFDA + PI	50	2.5×10^{3c}	1.6×10 (0.6%)
		3.5×10^{3b}	0

^a Calcein AM-stained; ^b PI-stained; ^c cFDA-stained

5.1.5 Nucleic Acid-Based Assays

Nucleic acid (NA-) based assays provide consistent and reliable results as the nucleic acids and their nucleotide sequences remain stable until variations due to mutation occur. These techniques are generally found to be more sensitive, specific, and rapid for the detection, identification, and quantification of bacterial pathogens present in seeds. Species/subspecies/pathovars share greater nucleotide similarity than those that are distantly related. After identifying a highly specific nucleotide sequence in the bacterial genome, probes or primers based on the specific nucleotide sequence in the DNA fragment are prepared for specific hybridization or amplification. Polymerase chain reaction (PCR) assay or its variants are the most widely employed procedure for the detection of bacterial pathogens in pure cultures or in single/multiple infections of crop plants. As the NA-based techniques have greater sensitivity, they have been successfully applied for the detection of bacterial pathogens in samples that have populations below the detection limits of immunoassays. However, the NA-based techniques involve the use of hazardous chemicals and are unable to differentiate the live and dead bacterial cells. Nonetheless, with the development of probes labeled with nonradioactive digoxigenin, NA-based techniques are preferred though these methods are relatively more expensive (Narayanasamy 2011).

5.1.5.1 Nucleic Acid Hybridization Methods

DNA probes specific to target pathogen(s) have been constructed and employed for rapid and reliable detection, identification, and differentiation of bacterial pathogens belonging to different species, subspecies, pathovars, or strains, especially those bacterial pathogens transmitted through seeds and propagules. The diagnostic probes show specificity at the genus, species, pathovar, or race level. For taxonomic comparisons, the probe is prepared by identifying a DNA fragment that is present only in the bacterial species to be detected/identified, but not in other closely related species. The DNA sequences of 16S ribosomal RNA of different bacterial species have been compared for selecting probes specific at genus level.

Sensitivity of dot-blot hybridization for detection of bacterial plant pathogens may show variations depending on the probe and target bacteria. The minimum number of bacterial cells required for detection may vary from 200 to 10^6 CFU mL⁻¹. Inclusion of an enrichment step for concentrating the bacterial pathogen on a semiselective medium before hybridization by spotting on the nitrocellulose membrane was advocated. *Pseudomonas syringae* pv. *phaseolicola*, causing bean halo blight disease, was detected by this pretreatment before detection by nucleic acid hybridization technique (Schaad et al. 1989).

A DNA probe for comparing partial sequences of 16S rRNA from 52 strains of the bacteria, including *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), causative agent of rice bacterial leaf blight (BLB) disease, was developed. The rRNA molecule is present in large numbers (>10,000 copies/cell) in actively growing bacterial cells. The sensitivity of the probe derived from unique 16S rRNA sequences was greatly enhanced due to the existence of a large number of copies of these sequences in a bacterial cell. The probe hybridized only with pathogenic pathovars of *Xoo* which could be identified consistently (De Parisi and Roth 1990). Nucleic acid hybridization methods were developed for the detection of *X. oryzae* pv. *oryzicola*, the incitant of rice bacterial leaf streak (BLS) disease (Cottyn et al. 1994). A probe specific for *Burkholderia (Pseudomonas) glumae*, causing bacterial

grain rot of rice, was designed and hybridization between the DNA of all strains of *B. glumae* and the probe occurred, but not with *Xoo* and several other bacterial species tested, indicating the specificity of the assay (Tsushima et al. 1994).

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) causes destructive bacterial wilt and canker in tomato. Southern hybridization with DNA probes derived from plasmid-borne genes *celA*, encoding an endocellulose and *pat-1* gene involved in pathogenicity of *Cmm*, was demonstrated to be effective in detecting the pathogens. Southern hybridization with *celA* probe allowed differentiation between *C. michiganensis* subspecies and other pathogenic corynebacterial species. This investigation revealed the specificity of *pat-1* probe and its correlation with virulence of *Cmm*. This region may be amplified by PCR amplification for providing a sensitive detection of virulent strains of *Cmm*. Further, the *celA* probe differentiated the *C. michiganensis* subsp. *michiganensis*, *insidiosus*, *nebraskensis*, *tesellarius*, and *sepedonicus*. Primers derived from the *pat-1* region were useful for detection of *Cmm* in homogenates from infected tomato plants and naturally contaminated tomato seeds (Dreier et al. 1995). The bacterial leaf spot pathogens of tomato and pepper *Xanthomonas axonopodis* pv. *vesicatoria* could be detected by employing a 1.75 kb fragment (KK1750) that preferentially hybridized to the pathogens (Kuflu and Cuppels 1997).

“*Candidatus Liberibacter asiaticus*,” the causal agent of huanglongbing (HLB) (earlier known as greening) disease of citrus, could be detected by using pathogen-specific DNA fragment (0.24 kb) labeled with biotinylated nucleotides in mandarins, tangerines, oranges, and pummelos (Hung et al. 1999). Possible vertical transmission of “*Ca. L. asiaticus*” through seed to seedling was investigated by applying fluorescence in situ hybridization (FISH) assay. Probes complementary to the “*Ca. L. asiaticus*” 16S rRNA were employed for FISH technique. The presence of bacterial cells was detected in the vascular tissue of intact seed coats of grapefruit and pummelo and in fragmented vascular bundles excised from grapefruit seed coats. The physical measurements and morphology of individual bacterial cells were consistent with those reported earlier for the pathogen. No bacterial cells were observed in the preparation of seed of fruit from uninfected trees. A small library of clones amplified from seed coats from a noninfected tree, using degenerate primers targeting the prokaryote 16S rRNA gene sequences, did not contain any pathogen sequences. On the other hand, 95% of the sequences in a similar library from DNA from seed coats from infected tree were identified as “*Ca. L. asiaticus*,” providing molecular genetic corroboration that the bacterial cells detected by transmission electron microscopic observations and FISH technique in seed coats from infected trees were “*Ca. L. asiaticus*” (Hilf et al. 2013).

5.1.5.2 Restriction Fragment Length Polymorphism

Restriction endonucleases are employed to cleave the genomic DNA of bacterial pathogens at specific sites, resulting in the production of unique banding patterns that can be visualized after gel electrophoresis. The restriction fragment length polymorphism (RFLP) analysis of pathogen DNA fragments has been useful for the detection and differentiation of certain bacterial species and their strains. Digestion of rice bacterial leaf blight (BLB) pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) with restriction endonuclease enzyme *Pst*I produced only a few high-molecular-weight DNA fragments which formed distinct patterns for different strains on electrophoresis. The strains of *Xoo* race 2 commonly prevalent in the Philippines could be detected and differentiated by RFLP analysis. The strains of *Xoo* existing in the USA were not considered to be closely

related to the Asian strains based on the differences in the RFLP patterns. It is possible that a distinct pathovar of *X. oryzae* may cause the BLB disease in the USA (Leach and White 1991).

The genetic diversity of *X. oryzae* pv. *oryzae* (*Xoo*) strains collected from 18 locations in India was assessed. Strains of *Xoo* belonging to a single lineage was found to be distributed in 16 of the 18 locations sampled. All strains with this lineage belonged to the pathotype 1b, as shown by pathotyping analysis (Yashitola et al. 1997). The population structure of *Xoo* from the main rice-growing region in China was evaluated by using RFLP and virulence analyses. Strains (103) of *Xoo* were classified into 61 pathotypes which formed three clusters. RFLP analysis differentiated four common bands in the DNA fingerprint patterns of *Xoo*. Each distinct RFLP banding pattern of each strain was considered as a haplotype. Forty-two haplotypes revealed by the probe were divided into four lineages. Some isolates with different pathotypes shared the same haplotype and others with different haplotypes harbored identical pathotype. A weak correlation between virulent pathotypes and molecular haplotypes was resolved in this investigation (Li et al. 2009a). The RFLP analysis of the ITS region (5S + ITS1 and ITS2) of rDNA repetitive units amplified by PCR was useful in detecting and distinguishing *Burkholderia* spp., causing diseases in rice. *B. glumae*, *B. gladioli*, *B. plantarii*, and *B. vandii* were identified based on distinct RFLP patterns. The DNA samples from rice leaf sheaths inoculated with *B. glumae* and *B. gladioli* and the cultured pathogen cells were subjected to digestion with *Hha*I and *Sau*3AI restriction enzymes followed by PCR-RFLP analysis. The patterns formed from the bacterial cells in culture and tissue extracts were identical, indicating that pathogens present in naturally infected rice plant tissues can be reliably identified by RFLP technique (Ura et al. 1998).

5.1.5.3 PCR-Based Assay

Polymerase chain reaction (PCR) is applied for amplification of the target DNA sequence resulting in the enhancement of its titer up to a million times, thereby transforming previously undetectable amounts into readily detectable quantities. The procedure involves essentially heat denaturation of the target ds-DNA and hybridization of a pair of synthetic oligonucleotide primer pairs to both strands of the target DNA, one to the 5' end of the antisense strand by an annealing step. By employing a thermostable *Taq* DNA polymerase enzyme from *Thermus aquaticus* (*Taq*), new DNA fragment is synthesized on templates to produce twice the number of target DNAs. Newly synthesized DNA strands are used as targets for subsequent DNA synthesis and the steps mentioned above are repeated up to 50 times or as required. There is an exponential increase in the number of target DNA molecules. DNA sequences between primers are therefore reproduced with high fidelity and an efficiency of up to 85% per cycle may be achieved. The procedure may be automated if necessary. *Taq* DNA polymerase is heat stable and can be employed at temperatures of 60–86°C (Weier and Gray 1988). The PCR products may be used (i) as a target for hybridization; (ii) for direct sequencing of the DNA to determine strain variations; and (iii) as a specific probe.

The PCR-based assays have several advantages over conventional methods of pathogen detection and disease diagnosis. For performing PCR assays, there is no need to isolate the bacterial pathogen in pure culture and it is enough to extract the DNA of the target pathogen. It is possible to detect single or multiple pathogen(s) from the samples, using appropriate primer(s). Primers in large numbers can be prepared at costs comparable to those methods for developing a few monoclonal antibodies used

Table 5.4 Detection by PCR assays using different target sequences of seedborne bacterial plant pathogens.

Bacterial pathogen	Target sequences	References
<i>Acidovorax avenae</i> ssp. <i>citrulli</i>	16S rRNA	Walcott and Gitaitis (2000)
<i>Burkholderia gladioli</i>	Primers PL-12f/PL11r and GL13f/GL14r	Takeuchi et al. (1997)
<i>B. glumae</i> and <i>B. plantarii</i>	ITS1 and 2 regions of rDNA	Ura et al. (1998)
<i>Candidatus Liberibacter</i>	16S rRNA	Coletta-Filho et al. (2005)
<i>Clavibacter michiganensis</i> ssp. <i>michiganensis</i>	Primers CM3 and CM4 pathogen-specific DNA <i>pat-1</i> gene sequences	Sousa Santos et al. (1997); Niepold (1999); Dreier et al. (1995)
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	Gene sequence of <i>argK</i> encoding ornithyl carbamoyl transferase	Mosqueda-Cano and Herrera-Esterella (1997); Rico et al. (2003)
<i>P. syringae</i> pv. <i>syringae</i>	<i>syfD</i> gene sequences	Bultreys and Gheysen (1999)
<i>Xanthomonas axonopodis</i> pv. <i>glycines</i>	DNA sequences encoding glycinecin	Oh et al. (1999)
<i>X. axonopodis</i> pv. <i>phaseoli</i> var. <i>fuscans</i>	Primers Xf1 and Xf2 with sequences conserved amplified region (SCAR)	Tóth et al. (1998)
<i>X. campestris</i> pv. <i>carotae</i>	350-bp pathogen DNA fragment	Meng et al. (2004)
<i>X. oryzae</i> pv. <i>oryzae</i>	Pathogen-specific DNA sequences	George et al. (1997)

in immunoassays which are more expensive and time-consuming and also comparatively less sensitive. A commercial product Gene Releaser could be employed to produce plant extracts suitable for PCR amplification and the use of organic solvents may be dispensed with. The simplicity and sensitivity of PCR-based assays have made them preferable for routine and large-scale applications in seed certification. Primer pairs based on the nucleotide sequences of genes coding for virulence, enzyme, or toxin production have been effectively used for detecting, quantifying, and differentiating the bacterial phytopathogens and their variants (Table 5.4). The inability to differentiate live and dead bacterial cells is the major flaw of PCR-based techniques, requiring the inclusion of biological methods for obtaining corroborative results, especially in epidemiological investigations (Narayanasamy 2001).

The detection of bacterial pathogens in seed may be difficult using the hybridization method or other techniques. PCR assays can help overcome such problems for the detection of bacterial pathogens effectively and in a highly sensitive manner as in the case of *Pseudomonas syringae* pv. *phaseolicola*, causing halo blight disease of bean. The PCR-based assay using the primers from DNA sequences of phaseolotoxin gene detected the pathogen efficiently, even in the presence of high populations of nontarget bacteria. The assay was highly sensitive with a detection limit of $1\text{--}5\text{ CFU mL}^{-1}$ of seed-soak wash (Prossen et al. 1991). *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*),

causative agent of tomato bacterial wilt and canker disease, contains plasmids pCM1 and pCM2. The strain NCPPB382 has the pathogenicity gene *pat-1* and the gene *CelA*, encoding endo cellulase. A PCR assay based on primers derived from the *pat-1* region was developed. The virulent strains of *Cmm* could be detected by the amplification of the *pat-1* sequences present in them. The PCR-based assay could be performed without prior isolation and enrichment of the virulent strains from plant tissues and the assay had a detection limit of 2×10^2 cells mL⁻¹ of tissue homogenate. At this level of sensitivity, it would be possible to detect latent infection by *C. michiganensis* subsp. *michiganensis* (Dreier et al. 1995).

A rapid PCR-based protocol was applied for concurrent detection of the causal agent of bean common blight disease, *Xanthomonas campestris* pv. *phaseoli* (*Xcp*), and bean halo blight pathogen, *Pseudomonas syringae* pv. *phaseolicola* (*Psp*), in bean seed. The G + C-rich oligonucleotide primers were designed from the phaseolotoxin gene cluster of *Psp*. The HB14 primer specifically directed the amplification of a 1.4 kb DNA fragment from 19 isolates of *Psp* but not from 62 other bacterial strains, including *Xcp* and other *Xanthomonas* species. The HB14 primers for *Psp* and X4 primers specific for *Xcp* were able to amplify discrete DNA fragments from bean seeds contaminated with these two pathogens. When the primers were used in combination, they successfully detected individual and mixed infection of bean common and halo blight diseases and yielded distinct DNA fragments from batches of seeds containing as few as one infected seed in 10,000 seed samples. Although the PCR assay did not discriminate between dead and live pathogen cells, this lacuna was considered to be of minor importance for the diagnosis of seasonally harvested seeds, since the common blight pathogen can survive in bean seeds for more than 30 years (Audy et al. 1996).

Among the isolates (152) collected from diseased beans in Spain, 91% of the strains (138) were identified as *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) and the rest as *P. syringae* pv. *syringae*. The *Psp* strains produced typical water-soaked lesions on bean pods. However, 95 of the *Psp* strains did not produce phaseolotoxin in vitro. In addition, the DNA of 94 of these isolates did not amplify the expected 0.5 kb product when PCR assay was performed using the primer specific for the open reading frame (ORF) 6 of the phaseolotoxin (*tox*) gene cluster. Further, they did not contain DNA homologous to ORF6 as revealed by Southern hybridization analysis. The widespread occurrence of nontoxigenic strains of *Psp* was first observed in the field. These strains lacked the *tox* cluster. The predominance of *tox*⁻ isolates indicated negative evidence against a role of phaseolotoxin in virulence. However, the nontoxigenic strains were pathogenic and their virulence was comparable to that of wild-type strain, except their inability to form chlorotic haloes. All *tox*⁻ isolates contain *avrPphF*, whereas the majority of the *Tox*⁺ isolates lacked this gene. The ELISA test employing the commercially available PAB failed to detect the *Tox*⁻ isolates, so the ELISA test was found to be unsuitable for certifying bean seed lots as free of *Psp*. Amplification of three avirulence genes by PCR allowed predictions of the *Psp* race structure to be made, as confirmed by plant tissue assays. Six races (race 1, 2, 5, 6, 7, and 9) were identified with race 7 being the most prevalent (46.1%), followed by race 6 (21.3%) and race 1 (9.0%). The PCR format developed in this investigation was valuable in detecting the *Tox*⁻ isolates of *P. syringae* pv. *phaseolicola* (Rico et al. 2003).

The ability of *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) to induce disease was attributed to the possession of a pathogenicity island (PAI) that include genes essential for pathogenicity on bean or soybean. In addition, the pathogenic strains have the

argK-tox gene cluster which controls biosynthesis of phaseolotoxin, facilitating identification of *Psp* strains. Over 60% of Spanish field isolates did not produce phaseolotoxin and they did not produce PCR amplification using primer pair ORF6 which amplified expected DNA fragment in phaseolotoxin-producing strains (Tox^+). Nonproducers of phaseolotoxin (Tox^-) did not show hybridization to an ORF6-specific DNA probe (Rico et al. 2003). The genetic variability within the Spanish *Psp* populations was analyzed in comparison with *Psp* and *P. syringae* pv. *glycinea* (infecting soybean) isolates from international collection. The Tox^+ and Tox^- isolates showed genomic divergence that included in ERIC-PCR and arbitrarily primed-PCR profiles. The Tox^+ isolates showed distinct patterns of IS801 genomic insertions that contained a chromosomal IS801 insertion that was absent in Tox^- isolates. Using heteroduplex mobility assay, sequence differences were observed only among the intergenic transcribed spacer of the five rDNA operons of the Tox^- isolates. The Tox^- isolates also lacked the characteristic plasmid that contains a pathogenicity island. The results provided the basis for the separation of the Tox^+ and Tox^- isolates of *P. syringae* pv. *phaseolicola* into two distinct genetic lineages, designated Pph1 and Pph2, respectively, that show relevant genomic differences that included the pathogenicity gene complement (Oguiza et al. 2004).

Xanthomonas campestris pv. *vitians*, causing lettuce bacterial spot disease, is seedborne and may survive in association with seed for extended periods (Sahin and Miller 1997). To determine the level of seed contamination and infection by *X. c.* pv. *vitians*, the seed samples (1000) were directly placed on XCS medium in petriplates. The number of seeds contaminated with the pathogen was determined by counting the number of seeds from which xanthomonads-like bacterial colonies developed. The identity of the bacteria developing from lettuce seeds was identified using the pathogen-specific primer pair B162 which directed the amplification of a c. 700 bp DNA fragment from all *X. campestris* pv. *vitians* strains. Seed-wash assays indicated a contamination level for seed recovered from spray-inoculated plants of c. 10^4 CFU g⁻¹ seed (Barak et al. 2002). *Xanthomonas campestris* pv. *campestris* (*Xcc*), causative agent of cabbage black rot disease, is seedborne. The presence of *Xcc* in plant tissues was detected using the primers XCF and XCR, designed on the sequences of *hrpF* gene of *Xcc*, homologous to *nolX*, host recognition protein. The primers amplified a specific fragment of 525 bp from the cabbage plants infected by *Xcc*. The specificity of the PCR assay was shown by the absence of amplifications from DNA of 40 isolates of *Xanthomonas* strains and other nontarget phytopathogenic bacterial species and *Escherichia coli* (Park et al. 2004).

A rapid and sensitive method of detecting the seedborne pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) was considered essential for producing disease-free seed stock. Initial inoculum carried by an infected seed is a critical factor determining the severity of the disease. *Xcc* may be carried within the seed, often attached to funiculus (infection) or simply associated with seed surface (infestation). Both infected and infested seeds are capable of producing disease in the developing seedlings and acting as reservoir of inoculum for surrounding plants. A PCR-based procedure was developed based on the primers designed to specifically amplify a 619 bp fragment of the *hrpF* gene from *X. campestris*. The specificity of the primers was revealed by the absence of amplification of expected product from the DNAs of other pathogens or epiphytic bacteria occurring on cruciferous plants. The primer pair DLH 120 and DLH 125 from the *hrpF* gene from *X. campestris* (*Xc*) amplified the 3' end of the *hrpF* gene. The primers amplified a 619 bp fragment of *hrpF* gene only from *Xcc* and not from other

Xanthomonas spp., indicating the primer specificity. Primers targeting a 360 bp section of the ITS region of *Brassica* spp. were used as internal control to avoid false-negative results. *Xcc* was readily detected in infected plants and was more sensitive and specific than the conventional plating methods or a commercially available ELISA method. Detection of *Xcc* in seeds of cabbage was accomplished by applying the seed-washing protocol which allowed detection of single artificially inoculated seed among 1000 healthy seeds, using the multiplex PCR format. The protocol developed in this investigation circumvents many of the problems associated with methods employed earlier, enabling detection of *X. campestris* pv. *campestris* in the presence of other microorganisms that may inhibit or overgrow the target pathogen on agar plates (Fig. 5.4) (Berg et al. 2005).

Xanthomonas campestris pv. *carotae*, a seedborne pathogen which causes carrot bacterial blight disease, has a worldwide distribution. The pathogen was detected in carrot seeds by applying the PCR assay with primer pairs developed from the sequences of cloned amplified polymorphic DNA (RAPD) fragments. The primer pairs 3S and 9B directed the amplification of c. 350 bp from all *X. campestris* pv. *carotae* strains. In addition, it provided greater sensitivity of detection by PCR assay. The presence of *X. campestris* pv. *carotae* was detected in seed-wash pellets from seed lots with contamination levels ranging from 2×10^2 to 2.3×10^8 CFU g⁻¹. All strains (785) of *X. campestris* pv. *carotae* collected from carrot plants and seeds from California, Oregon, and Washington were detected by the PCR format using the 3S primer pair. *X. campestris* pv. *carotae* was detected by PCR format from hot-water-treated seeds from which viable bacteria were not recovered with the seed-wash dilution plating assay. The PCR assay was found to be more sensitive than the ELISA-based assay (Meng et al. 2004; Section 5.4.5).

Xanthomonas campestris pv. *vesicatoria* (*Xcv*), a seedborne pathogen, causes the bacterial spot which is one of the most important diseases of pepper and tomato, occurring in most of the countries in the world. Due to the heterogeneous nature of the antigenic determinants in immunogens of *Xanthomonas* spp., the usefulness of immunoassays for the detection of *Xcv* in seeds was not found to be up to the expected level. Polyclonal antibodies raised against strains of *Xcv* cross-reacted with other pathovars of *X. campestris* (O'Brien et al. 1967). A method of detection of *Xcv* in seeds was therefore developed based on amplification of a specific DNA fragment of *Xcv* by polymerase chain reaction (PCR). Oligonucleotide primers specific for hypersensitive reaction and pathogenicity (*hrp*) gene cluster were amplified from DNA preparations containing seed washes with cells of *Xcv*. Identification of a *hrp* fragment (840 bp) such as that from *Xcv* was accomplished by restriction enzyme analyses of the amplified fragment. The threshold of detection of *Xcv* in seed washes from pepper or tomato seed was 10^2 – 10^3 CFU mL⁻¹. The primers RTS9 + RTS10 were more sensitive than the primers RTS2 + RTS3. The sensitivity level of RTS2 + RTS3 was determined with seed washes from both pepper and tomato. The limit of detection of *Xcv* by PCR assay was about 1000 times less than that of ELISA test with seed washes. *X. campestris* pv. *vesicatoria* could also be detected in washes obtained from several seed lots of naturally contaminated pepper and tomato, one of which contained background bacterial microflora greater than 10^7 CFU g⁻¹ of seed. The results showed that PCR assay might be employed for detecting *X. campestris* pv. *vesicatoria* in pepper and tomato seeds because of its higher sensitivity of detection of the pathogen, even in the presence of heavy populations of nontarget bacteria (Leite et al. 1995). In another investigation, the primer pair prepared from the nucleotide sequences of *rhs* family gene was shown to be useful for the specific detection and identification of *X. campestris* pv. *vesicatoria* (*Xcv*), employing the PCR-based assay.

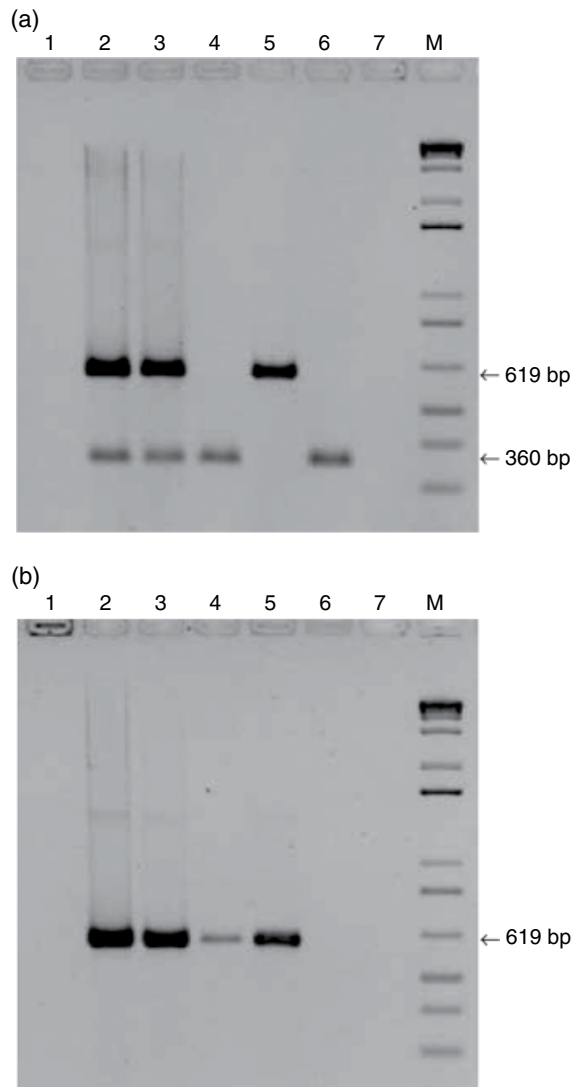


Figure 5.4 Detection of *Xanthomonas campestris* pv. *campestris* (Xcc) in *Brassica* seed using multiplex PCR assay targeting sequences of *hrpF* gene. (a) Multiplex reactions for amplification of pathogen-specific 619 bp and 360 bp products from *Brassica* samples and (b) pathogen-specific *hrpF* product of 619 bp amplified from *X. campestris*. Source: Berg et al. (2005). Reproduced with permission of the American Phytopathological Society, MN, USA.

The 20-mer oligonucleotide XCVF and XCVR were able to amplify the expected 517 bp fragment from all six strains of Xcv tested. The presence of *rhs* family gene homolog in *Xanthomonas* strain was confirmed by dot-blot analysis. *Xcv* strains showed high hybridization signal to the probe. *X. campestris* pv. *vesicatoria* and *X. axonopodis* pv. *vesicatoria* could not be easily distinguished, since both are pathogenic to pepper and tomato, causing the bacterial spot disease. This investigation revealed the specificity of XCVF and XCVR primers which amplified the expected 517 bp fragment only from

Xcv, but not from other *Xanthomonas* spp. This primer could therefore be employed as a rapid tool for detection and identification of *X. campestris* pv. *vesicatoria* consistently and reliably (Park et al. 2009).

Isolates of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) developed resistance to copper. The genes governing copper resistance were located on 188–200 kb self-transmissible plasmid in strains occurring in the USA (Bender et al. 1990). Later, chromosome-encoded copper resistance in a strain of *Xcv* (strain XvP26) was detected in Taiwan. The copper resistance genes in XvP26 strain was characterized. The determinants of copper resistance were identified on a 7652 bp *Xba*I/*Eco*RI chromosomal fragment of XvP26 strain of *Xcv*. The copper resistance genes were localized in the specific region (7652 bp) which was determined to be located on the chromosome, based on pulsed-field gel electrophoresis (PFGE) and Southern hybridization. PCR analysis of 51 pepper and 34 tomato copper-resistant strains of *Xcv* collected from several regions in Taiwan between 1987 and 2000 and nine copper-resistant strains from USA and South America showed that successful amplification of DNA occurred only in strain XvP26. The results indicated that none of the strains from USA and South America contained the uniquely oriented copper gene cluster found in XvP26 strain of *Xcv*, indicating that this strain may represent a rare introduction into Taiwan or chromosomal transfer from other organism(s) (Basim et al. 2005).

Bean crops are seriously affected by the bacterial wilt disease caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) which is transmitted through seeds. As the use of disease-free seed is one of the important strategies for the management of the bacterial wilt disease, detection of the pathogen in the seed as well as in the mother plants rapidly is required for the production of healthy seed stocks. Since *Cff* is a vascular pathogen, differences in the transmission capacity of the pathogen from diseased plant to seed are likely to exist among dry bean cultivars with differing levels of resistance to bacterial wilt disease. Two pathogenic isolates of *Cff* were identified through sequencing of the 16S rRNA gene. The genes were amplified by PCR and the nucleotide sequences (1418 bp) were identical to each other. These sequences were also similar to the sequences in the databases (Gonzalez et al. 2005). Bacterial isolates obtained from bean seeds were consistently identified by PCR assay by employing *Cff*-specific primers which amplified a specific product of 306 bp from all isolates obtained from infected seeds. The isolates showed similarity indices over the range 51–86%. In addition, the Microbiolog 2TM was also used to identify *Cff* from naturally infected bean seeds (Camara et al. 2009). The performance of two PCR formats for the detection of *C. flaccumfaciens* pv. *flaccumfaciens* in the bean seed was determined. In the case of positive results, direct isolation of viable *Cff* cells on agar media should be taken up to confirm the results of PCR since this PCR assay is not able to differentiate dead and live cells of the pathogen (EPPO 2011).

Rice bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) has a worldwide distribution as the pathogen is seedborne. In order to detect the pathogen reliably and rapidly a triplex PCR technique employing three primer sets was developed for internal regions of two genes (*hpaA* and *Xor11*, very short-patch-pair endonuclease) and for a genomic locus derived from an amplified fragment length polymorphism (AFLP) fragment specific to the K3 and K5 races. The sizes of the PCR products amplified were 327 bp, 427 bp, and 1.0 kb by XOOF/XOOR, XRMF/XRMR, and XAF3F/XAF3R, respectively, when the assay was performed to detect *Xoo* in solution and lesion exudates and as a template. The PCR could be applied directly without the

need for prior processing to obtain the DNA from substrates. In addition, the presence of *Xoo* in asymptomatic rice leaf was detected at 3 days after inoculation and at a distance of 6 cm from the lesion site. This PCR format has the potential for large-scale application for the detection and identification of rice BLB pathogen under field conditions, as required for epidemiological investigations (Song et al. 2012).

Burkholderia glumae, a seedborne pathogen, causes bacterial seedling rot and grain rot diseases of rice. The pathogen isolates developed resistance to oxolinic acid (OA) which was applied traditionally for suppressing the development of these seedborne diseases. Mutants of *B. glumae* with different levels of resistance to OA arose due to substitutions in the amino acid sequences. Ser83Arg and Ser83Ile substitutions in GyrA protein were responsible for moderate and high resistance to OA, respectively, in field isolates of *B. glumae*. A mismatch amplification mutation assay PCR protocol was developed using DNA from bacteria incubated in modified S-PG medium for the detection of isolates resistant to OA in infested rice seeds. Primers based on the amino acid substitutions at position 83 in GyrA protein were employed in this PCR format (Maeda et al. 2004a). Bacterial strains were isolated from bacterial panicle blight (BPB-) infected rice sheath, panicle, or grain samples from the southern USA. Pathogenicity of the strains was established by greenhouse inoculation tests. Identification of the strains was carried out by growth of the strains on S-PG medium, carbon source utilization profile (Biolog), cellular fatty acid methyl esterase (FAME) analysis, and polymerase chain reaction (PCR) assay. Among the strains analyzed, 76% and 56% of the strains were identified as *Burkholderia glumae* and *B. gladioli*, respectively. PCR assay was found to be the most sensitive and accurate technique, using 16S rDNA gene and 16S-23S rDNA ITS region of the pathogens. The strains of *B. glumae* and *B. gladioli* showed more than 99% sequence homology with published sequences (Nandakumar et al. 2009). Differentiation of the pathovars of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and pv. *oryzicola* is necessary to apply effective management practices, since both of them infect rice crops either singly or in combination. A novel SYBR Green real-time PCR assay was developed to target a putative membrane protein gene of *X. oryzae* pv. *oryzicola* strains and 26 other reference pathogenic bacteria were tested to determine the specificity of the primer set. A single band of the expected size was amplified by the primer set only from the strains of *X. oryzae* pv. *oryzicola*, but not from the strains of *Xoo* and other reference bacterial species tested. The assay detected at least two genomic equivalents of cloned amplified target DNA using purified DNA or 0.1×10^{-5} OD₆₀₀ unit of cells per reaction respectively. The PCR format developed in this investigation may be useful to differentiate the populations of *Xoo* and *X. oryzae* pv. *oryzicola*, infecting rice plants simultaneously or individually (Kang et al. 2012).

Different variants of polymerase chain reaction (PCR) assay have been developed to improve the sensitivity and specificity of pathogen detection and identification. Repetitive-sequence (rep-) PCR genomic fingerprinting, used often as the adaptation of the conventional PCR assay, is based on PCR-mediated amplification of DNA sequences located between specific interspersed repeated sequences in prokaryotic genomes. These repeated sequences are designated REP (repetitive extragenic palindromic sequences), BOX (DNA sequences of the BOXA subunit of the BOX element *Streptococcus pneumoniae*), and ERIC (enterobacterial repetitive intergenic consensus) elements. Amplification of the DNA sequences between primers based on these repetitive elements generates an array of differently sized DNA fragments from the genomes of different strains. Separation of these DNA fragments on agarose gels yields highly

specific DNA fingerprints. The rep-PCR genomic fingerprinting protocol may be carried out on whole cells (individual colonies of bacteria), bypassing the need for DNA extraction. This technique may also be applied directly to cell suspensions obtained from symptomatic plant tissues (Louws et al. 1994, 1996). The genetic diversity of strains of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* was assessed by rep-PCR assay. Phylogenetic analysis of the rep-PCR genomic fingerprints of *Curtobacterium*, *Clavibacter*, and *Rathayibacter* species grouped all the *C. flaccumfaciens* strains and pathovars together, but *C. flaccumfaciens* pv. *flaccumfaciens* did not form a single cluster. Rather, they dispersed into two clusters along with strains of other pathovars of this species (McDonald and Wong 2000).

Subspecies of *Clavibacter michiganensis* were detected and differentiated by using the rep-PCR assay in comparison with *C. michiganensis* subsp. *michiganensis* (*Cmm*), causal agent of tomato bacterial canker disease. DNA primers (REP, ERIC, and BOX) corresponding to conserved repetitive element motifs in the genomes of diverse bacterial species were employed to generate genomic fingerprints of *Cmm*, *C. michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *nebaraskensis*, *C. michiganensis* subsp. *tessellarius* and *C. michiganensis* subsp. *insidiosum*. The rep-PCR generated patterns of DNA fragments after agarose gel electrophoresis support the division of *C. michiganensis* into five subspecies. Further, the rep-PCR fingerprints identified at least four types, namely A, B, C, and D, within *Cmm*, based on limited DNA polymorphism. The ability to differentiate individual strains might be useful for investigations on the epidemiology and host–pathogen interactions. In addition, a relatively large number of naturally occurring avirulent strains of *Cmm* could be detected and identified by employing rep-PCR fingerprints identical to those of virulent strains of *C. michiganensis* subsp. *michiganensis* (Louws et al. 1998).

Acidovorax avenae subsp. *citrulli* (*Aac*) causes the bacterial fruit blotch (BFB) disease in watermelon and other cucurbitaceous crops. The strains (64) of *Aac* collected from a range of cucurbitaceous hosts in the USA, China, Taiwan, Thailand, Canada, Australia, Brazil, and Israel were subjected to DNA fingerprinting by pulsed-field gel electrophoresis and rep-PCR assay to assess their genetic diversity. These strains clustered into two distinct groups. Group I strains (82%) were recovered from nonwatermelon hosts and the subspecies type strain was the only member of this group that utilized I-leucine as a sole carbon source. In contrast, 94% of the group II strains were recovered from watermelon and 96% of them utilized I-leucine. Group II strains were more aggressive on watermelon than on other host plant species. On the other hand, group I strains were moderately aggressive on all cucurbit hosts tested (Walcott et al. 2004). In another investigation, isolates (12) of *A. avenae* subsp. *citrulli* strains were found to be associated with serious outbreaks of bacterial fruit blotch (BFB). They were identified as *Aac* by fatty acid methyl esters (FAME) and substrate utilization profiles. Rep-PCR assay and pulsed-field gel electrophoresis (PFGE) approaches were applied to characterize and differentiate the isolates of *Aac*, based on their DNA fingerprinting profiles. ERIC- and rep-PCR assays efficiently distinguished the isolates from melon (M) and watermelon (W) classified into group I and II, respectively. DNA fingerprinting by PFGE following *SpeI* digestion revealed that the 12 isolates belonged to five different haplotypes which could be grouped into the two defined groups of *A. avenae* subsp. *citrulli*. Isolates from watermelon showed a higher degree of heterogeneity than melon isolates. The most significant advantage of PFGE resides in its reproducibility, whereas rep-PCR provides the advantage of being more accessible, less expensive, and faster than PFGE.

The rep-PCR may be employed to distinguish between group I and group II isolates, but PFGE analysis may be preferable if the genetic diversity of the isolates/strains is to be assessed (Burdman et al. 2005).

Polymerase chain reaction (PCR-) based DNA fingerprinting methods, such as rep-PCR assay, provide practical and effective means of examining genotypic diversity among *Xanthomonas* spp. The rep-PCR format correlates well with other techniques such as DNA-DNA hybridization for assessing genetic relatedness in bacteria. Genetic analysis of *Xanthomonas campestris* pv. *phaseoli* (*Xcp*) and *X. campestris* pv. *phaseoli* var. *fuscans*, causative agents of bean common bacterial blight (CBB) disease, was carried out by rep-PCR and random amplified polymorphic DNA (RAPD) methods. Relatively little genetic diversity was observed among spinach strains of *Xcp* and these strains were grouped with the New World strains into a large cluster. Genetic analyses and pathogenicity tests confirmed and indicated that the East African strains represented distinct *Xanthomonas* spp. that independently evolved to be pathogenic on common bean. Strains of *X. campestris* pv. *phaseolicola* var. *fuscans* were more closely related and genetically distinct from *Xcp* strains. However, two distinct clusters of *X. campestris* pv. *phaseoli* var. *fuscans* strains were distinguished. One cluster enclosed the most New World strains and the other cluster included the most African strains. Spanish strains were distributed in both clusters, but all strains were highly pathogenic on bean cultivars of both gene pools (Andean and Middle American). These strains were introduced into Spain through multiple introductions of CBB pathogen (López et al. 2006). *Xanthomonas campestris* pv. *malvacearum* (*Xcm*), causal agent of angular leaf spot, black arm, and boll rot diseases of cotton, significantly reduces the yield and quality of the lint. The pathogen exists in the form of several strains that differ in their virulence on cotton cultivars. Highly virulent strains (HVSs) of *Xcm* could infect all commercial cultivars, including the resistant cultivar 101-102 R. The global genetic diversity of *Xcm* as well as HVS (38 strains) were analyzed by repetitive-sequence (rep-) PCR with ERIC and BOX primer sets. The rep-PCR assay revealed abundant polymorphism of fingerprinting. The strains were assigned to different genotypic groups that showed a similarity coefficient of 0.76 using UPGMA analysis. The strains with low or moderate virulence were clustered in one genogroup. All HVSs collected from Africa were placed in another genogroup. Further, the HVSs formed two subgroups which showed a similarity coefficient of 0.80, indicating the genetic diversity of the highly virulent strains of *Xcm*. The results suggested that the virulence of *X. campestris* pv. *malvacearum* was highly related to genotype and/or geoclimatic origin of strains (Zhai et al. 2010).

Bacterial populations associated with rice seed collected from farmers' fields in the Philippines were characterized by BOX-PCR fingerprinting of total genomic DNA and represented 151 fingerprint types (FPTs). On the basis of BOX-PCR fingerprinting of genomic DNA, 82 FPTs were distinguished among Gram-negative bacteria and 69 FPTs among Gram-positive bacteria. The BOX-PCR fingerprint patterns consisted of 10–23 PCR products ranging in size from c. 200 bp to 4.5 kb. The BOX-PCR technique clearly distinguished different bacterial species, but the diagnostic value of patterns varied according to the species. Within some species such as *Burkholderia glumae* and *B. plantarii*, nearly identical fingerprint patterns were produced for multiple reference strains. Seven pathogenic isolates were found in six samples and were clearly virulent on rice. Five isolates had a uniform BOX-PCR profile similar to that of the type strain of *B. glumae* strain LMG2196, whereas two isolates showed similarity to the profile of

B. gladioli pv. *gladioli* LMG2216. Two percent of isolates were seedborne pathogens identified as *Burkholderia* spp. and were found in 55% of the rice seed samples. The BOX-PCR analysis of a collection of rice seed isolates revealed a high heterogeneity within the bacterial communities associated with rice seed (Cottyn et al. 2001).

Bacterial seedling rot and grain rot disease of rice is incited by *Burkholderia glumae* in Japan and Korea. Isolates, exhibiting insensitivity to oxolinic acid (OA) used for seed treatment, were isolated from spray inoculated on rice plants at heading stage. Amino acid substitutions with arginine (Arg) and isoleucine (Ile) at position 83 in GyrA (GyrA83) were reported to be responsible for OA resistance in field strains of *B. glumae* (Maeda et al. 2004a, b). In a later investigation, the phylogenetic diversity of *B. glumae* field strains was assessed using rep-PCR with ERIC and BOX as primers. In order to elucidate the sources of oxolinic acid (OA) resistance in field strains of *B. glumae*, the amino acid at position 83 of GyrA (GyrA83) and nucleotide sequences of *gyrB* and *rpoD* from various strains were analyzed. Ten strains from Mie Prefecture, in which GyrA83 was isoleucine (Ile), were divided into two groups based on band patterns in rep-PCR analysis. However, the nucleotide sequences of *gyrB* and *rpoD* were identical among the strains. Based on the band patterns in the rep-PCR analysis and the *gyrB* and *rpoD* sequences, two highly OA-resistant strains from Toyama Prefecture, Pf-13 and Pg14, were identified. The results suggested that these two strains might have separately acquired OA-resistance. The bacteria might acquire OA resistance faster than they might acquire phylogenetic diversity via the repetitive sequences of ERIC and Box and via *gyrB* and *rpoD*. These strains from Iwate Prefecture, H95, H101, and H104 isolated from different rice cultivars, had Ile at GyrA83, suggesting that OA-resistant bacteria might be transferred among different rice cultivars (Maeda et al. 2007).

5.1.5.4 Real-Time Polymerase Chain Reaction Assay

The standard polymerase chain reaction (PCR) assay has certain limitations. The detection of all strains of a bacterial species could not be accomplished, as in the case of *Xanthomonas axonopodis* pv. *citri* (Hartung et al. 1996). In addition, the post-PCR steps involving the use of hazardous chemicals have to be performed, extending the period needed to obtain the results. An improved detection system had to be developed to overcome the limitations of standard PCR format, to make the system portable, facilitating its use under field conditions away from the laboratory, and to obtain the results rapidly. Real-time PCR technology has been demonstrated to be sensitive, reliable, and rapid for the detection and identification of microbial plant pathogens, including bacterial pathogens. Real-time PCR technique allows real-time fluorescence detection of PCR amplicons and records a specific cycle number, as measured by cycle threshold (Ct) at which a statistically significant increase in the fluorescence (greater than background) can be detected. Real-time PCR provides accurate quantification, as PCR products are monitored as they accumulate in the exponential phase before the PCR agents become a limiting factor. It also allows the melting point of the PCR products to be measured, ensuring the specificity of the amplicon. Real-time PCR assay is simpler to perform, less labor-intensive, and much faster (providing results in 60 min) compared to standard PCR format.

Xylella fastidiosa (Xf), a xylem-limited, Gram-negative bacterium, causes citrus variegated chlorosis (CVC) disease of sweet orange (*Citrus sinensis*). The distribution of *X. fastidiosa* in various parts of sweet orange fruit and seeds, and the transmission of Xf through seed to seedlings, were investigated by employing standard PCR and

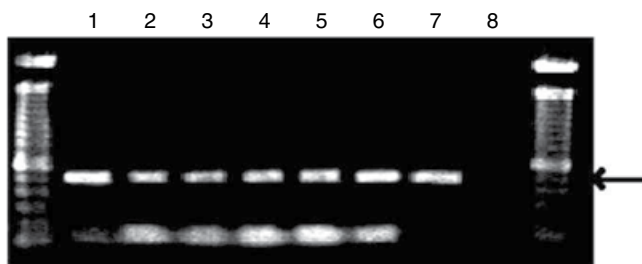


Figure 5.5 Detection of *Xylella fastidiosa* (Xf) in citrus fruits by polymerase chain reaction (PCR). Note the presence of pathogen-specific 472 bp amplicon (arrow) in different fruit tissues. Lane 1: peduncle; lane 2: exocarp; lane 3: mesocarp; lane 4: endocarp; lane 5: septa; lane 6: central axis; lane 7: Xf strain 9a5c; lane 8: central axis of healthy fruit (negative control). DNA ladder (100 bp) is positioned at the extremities. Source: Li et al. (2003). Reproduced with permission of the American Phytopathological Society, MN, USA.

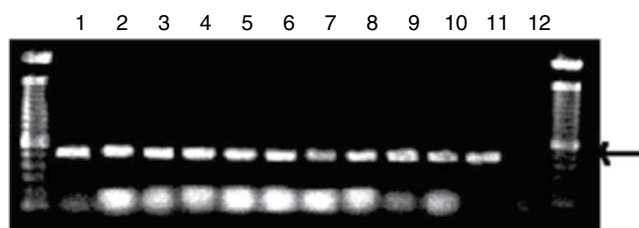


Figure 5.6 Detection of *Xylella fastidiosa* (Xf) by PCR assay in different seed tissues and emerging seedlings, as revealed by Xf-specific product of 472 bp (arrow). DNA ladder (100 bp) is positioned at the extremities. Lane 1 and 11: Xf strain 9a5c DNA (positive controls); lane 2: seed coat; lanes 3 and 4: embryos; lanes 5–7: in vitro isolates from cultivars Valencia, Natal, and Pera; lanes 8–10: seedlings of cultivars Pera, Natal, and Valencia; lane 12: water (negative control). Source: Li et al. (2003). Reproduced with permission of the American Phytopathological Society, MN, USA.

real-time PCR assays. Citrus fruits have a well-developed vascular system which is continuous with the vascular system of the plant. Citrus seeds have prominent vascular bundles which are attached through ovular and seed bundles to the xylem system of the fruit. *X. fastidiosa* was detected by PCR in 20–22 % of seed coat samples and in 15–16% of the embryo samples from seeds of CVC-affected sweet orange fruits. *X. fastidiosa* was not detected in any part of the seeds from healthy sweet orange fruits (Fig. 5.5). Ten PCR-positive seed coats and embryos of three cultivars were analyzed by quantitative real-time PCR assay. Extracts of seed coats (50 µL/sample) or embryos contained approximately 1250 cells of *X. fastidiosa* and a contaminated seed might have a minimum of 2500 *X. fastidiosa* cells. No significant differences in the number of *X. fastidiosa* cells among the seeds from three sweet orange cultivars could be noted. Apparently healthy seed extracts did not have a detectable quantity of the pathogen (Fig. 5.6). *X. fastidiosa* was also detected in and recovered from seedlings grown from infected seeds for isolation in vitro (Table 5.5; Li et al. 2003).

Rice panicle blight disease caused by *Burkholderia glumae* has great economic importance because of the frequent losses caused in the southern USA. As the standard PCR assay was inadequate to detect all strains of the pathogen, a real-time PCR procedure was developed for detection of *B. glumae* in seeds and flag leaf sheath of rice plants.

Table 5.5 Detection of *Xylella fastidiosa* (Xf) by PCR and isolation of Xf in culture from sweet orange seedlings obtained from seeds extracted from CVC-affected fruits (Li et al. 2003).

Cultivar	No. plants tested	No. of positive reactions				
		Plants*	Primary root	Aerial parts	Roots and aerial parts	
Pera	87	14 (16.1%) 2	5	12	3	
Natal	80	26 (32.5%) 2	6	22	2	
Valencia	83	19 (22.9%) 3	3	17	1	
Total	250	59 (23.6%) 7	14 (5.6%)	51 (20.4%)	6 (10.1%)	

* The first number indicates the number of + ve PCR reactions; the number in parenthesis represents the percentage of PCR + ve seedlings; and the third number is the number of seedlings from which Xf was isolated.

Based on the results of Biolog GN microplate system, whole cell fatty acid methyl ester (FAME) analysis, rep-PCR genomic fingerprinting, and 16S-23S rDNA intergenic transcribed spacer (ITS) sequence analysis, the isolates of *B. glumae* from Arkansas were identified and divided into two major groups. However, their genetic diversity was relatively low as revealed by 16S-23S rDNA ITS sequence analysis. The ITS region of all isolates of *B. glumae* from Arkansas and Japan were found to be identical. The real-time procedure developed was effective in detecting as few as 1–10 cells in water, and identifying the pathogen in seed lots and in whole plants. The specific primers were designed based on the 16S-23S rDNA ITS sequence of several representative isolates from Arkansas and Japan. The real-time PCR assay employed in this investigation detected *B. glumae* in seeds using seed wash and plant extracts directly without the preliminary extraction step. Further, the real-time PCR format did not require the need for visualization of PCR products on agarose gels, consequently reducing assay time from 6 h to 2 h with the availability of real-time data. In addition, the real-time assay could provide both qualitative and quantitative assessments of *B. glumae* infection on seed and plant tissues. Use of this real-time PCR format appeared to be a vital step for implementing a clean seed program aimed at preventing crop loss through improved seed sanitation (Sayler et al. 2006; Section 5.4.6).

Burkholderia glumae and *B. gladioli* are seedborne and associated with rice bacterial panicle blight (BPB) disease which has become one of the most limiting rice diseases in Louisiana and the southern USA. As the use of disease-free seeds forms the principal disease management strategy, development of a sensitive and rapid real-time PCR format was considered essential to detect and quantify the populations of *B. glumae* and *B. gladioli* in seed lots. By applying species-specific PCR and sequencing of the 16S rDNA and ITS regions of the 16S-23S rDNA, the bacterial strains isolated from rice seeds were identified as *B. glumae* and *B. gladioli*. The real-time PCR assay was employed to quantify *B. glumae* from seed samples, using TaqMan probe and primers designed for the 16S-23S rDNA ITS region of *B. glumae*. The primers and probes were highly specific and quantified the populations of *B. glumae* present in a single seed, indicating that the asymptomatic seeds might carry the pathogen. Seed from panicles were freshly collected from the field with high levels of *B. glumae*, and varying numbers of seeds (1–10 seeds). The results clearly showed that the real-time PCR protocol developed in

this investigation could be used to identify and quantify the bacterial populations in both field-infected tissues and commercial seed lots (Nandakumar et al. 2009; Section 5.4.7).

For the detection of *Burkholderia glumae*, causal agent of rice grain rot disease, a reliable, rapid, sensitive, specific, and quantitative diagnostic technique was developed. The available genomic information in public databases was used to develop specific PCR primers for accurate detection and identification of *B. glumae*. An SYBR Green real-time PCR primer set was designed based on *rhs* family genes (YD repeat protein) of *B. glumae* BGR1 strain as these genes are structurally diverse. The specificity of the primers was evaluated using purified DNA from five isolates of *B. glumae*, six different species of *Burkholderia*, and 18 other reference pathogenic bacterial species. Using this protocol it was possible to detect at least one genome equivalent of cloned amplified target DNA, using purified DNA or 1 CFU/reaction, when calibrated bacterial cell suspension was used. The procedure developed in this investigation has the potential for analyzing large numbers of rice seed samples rapidly and reliably (Kim et al. 2012). *Burkholderia glumae* and *B. gladioli* were isolated from discolored rice seed collected from Colombian fields. *B. glumae* was more aggressive, causing greater yield reduction and higher level of grain sterility, compared with *B. gladioli*. In order to identify putative virulence gene differing between *B. glumae* and *B. gladioli*, four sequenced genomes of Asian and US strains of the two pathogens, published earlier, were compared with each other. Two draft genomes of Colombian strains of *B. glumae* and *B. gladioli* were predicted to encode distinct groups of genes encoding type IV secretion systems, transcriptional regulators, and membrane-sensing proteins. The results indicated that *B. glumae* and *B. gladioli* might affect grain quality. *B. glumae* reduced the yield significantly. Further, the genotypic difference between the two strains might reflect on the differences in disease phenotypes (Fory et al. 2014).

Pantoea stewartii subsp. *stewartii* (*Pss*) (= *Erwinia stewartii*), causal agent of Stewart's wilt disease of maize (corn), is responsible for significant crop losses. *Pss*, indigenous to America, has been introduced to other parts of the world through maize seeds (EPPO 2006). Detection and identification of *Pss* by enzyme-linked immunosorbent assay (ELISA) and other conventional methods were time-consuming and less sensitive compared with nucleic acid-based techniques. A TaqMan® real-time PCR assay based on the *cpsD* gene for detection and identification of *P. stewartii* subsp. *stewartii* in pure cultures, infected maize seeds, and leaf was therefore developed. The *cps* gene cluster is one of the two genes that may play an important role in pathogenicity and virulence of *Pss*. The *cps* gene is required for the production of exopolysaccharide (EPS) stewartan (Coplin and Majerczak 1990). Under optimal conditions, the selected primers CPSL1 and CPSR2c for the *cpsD* genes amplified a 1.1 kb fragment. The primers and probes were specific for the detection of all 14 reference strains of *P. stewartii* subsp. *stewartii* by real-time PCR format. No amplification occurred in the DNA of 32 non-*Pantoea* and eight other *Pantoea* strains tested. The TaqMan® PCR assay detected 1 pg of purified DNA and 10^4 *Pss* CFU mL⁻¹ (10 cells/reaction) in pure cultures consisting of 92.0% intact (viable) pathogen cells. By directly processing leaf lesions and seeds, it was possible to detect 10 and 50 *Pss* cells/reaction respectively. The higher level of sensitivity was revealed by the ability of the real-time PCR assay to detect the pathogen in three of the four samples that were ELISA-negative. Seeds from control (healthy) plants did not show any detectable fluorescence. TaqMan® real-time detection of *P. stewartii* subsp. *stewartii* was validated by the PCR-based cloning and colony isolation (from fresh seeds)

followed by DNA sequencing. The TaqMan® PCR assays offer real-time monitoring of the targeted amplicon with high specificity without the requirement of any post-amplification analysis. The time required for processing samples by real-time PCR is shorter and specificity of the assay may be enhanced by the use of probe hybridization. The procedure is robust, rapid, and can be automated with high sample throughput potential, permitting analysis of up to 96 samples within 24h of receiving samples, facilitating early certification of seed consignments (Tambong et al. 2008).

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), the incitant of tomato bacterial canker disease, is seedborne and a pathogen of quarantine importance. Infected seeds and seedlings form the primary sources of inoculum for disease incidence and spread over short and long distances. A sensitive and rapid method of detecting and identifying the pathogen in the asymptomatic seeds and plants is therefore critical for the management of the bacterial canker disease. A one-step procedure was developed for the detection of *Cmm* in symptomless seeds directly using a TaqMan probe. The primers and probe were designed based on the conserved sequence of the ITS gene with an expected size of 270bp. Strong fluorescence signals (R_n value of c. 9000) could be recorded only in infected seeds or culture of *Cmm*. The absolute sensitivity threshold was approximately 10 CFU mL^{-1} . In the tomato seeds, the pathogen could be detected in 2h without the need for DNA extraction. The real-time PCR protocol was specific in detecting *C. michiganensis* subsp. *michiganensis* and it did not react with 11 other plant pathogenic bacterial species or subspecies. The pathogen was detected in tomato seeds if the contamination level was more than 1.0%. The addition of reagents that arrested the activities of endogenous PCR inhibitors resulted in reduction of time, test costs, and the chances of cross-contamination across samples (Zhao et al. 2007).

The detection of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in tomato seeds precisely and rapidly, accurate quantification of the pathogen, and ability to discriminate viable cells may play an important role in assessing the potential risks of spreading the bacterial canker disease via seed lots. A DNA binding dye ethidium monoazide (EMA) was used to differentiate between viable and dead bacterial cells (Nogva et al. 2003). The mechanism of action of EMA is by penetration into dead cells with damaged membranes, as viable cells with intact membranes are resistant to the entrance of the dye. A quantitative real-time PCR format involving the use of the DNA-binding dye EMA was developed for the detection and quantification of *Cmm* in tomato seeds. The bacterial suspension was treated with EMA with varying concentrations of the dye prior to performing the real-time PCR assay. The primers and TaqMan probe, based on 16S-23S rDNA spacer sequences, were highly specific for *Cmm* at subspecies level. The detection limit of the direct real-time PCR was 10^3 CFU mL^{-1} in samples, with an apparent sensitivity of 2 CFU of target cells in PCR solution. The viable cells of *Cmm* could be selectively quantified by the EMA real-time PCR assay. The viable cells of *C. michiganensis* subsp. *michiganensis* could be discriminated from dead cells with $1\text{ }\mu\text{g mL}^{-1}$ of EMA in the sample with bacterial concentration of 10^7 CFU mL^{-1} or lower. The EMA PCR appeared to be a promising method for detecting the existence and levels of viable bacterial populations in seed extracts or plant tissues in a real-time assay (Luo et al. 2008).

Strains of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) collected from nine prefectures in Japan were fingerprinted by intersimple sequence repeats-PCR and repetitive-sequence PCR formats. Forty-three strains corresponded to 22 DNA

fingerprinting patterns, indicating that *Cmm* populations in Japan had a high genetic diversity. Tomato plants were inoculated with *Cmm* by defoliating using contaminated scissors and planting in soil containing infected plant debris. Defoliation resulted in a larger number of diseased plants; disease developed earlier and plants survived for significantly fewer days. Upon planting in the infected soil, 33.3% of plants were infected and 6.7% showed disease symptoms (21.7% of infected plants developing symptoms). The results indicated that tomato plants might be infected by *C. michiganensis* subsp. *michiganensis* through plant debris in the soil as the primary inoculum and most plants might remain asymptomatic. The growers may unwittingly spread the disease while debudding and defoliation of latently infected plants, resulting in the secondary spread of the disease (Kawaguchi and Tanina 2014).

The influence of the location of *Acidovorax avenae* subsp. *citrulli* (*Aac*) in watermelon seeds on the effectiveness of pathogen extraction and detection was assessed by using seed lots generated by inoculating watermelon stigmas (pistil inoculation) or ovary pericarp (pericarp inoculation) with *Aac* strain AAC00-1. Pistil inoculation resulted in bacterial localization primarily in the seed embryo, whereas pericarp inoculation led to bacterial deposition under the seed coat. The quantitative real-time PCR format was applied for the detection of *Aac* in purified total genomic DNA. *A. avenae* subsp. *citrulli* populations from pericarp- and pistil-inoculated seed lots were enumerated to determine *Aac* recovery from seed washes and seed macerates by real-time PCR and plating on semiselective medium. The distribution frequency of *Aac* population ($\log_{10}$ CFU g^{-1}) in seed wash versus seed macerate was not significantly different for pericarp-inoculated seed lots ($P=0.070$). On the other hand, for pistil-inoculated seed lots the difference between distribution frequency was significant ($P<0.001$), indicating the influence of location of the pathogen in the seed tissues on the efficacy of pathogen detection. However, use of macerates provided greater level of detection of *A. avenae* subsp. *citrulli*, irrespective of pathogen location in watermelon seeds (Dutta et al. 2012).

The loop-mediated isothermal amplification (LAMP) assay employs a strand-displacement DNA polymerase and four to six primers targeting specific DNA regions with predesigned secondary structures. The LAMP reaction is initiated by hybridization of one set of primers, the inner primers, to specific sites on the target (pathogen) DNA, followed by extension in the presence of a DNA polymerase. A second set of primers, the outer primers, complementary to specific priming sites just outside the inner primer binding sites, initiates synthesis of new complementary strands that displace the DNA strands initiated from the inner primers, resulting in the release of those strands. The displacement reaction which does require the use of a strand displacement polymerase precludes the need for temperature cycling, as required for conventional PCR assay. The first amplification products form stem-loop structures at each end as a result of the unique design of the inner primers and are autoprimed, driving the amplification of a large quantity of DNA. The LAMP reaction may be further accelerated using a third set of primers, targeting the loops of the first DNA amplification. LAMP-amplified DNA forms a ladder of multiple bands in gel electrophoresis and can also be visualized directly in the amplification tube by adding SYBR Green I, a fluorescent DNA-binding dye, and observing under UV-light. In addition, positive reactions may be visually confirmed by the formation of magnesium pyrophosphate precipitation (Mori et al. 2001; Kubota et al. 2008).

Pseudomonas syringae pv. *phaseolicola* (*Psp*), the causative agent of bean halo blight disease, may not be readily distinguished from other pathovars using routine

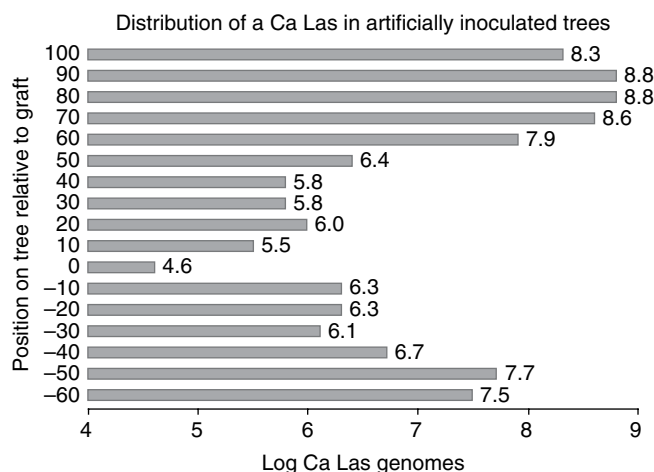


Figure 5.7 Detection of “*Candidatus Liberibacter asiaticus*” in greenhouse-grown sweet orange trees inoculated by bud grafting. Monitoring pathogen distribution by determining populations of pathogen (genomes) in bark tissues of stem and root at different positions by qPCR assays. Source: Li et al. (2009c). Reproduced with permission of the American Phytopathological Society, MN, USA.

microbiological methods because of overall similarity, despite their different pathogenicity profiles. Specific pathovars of *P. syringae* such as *phaseolicola* have to be identified and differentiated for quarantine and/or regulatory reasons. Loop-mediated isothermal amplification (LAMP) assay was developed for specific and rapid detection of *Psp* and possible adaptation for onsite testing. LAMP reactions were optimized by changing each key component of the reaction mixture through a direct optimization process. Best results were consistently obtained using 8 U of Bst enzyme in a 25 μ L reaction with 4 mmol L⁻¹ of Mg and 1 mmol L⁻¹ of betaine for 50 min at 65°C, followed by deactivation at 80°C for 10 min. *Psp* could be differentiated from all other fluorescent *Pseudomonas* spp. representing 12 different pathovars of *P. syringae*, two other *Pseudomonas* spp. and nine other plant pathogenic bacteria tested. Only three strains of *P. syringae* pv. *phaseoli* showed positive amplification under standard LAMP reaction conditions. The sensitivity of LAMP assay was 6.9×10^3 CFU mL⁻¹ when amplification was visualized by gel electrophoresis (Fig. 5.7). The addition of the SYBR Green I dye to the reaction mix after completion of LAMP reaction enabled the test result to be visualized under UV-light. LAMP assay may be performed with a minimum of laboratory equipment and supplies. LAMP technique has been demonstrated to be a simple, efficient, and sensitive tool for the detection, identification, and differentiation of *P. syringae* pathovars (Li et al. 2009b).

It has been observed that *Xanthomonas campestris* pv. *campestris* (*Xcc*) did not develop on nutrient-rich medium when inoculated cabbage seeds were plated after washing and soaking. Plantlets developed symptoms at 7–14 days after germination, indicating that not all cells of *Xcc* were able to grow on the medium. A technique designated seed-qPCR was therefore developed for the detection of living cells of *Xcc* present in seed lots. This procedure, based on an enrichment of pathogen population associated with infected seeds following germination coupled with real-time TaqMan PCR after extraction of target DNA, was employed. This technique allowed detection of one contaminated seed among 10,000 healthy seeds. This seed-qPCR procedure combined an efficient extraction based on bacterial multiplication on seedlings with sensitive qPCR

format for the detection of *X. campestris* pv. *campestris* in seed lots (Laala et al. 2015). A real-time PCR procedure was developed for specific detection and quantification of “*Candidatus Liberibacter solanacearum*” in carrot seeds. The pathogen was detected in 23 of 54 carrot seed lots during 2010–2014, including seeds collected from infected plants. Although the population of “*Ca. L. solanacearum*” in individual seed varied from 4.8 ± 3.3 to 210 ± 6.7 cells in three lots, 95% of the pathogen cells were dead as indicated by propidium monoazide staining assay. Presence of Liberibacter-like cells was observed in phloem sieve tubes of the seed coat and in the phloem of carrot midrib from seedlings. Real-time PCR assay detected the pathogen as early as 30 days post-germination, but more consistently after 90 days in seedlings in PCR-positive seed lots in an insect-proof P2-level containment greenhouse. Seedlings were infected to an extent of 12–42%, as revealed by the assay. “*Ca. L. solanacearum*” haplotype E was identified in seeds and seedlings of cultivar Maestro. Use of disease-free seed was suggested as an important disease management strategy for reducing infection by “*Ca. L. solanacearum*” in carrot (Bertolini et al. 2015).

5.1.5.5 Integration of PCR with Other Detection Methods

Polymerase chain reaction (PCR) assay using pathogen-specific primers targeting selected DNA sequences has been widely employed for the detection and identification of several microbial plant pathogens, including bacterial pathogens. However, it requires time-consuming, cost-adding procedures such as Southern blots to detect a small population of pathogens in some seed extracts which contain inhibitors of PCR. Further, the PCR assay may not differentiate live and dead cells of bacterial pathogens. It is therefore likely that false-positive results may be obtained, making it difficult for use in phytosanitary applications. BIO-PCR and immunomagnetic separation (IMS-) PCR assays have been shown to significantly enhance the sensitivity of detection of bacterial pathogens present in seed wash/macerate and other plant tissues. The BIO-PCR assay integrates the agar plating as a preliminary step, depending on the biological characteristics of the pathogen, with the enzymatic amplification of PCR targets in the seed extracts. Aliquots of a seed extract are first placed onto pooled washings from the plates and used for direct nested PCR assay for the detection in bean seeds of *Pseudomonas syringae* pv. *phaseolicola* (*Psp*), causative agent of halo blight disease. The PCR technique effectively amplified a segment of the genomic phaseolotoxin gene cluster. Two consecutive rounds of PCR were conducted without prior DNA extraction, using nested pairs of primers designed to amplify a segment of *tox* gene (phaseolotoxin) region of *Psp*. Both standard and nested PCR formats yielded a single discrete product band (0.5 kb with the external primers and 0.45 kb with internal primers). The detection threshold for serially diluted cells was 1200 cells for the standard PCR format with external primers and 1–2 cells for the nested PCR format, based on Southern hybridization. For purified genomic DNA of *Psp*, the detection thresholds were 250 pg with external primers and 0.1 pg (2500-fold lower) with the nested PCR primers, based on EtBr (ethidium bromide) staining and Southern hybridization (Fig. 5.8). The elimination of false positives because of the presence of dead cells of *Psp* in the seed and false negatives due to potential inhibitors in seed extracts increased the sensitivity of detection; this increased sensitivity and nonrequirement of DNA extraction prior to PCR amplification are the advantages of BIO-PCR assay over the standard PCR format for the detection of *P. syringae* pv. *phaseolicola* in bean seeds (Schaad et al. 1995). A modified BIO-PCR protocol, allowing *Psp* in the bean seed extract to multiply in a semisolid

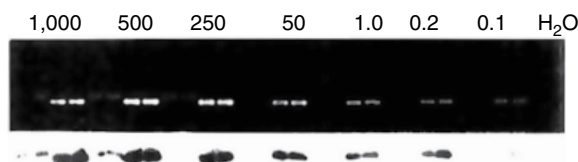


Figure 5.8 Determination of detection threshold of *Pseudomonas phaseolicola* pv. *phaseolicola*, using standard PCR and nested PCR formats. Nested PCR assay was more sensitive than standard PCR assay. Ethidium bromide-stained bands (white on black background, upper panel) and bands detected by Southern hybridization (black bands on white background, lower panel) represent pathogen DNA concentrations in a descending order; two left lanes in each group correspond to duplicate PCR reactions with external primers and two right lanes to duplicate reactions with nested primers. Source: Schaad et al. (1995). Reproduced with permission of the American Phytopathological Society, MN, USA.

medium for 18 h prior to PCR amplification, was more sensitive in detecting *P. syringae* pv. *phaseolicola* (Mosqueda-Cano and Herrera-Estrella 1997).

A high-throughput 96-well membrane BIO-PCR technique was developed to increase the sensitivity and to reduce the labor required for BIO-PCR assay used earlier for the detection of *Pseudomonas syringae* pv. *phaseolicola*, the halo blight pathogen in bean seeds and plants. Available classical PCR primers and newly designed real-time primers and probes were employed for the ultra-sensitive detection of *Psp* in washings of seeds and leaves of bean. The primers and probe, designed from a *tox-argK* chromosomal cluster of the *Psp*-specific phaseolotoxin gene, were confirmed to be specific to *Psp*. Samples (1–2 mL) were filtered under vacuum in 96-well membrane plates. After incubating on soft agar medium for 48–52 h, each well was washed with sterile water and used immediately for nested (two-step) PCR or real-time PCR assays. The standard PCR assay could not detect the pathogen at mean concentrations of 40 CFU mL⁻¹. The BIO-PCR detected *Psp* in five of six samples at 40 mean CFU mL⁻¹ concentration, but none at the mean concentrations of 4.2 and 0.4 mean CFU mL⁻¹. On the other hand, membrane BIO-PCR format could detect *Psp* in all six samples tested containing as few as 0.4 mean CFU mL⁻¹. The BIO-PCR and standard PCR formats gave negative results for all three levels of inoculum, whereas membrane BIO-PCR format provided positive results for all three samples at a mean concentration of 80 CFU mL⁻¹ and one of three at 40 mean CFU mL⁻¹, revealing higher level of sensitivity of the membrane BIO-PCR assay (Table 5.6). As the halo blight pathogen *P. syringae* pv. *phaseolicola* has a zero-tolerance limit for the infection of bean seed lots, the application of highly sensitive methods such as membrane BIO-PCR technique might be the technique of choice to avoid rejection of seed consignments (Schaad et al. 2007; Section 5.4.8).

Tomato seeds infected by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causing the bacterial canker disease, form the primary sources of inoculum for disease incidence and spread. For the detection of *Cmm* in tomato seeds, agar plating assay, direct PCR assay, and BIO-PCR format were tested using 10,000 seed samples containing one infected seed. The pathogen could be detected in these seed samples only by the BIO-PCR assay, but not by agar plating and direct PCR methods, indicating the higher level of sensitivity of BIO-PCR format. A high correlation ($r^2=0.9448$) was recorded between seed contamination level and disease rating. Seed lots infested with less than 58 CFU g⁻¹ did not cause disease. Seed lots infested with about 1000 CFU g⁻¹ resulted in 78 diseased tomato seedlings out of 2000 seedlings observed. *Cmm* was detected in all

Table 5.6 Comparative sensitivity of PCR formats determined by using different CFUs of *Pseudomonas syringae* pv. *phaseolicola* (Schaad et al. 2007).

PCR format ^b	Treatments ^a				
	Negative control	910 CFU	26 CFU	2.0 CFU	<1.0 CFU
Direct PCR					
Real-time	—	—	—	—	—
Nested	—	+	—	—	—
BIO-PCR					
Real-time	—	+	—	—	—
Nested	—	+	+	—	—
Membrane BIO-PCR					
Real-time	—	+	—	+	—
Nested	—	+	—	+	+

^a Seed washings containing different populations of *P. syringae* pv. *phaseolicola* expressed as CFU mL⁻¹; ^b PCR formats tested for their efficiency for detecting the pathogen present in different concentrations in seed washings

diseased seedlings (Hadas et al. 2005). Bacterial wilt disease of alfalfa caused by *Clavibacter michiganensis* subsp. *insidiosus* (*Cmi*) spreads to new production areas mainly via infested seed. A conventional PCR format targeting the internal transcribed spacer region of the *rrn* operon and a real-time PCR assay based on published primers targeting a genomic insertion element were evaluated for their effectiveness for detection and identification of *Cmi* in alfalfa seed. The real-time TaqMan BIO-PCR assay and subsequent confirmation of amplicon identity by melting peak analysis was the most useful for routine diagnosis of infection by *C. michiganensis* subsp. *insidiosus*. The PCR-positive samples were tested by bioassay on alfalfa seedlings for confirmation of seed infection by the pathogen (Ward et al. 2008).

Rice seeds contaminated with *Acidovorax avenae* subsp. *avenae* (*Aaa*), causative agent of rice bacterial stripe disease, form important primary sources of inoculum and a means of dissemination of the disease to new locations. A semiselective liquid medium based on D-sorbitol and L-pyrogutamic acid (SP medium) was employed for isolating *Aaa* and two sets of PCR primers were designed for use in BIO-PCR assay for detection of *Aaa* in rice seeds. An external primer set (Aaaf3 and Aaar2) and an internal primer set (Aaaf5 and Aaar2), designed from a 619 bp fragment of the internal transcribed spacer region of the 16S-23S rDNA of *Aaa* strain CAa4, were specific at the subspecies level (Fig. 5.9). A nested PCR assay produced the expected DNA product from 58 rice strains tested, but not with DNA from 27 strains of *A. avenae* subsp. *avenae* from corn and other hosts. In addition, the specificity of the primers was revealed by the lack of amplification of DNA from 10 strains of *A. avenae* subsp. *Citrulli*, two strains of *A. avenae* subsp. *cattleyae*, or 64 other bacterial species tested. When *A. avenae* subsp. *avenae* was enriched prior to PCR amplification by incubating washings of naturally contaminated rice seeds for 12 h in SP liquid medium, populations of the pathogen increased by over 1000-fold whereas populations of saprophytic bacteria remained the same. Populations

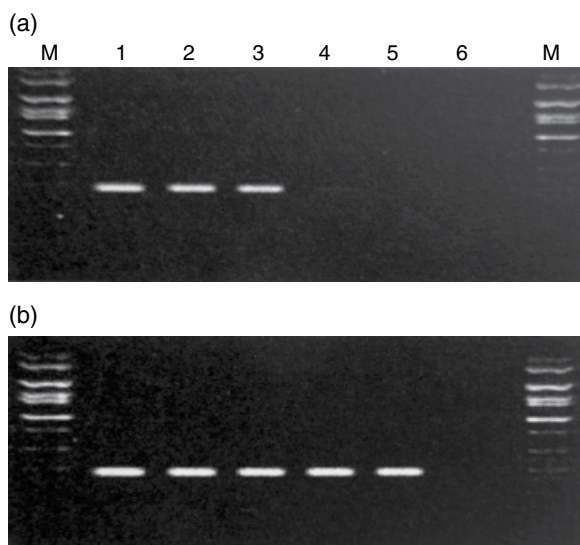


Figure 5.9 Detection of *Acidovorax avenae* subsp. *avenae* by nested PCR assay in spiked rice seed samples (a) without enrichment and (b) with enrichment in SP liquid medium. Note the presence of amplified product of 224 bp from pathogen DNA at different concentrations. Lane 1: $(1-2) \times 10^4$; lane 2: $(1-2) \times 10^3$; lane 3: $(1-2) \times 10^2$; lane 4: 10–20; lane 5: 1–2 CFU g^{-1} of seeds; lane 6: uninoculated seed extract. Source: Song et al. (2004). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

of the target bacterium routinely reached PCR-detectable levels after seed samples containing as few as 1–2 target cells and up to 10^4 CFU of other bacteria were soaked overnight in phosphate buffer and then enriched for 12 h in SP liquid medium. Assays of 64 naturally infected rice seed samples resulted in 53 being positive by BIO-PCR assay and 34 by standard PCR format. The BIO-PCR assay was found to be a sensitive, reliable tool for specific detection of *A. avenae* subsp. *avenae* in rice seeds. In addition, BIO-PCR assay might be useful for monitoring the natural spread of the pathogen, tracking the pathogen for epidemiological investigations and detecting waterborne or airborne bacterial cells caught on filters. The BIO-PCR assay could be completed within 16–18 h, including sampling and a 12-h enrichment step, by using a real-time PCR and portable rapid cycling PCR platforms (Song et al. 2004).

A combined agar plating, real-time PCR assay was developed for detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*), causal agent of bacterial seedling blight and fruit blotch disease of watermelon and other cucurbits. The bacteria were extracted by soaking seeds in buffer containing vancomycin and assayed by dilution plating onto semiselective ethanol bromocresol purple/brilliant blue R (EBB) agar and onto EBB agar containing ampicillin (EBBA). Direct real-time PCR and real-time BIO-PCR, using EBBA agar, detected *Aac* in extracts containing as few as 1 cell mL^{-1} . All assays including plating onto EBB and EBBA agar, direct PCR, and BIO-PCR formats successfully detected *A. avenae* subsp. *citrulli* in seed samples, each consisting of 1000 healthy seeds and one naturally infected seed. However, combined agar-plating–real-time PCR format showed potential for use as a routine procedure for detection of *A. avenae* subsp. *citrulli* in watermelon and melon seeds, even when the seed samples contained high levels of saprophytic bacteria (Zhao et al. 2009).

Burkholderia glumae, causative agent of rice bacterial grain rot/panicle blight disease, is responsible for significant yield losses in Korea, Japan, USA and other rice-growing countries. As the use of disease-free seed was considered as the only practical method of managing the grain rot disease, a reliable, quick, and accurate quantitative procedure had to be developed to assess the extent of seed infestation by *B. glumae*. An SYBR Green real-time PCR primer set BG1F/R was designed based on the *rhs* family gene (YD repeat protein) of *B. glumae* BGR1, as these genes were structurally diverse. The primer set amplified a 138 bp DNA fragment in the conventional PCR format against the five isolates of *B. glumae*. A large collection of other bacterial species including other *Burkholderia* spp. was tested by SYBR Green real-time PCR to check the specificity of the primer set. The expected DNA fragment was amplified only from the isolates of *B. glumae*, revealing the primer specificity. Naturally infected seed lots were tested by BIO-PCR assay. The specific amplicon (138 bp DNA) was detected by SYBR Green real-time BIO-PCR assay in the infected rice samples, but not in the healthy control seed samples. The primer set developed through the bioinformatics method was highly specific to *B. glumae* and the procedure was reliable and rapid for the detection of the pathogen *B. glumae* and diagnosis of rice grain rot disease (Kim et al. 2012).

Xanthomonas campestris pv. *campestris* (*Xcc*), a seedborne pathogen, causes black rot disease of cruciferous crops and has a worldwide distribution, accounting for about 50% losses under conditions favorable for disease development and spread. In order to detect the pathogen in seeds and seedlings a BIO-PCR format was employed, using the primer set Dhrrp_Xcc_F/DhrrpXccR, which amplified a 769 bp DNA fragment of *Xcc*. The primers were highly sensitive and capable of detecting $0.1 \text{ ng } \mu\text{L}^{-1}$ of bacterial DNA. The BIO-PCR assay detected the pathogen in 94% of the 136 black-rot-infected samples of cauliflower and cabbage plants collected from different states of India. Cauliflower seeds were contaminated by dipping in the broth culture containing 10^9 CFU mL^{-1} for 2.5 or 12 h to obtain different levels of contamination ranging from 0.01 to 100%. *X. campestris* pv. *campestris* could be detected by using BIO-PCR assay up to 0.01% contaminated seeds, whereas the conventional agar plating technique was able to detect the pathogen only up to 1.0% of seed samples dipped for 12 h in broth culture. The results indicated clearly the higher levels of effectiveness and sensitivity of the BIO-PCR assay for the detection of *X. campestris* pv. *campestris* present in seeds of cauliflower (Singh et al. 2014).

The sensitivity of detection and reliability of identification of bacterial pathogen have been improved by integrating the PCR assay with other methods, depending on the biological and immunological properties of the target pathogen. Immunomagnetic separation and polymerase chain reaction (IMS-PCR) were combined for improving the sensitivity of detection of *Acidovorax avenae* subsp. *citulli* (*Aac*), the causative agent of bacterial fruit blotch (BFB) disease infecting watermelon and other cucurbits. The antibodies are covalently attached to the magnetic beads which bind target bacterial cells by specific antibody–antigen interactions during incubation with cell suspensions. The immunomagnetic bead (IMB-) bacterial complexes are held together by a magnetic force, while nontarget cells and inhibitory compounds are removed by rinsing with buffer solutions. Bound cells remain viable and can be recovered on semiselective media. Super-paramagnetic beads precoated with sheep antirabbit antibodies were coated with purified IgG fractions of anti-Aac, as per manufacturer's recommendations. Immunomagnetic separation of *Acidovorax avenae* subsp. *citulli* (*Aac*) from the suspension yielded a 10-fold increase in the recovery of the pathogen over direct spread

plating on King's medium B. Synthetic oligonucleotide primers were designed based on the 16S rRNA gene of *A. avenae* subsp. *citrulli* and tested for specific DNA amplification by PCR assay. The primers amplified DNA from all *Aac* strains and also from closely related bacterial species. IMS-PCR combination provided a 100-fold increase in the sensitivity of *Aac* detection over standard PCR format. Further, IMS-PCR format was not affected by PCR inhibitors present in the watermelon seeds. The threshold of detection of *Aac* by IMS-PCR format was 10 CFU mL^{-1} in watermelon seed wash and seed lots with 0.1% infestation could be located consistently. Further, IMS-PCR assay could detect *Aac* in 100% of seed lots with 10% infestation, while seed lots with 5% and 0.1% infestation showed positive reaction in 80% of the tests. Seed treatment with fungicides had no influence on the assessment of seed infestation by IMS-PCR assay. Despite the possibility of improving the sensitivity of pathogen detection by IMS, the results indicated that the assay reliability might be reduced as the pathogen population decreased. IMS-PCR must be validated using naturally infested seed lots before it is adopted for large-scale field application (Walcott and Gitaitis 2000).

The influence of blossom inoculation in watermelon with *Acidovorax avenae* subsp. *citrulli* (*Aac*) was investigated. The majority of fruits (98%) developed from inoculated blossoms with 1×10^7 or 1×10^9 CFU of the pathogen per blossom were asymptomatic. By employing IMS-PCR assay, infection by *Aac* of 44% of seed lots from the asymptomatic fruits was detected. Viable colonies were recovered from 31% of the seed lots on semisynthetic medium. *A. avenae* subsp. *citrulli* was detected and recovered from the pulp of 33% and 19% of symptomless watermelon fruit whose blossom were inoculated with *Aac*. IMS followed by plating on agar medium revealed a positive relationship between pulp and seed infestation by *A. avenae* subsp. *citrulli*. IMS-PCR assay was useful to establish the role of watermelon blossoms as a potential site of ingress of fruit and seed infestation by *A. avenae* subsp. *citrulli* (Walcott et al. 2003).

A commercial-scale PCR-based assay for detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*) in watermelon seed was developed by optimizing parameters for immunomagnetic separation (IMS). Optimal conditions for target cell recovery included $40 \mu\text{g}$ of polyclonal anti-*Aac* antibody per 10^8 immunomagnetic beads (IMBs) for coating c. 2.5×10^7 IMBs/sample and immunocapture for 1 h at 4°C . These parameters consistently enhanced the recovery of *Aac* cells from suspensions containing c. 10 CFU mL^{-1} . Using lots contaminated with artificially infested seeds (10^4 *Aac* CFU/seed), IMS-PCR assay detected *Aac* in 25% and 87.5% of samples ($n = 10,000$ seeds) with 0.01 and 0.1% infestation, respectively. Detection frequencies for seedling grow-out assay (SGO) were 12.5% and 37.5% for seed lots with similar infestation levels. However, the differences in the detection frequencies of SGO and IMS-PCR were not statistically significant. Liquid enrichment improved the detection sensitivity of IMS-PCR assay by 100-fold, but the difference in detection frequency between enrichment IMS-PCR and IMS-PCR was not statistically significant. The IMS-PCR might be an effective alternative to SGO, which requires a long time to provide results of detection of *A. avenae* subsp. *citrulli* (Walcott et al. 2006). Another bacterial pathogen *Pantoea ananatis*, causal agent of center rot of onion, was also detected efficiently from the seeds by using IMS-PCR protocol. Species-specific primers and PABs were employed in this assay with a detection limit of $10\text{--}10^3 \text{ CFU mL}^{-1}$ of seed wash (Walcott et al. 2002).

Immunomagnetic separation (IMS-) PCR assay approach was developed for the detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*) in seeds of watermelon. New species-specific primers were designed based on PCR fragments obtained in ERIC- and

BOX-profiles of *Aac* strains. The sequence specificity of the BX and BX-S primers allowed detection of all *Aac* strains (30) tested from various locations and from different hosts, but did not amplify DNA from closely related species and subspecies, with the exception of DNA of one strain of *A. avenae* subsp. *avenae* which was amplified by one of the primer sets, named BX-S. This primer set, based on a BOX-PCR fragment, performed under highly stringent conditions without losing its detection sensitivity. The applicability of the BX-S primer set for IMS-PCR assay was assessed and compared to that of the *Aac* primer set (AACF2/R3). The BX-S set detected contaminated seeds in three independent trials in which two levels of infestation (0.1% and 0.02%) were used. The results obtained with the BX-S set were comparable to those of *Aac* set (Table 5.7), while no amplification occurred in control seed lots. Both primer sets used in IMS-PCR facilitated detection of the pathogen in seed lots with 0.1% and 0.02% infestation. For seed lots with 0.02% infestation, the two primer sets displayed similar levels of sensitivity as indicated by mean Ct values of 27.9 and 27.3 for BX-S and *Aac* primer sets, respectively. The BX-S set was 100-fold more sensitive than the *Aac* primers, although a lower annealing temperature was used with the latter. The primers were evaluated for their ability to detect *A. avenae* subsp. *citrulli* in contaminated watermelon and melon seed samples. The BX-S primers detected the pathogen efficiently in seed washing of 5000-seed samples with 0.02% infestation. The results indicated that BX-S primer set might improve IMS real-time PCR detection of *A. avenae* subsp. *citrulli* in naturally infested watermelon seeds. As the BX-S primer set has not yet been patented, it can be integrated into diagnosis by industry without any legal limitation (Bahar et al. 2008; Section 5.4.9).

Immunocapture (IC-) PCR technique is based on the combination of the ability of antibodies to capture target bacterial pathogen and the sensitivity and specificity of the

Table 5.7 Comparative efficacy of the BX-S and *Aac* primer sets in IMS-real-time PCR assay for the detection of *Acidovorax avenae* ssp. *citrulli* (*Aac*) in seed lots of watermelon with different levels of infestation (Bahar et al. 2008).

Real-time PCR threshold value	<i>A. avenae</i> ssp. <i>citrulli</i> seed infestation (%)			
	0 (0/5000)	0.02 (1/5000)	0.1 (5/5000)	Positive control ^a
BX-L1 and BX-S-R2 primers				
Rep 1	0.00	32.31	27.89	30.44
Rep 2	0.00	21.89	21.79	31.21
Rep 3	0.00	29.49	27.84	ND ^b
Mean	0.00	27.89	25.84	30.82
<i>Aac</i> 3 and <i>Aac</i> 2 primers				
Rep 1	0.00	29.18	29.50	ND
Rep 2	0.00	24.71	24.68	ND
Rep 3	0.00	27.97	27.70	33.66
Mean	0.00	27.28	27.29	33.66

^a Tested on a cell suspension containing a population of 10^3 cells CFU mL⁻¹ of *Aac*; ^b ND: not determined

PCR primers for amplification of the DNA fragment of the captured pathogen. The IC-PCR format was developed for the detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*), the incitant of bacterial fruit blotch (BFB) disease of watermelon, using the primers WFB1 and WFB2. The PCR amplification product of 360bp DNA fragment was detected only in four strains of seven different genera by both direct PCR and IC-PCR formats. The detection thresholds of direct PCR and IC-PCR assays were about 10^3 – 10^4 CFU mL⁻¹ and 10^1 – 10^2 CFU mL⁻¹, respectively, indicating that IC-PCR format was 100 times more sensitive than direct PCR assay. The minimum concentration of *Aac* from seed washes that could be detected by IC-PCR assay was about 5×10^1 – 1×10^2 CFU mL⁻¹. IC-PCR assay could detect the pathogen in one batch of melon seeds in about 8 h with great accuracy and at low cost per test. Seven batches of different melon seeds from the market were indexed by IC-PCR. One cantaloupe, two honeydew melon, and two watermelon seed varieties showed infection by *A. avenae* subsp. *citrulli*. The results of grow-out test were closely related to those of IC-PCR assay which was found to be accurate, sensitive, and rapid (Xiao et al. 2007; Section 5.4.10).

Magnetic capture hybridization (MCH) was combined with multiplex real-time PCR technique for simultaneous detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*) and *Didymella bryoniae*, incitant of gummy stem blight disease in cucurbit seed samples. Sequestered template DNAs were subsequently amplified by multiplex real-time PCR format, using pathogen-specific TaqMan PCR assays. Detection thresholds of 10 CFU mL⁻¹ for *Aac* and 10^5 conidia mL⁻¹ for *D. bryoniae* could be attained in mixtures of pure cultures of the two pathogens, representing a 10-fold increase in sensitivity over the direct real-time PCR assays for these two pathogens separately. The comparative frequencies of detection of *Aac* for direct real-time PCR assay and MCH real-time PCR formats were 25% (1 of 4 samples) and 100% (4 of 4 samples), respectively, at the same DNA concentrations. However, MCH did not improve detection sensitivity for *D. bryoniae* relative to the direct real-time PCR format, using conidial suspensions or seed washes from *D. bryoniae*-infested cucurbit seed. MCH real-time PCR procedure could be effectively applied for the detection of pathogens present in watermelon and melon seed samples ($n=5000$ seeds/sample) in which 0.02% of the seeds were infested with the bacterial pathogen *A. avenae* subsp. *citrulli* and 0.02% by the fungal pathogen *D. bryoniae* (Ha et al. 2009).

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) and *Pepino mosaic virus* (PepMV) infecting tomatoes are important quarantine pathogens. These bacterial and viral pathogens could be introduced into tomato transplant houses and fields on infested seeds, resulting in substantial yield losses due to diseases induced by them. To overcome the necessity of using separate detection methods for these pathogens, the effectiveness of the combination of magnetic capture hybridization (MCH) step to concentrate and purify the target nucleic acids of the pathogen(s) prior to real-time PCR assay was evaluated for the detection of both *Cmm* and PepMV in tomato seeds. The combination of MCH with multiplex real-time PCR resulted in a 10^2 – 10^3 -fold increase in detection sensitivity for both pathogens compared to DNA extraction and direct multiplex real-time PCR format. The detection threshold for the MCH multiplex PCR assay was a combination of 10^5 *Cmm* cells mL⁻¹ plus a 10^4 -fold dilution of total RNA extracted from PepMV-infected tomato leaf tissue. The results indicated that MCH could facilitate the simultaneous detection of *C. michiganensis* subsp. *michiganensis* and *Pepino mosaic virus* by multiplex real-time PCR format (Johnson and Walcott 2012).

Infested cucurbit seed lots form the most important sources of primary inoculum for the spread of bacterial fruit blotch (BFB) disease caused by *Acidovorax avenae* subsp. *citrulli* (*Aac*) (= *Acidovorax citrulli*). The conventional isolation method, seedling grow-out tests, immunoassays, and polymerase chain reaction (PCR-) based techniques were found to be time-consuming and variable in reliability, sensitivity, and consistency; they sometimes also gave false-negative results or -positive results. As effective management of BFB disease relies on effective detection technique with required level of sensitivity, rapidity, and reliability, a diagnostic procedure based on padlock probes (PLPs) was developed for the detection of *Aac* in watermelon, melon, and cantaloupe seeds. The PLPs are long oligonucleotides (approximately 100 bp) whose ends are complementary to sequences adjacent to the site targeted for PCR amplification. Upon hybridization to the target, the 5' end and 3' end of PLPs are brought into contact allowing circularization by ligation, which is possible only when both flanks hybridize to their target sequences correctly. PLPs provide extremely specific target sequence recognition which is followed by universal amplification and detection by microarray analysis. PLPs combined with universal PCR amplification and microarray detection may be applied for successful detection of several microbial plant pathogens, including bacterial plant pathogens (Tsui et al. 2010).

In a later investigation, a method using padlock probe (PLP) and dot-blot hybridization was developed for the detection of *Acidovorax citrulli* (= *A. avenae* subsp. *citrulli*), infecting cucurbits. The PLP was designed based on the 16S-23S ITS rDNA sequence from *A. citrulli*. Strains (75) of the pathogen from six countries around the world were detected by PLP assay and it did not cross-react with closely related *A. avenae*. Ligation to the PLP-AC did not occur even with high concentration (100 ng) of *A. avenae* genomic DNA, indicating the high specificity of the PLP. The PLP assay detected the target DNA in solutions containing 1 pg of genomic DNA by agarose gel electrophoresis and 100 fg of genomic DNA by dot-blot hybridization. Conventional PCR format failed to detect *A. citrulli* in artificially infested cantaloupe seed with varying levels of inoculum. In contrast, the PLP assay could detect the pathogen in 100% of the seed lots with 10, 5, 1, and 0.1% infestation levels. In the naturally infested seed lots, the PLP assay detected *A. citrulli* in 4 of 8 cantaloupe seed lots and 15 of 47 watermelon seed lots. Isolation of *A. citrulli* in seed wash from PLP positive seed lots confirmed the results of PLP assay. On the other hand, conventional PCR format with WFB1 and WFB2 primers detected *A. citrulli* only in 1 of 8 cantaloupe and 5 of 47 watermelon seed lots. The results revealed the higher levels of sensitivity and specificity of PLP assay relative to the conventional PCR format for the detection of *A. citrulli* in watermelon and cantaloupe seed lots. The PLP assay required less time (13–19 h) to test seed lots (1000/sample). The combination of PLP and dot-blot hybridization provided more effective and sensitive detection of *A. citrulli* (Tian et al. 2013).

For the detection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (causal agent of rice bacterial leaf blight) and *X. oryzae* pv. *oryzicola* (incitant of rice bacterial leaf streak), two padlock probes (PLPs), P-*Xoo* and P-*Xoc*, were designed based on the 16S-23S internal spacer ribosomal DNA (rDNA) sequences. These PLPs were combined with dot-blot hybridization to detect the bacterial pathogens individually in rice seed. The detection sensitivity of this technique was 1.0 pg of *X. oryzae* pv. *oryzae* genomic DNA. This technique was also effective for the detection of *X. oryzae* pv. *oryzae* in rice seed lots with 2% artificially infested seed. It was possible to detect *X. oryzae* pv. *oryzicola* with a detection threshold of 1.0 pg of pathogen genomic DNA and in seeds spiked with 0.2%

artificially infested seed. The PLP assays detected *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in 39.3% and 21.3% of naturally infested commercial rice seed lots, respectively. On the other hand, standard PCR format using OSF1/OSR1 and XocF/XocR primer sets detected *X. oryzae* pv. *oryzae* in 9.1% and *X. oryzae* pv. *oryzicola* in 8.5% of the same rice seed lots tested with PLP assays, revealing the greater sensitivity of PLP assay. Both pathogens could be detected simultaneously by PLP assays in two seed lots, indicating the ability of PLP probes to detect both pathogens efficiently in rice seed lots. The results showed the usefulness of PLPs combined with reverse dot-blot hybridization for detecting multiple pathogens simultaneously (Tian et al. 2014).

5.1.5.6 Assessment of Genetic Diversity of Bacterial Pathogens

Detection, identification, and differentiation of bacterial plant pathogens is vital to understand the importance of the existence of a pathogen and its variants whose pathogenic potential varies, resulting in different magnitudes of yield losses in quantity and quality. Nucleic acid-based techniques have been employed to detect and differentiate several bacterial pathogens with greater accuracy, sensitivity, and reliability. Restriction fragment length polymorphism (RFLP), PCR either alone or in combination with random amplified fragment length polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) methods, have been demonstrated to be useful for the differentiation of strains of bacterial plant pathogens. Restriction endonucleases have the ability to cleave bacterial DNA at specific sites, resulting in different number of DNA fragments which migrate to different distance in gels depending on their size, forming a characteristic pattern for the target bacterial species/subspecies/strains. The RFLP patterns generated by digestion with *Pst* restriction enzyme for *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains of race 2 frequently observed in the Philippines formed the basis of differentiation of the strains (Leach and White 1991). *Burkholderia glumae*, *B. gladioli*, and *B. vandii*, infecting rice panicles and grains, could be differentiated based on their RFLP patterns generated, following digestion of genomic DNA with specific restriction enzymes (Ura et al. 1998).

Random amplified polymorphic DNA (RAPD) technique has been shown to be useful for the differentiation and establishment of relationship between bacterial pathogen species and strains/pathovars. The detection of polymorphism throughout the entire bacterial genome may be more useful for differentiation of bacterial pathogens than other procedures. RAPD markers are useful for the reliable strain differentiation and identification, in addition to other genetic analysis of microbial plant pathogens. *Erwinia* spp. cause soft rot diseases of vegetable crops such as potato, cabbage, cucumber, and tomato. Pepper (*Capsicum annuum*) fruits are infected by *E. carotovora* subsp. *carotovora* (*Ecc*). A significant correlation was observed between the level of *Ecc* on pepper seeds and percentage of diseased seedlings (Fig. 5.10). Surface sterilization of pepper seeds with sodium hypochlorite eliminated the pathogen, indicating that *Ecc* was located on the seed surface. As the pathogen infected several vegetable crops, isolates of *Ecc* from different host species were subjected to RAPD-PCR analysis. DNA fingerprints were generated from total chromosomal DNA extracted from 14 isolates of *Ecc*. Twenty-six different primers were screened with DNA obtained from eight isolates (two each from potato, tomato, pepper, and cabbage). Primers (A2, A4, B1, C2, C12, C19, G10, and G17 from operon and CGTAGTGGTG) generated reproducible banding patterns. These primers were selected for cluster analysis of all isolates and produced 150 distinct bands, demonstrating polymorphism among various isolates. RAPD

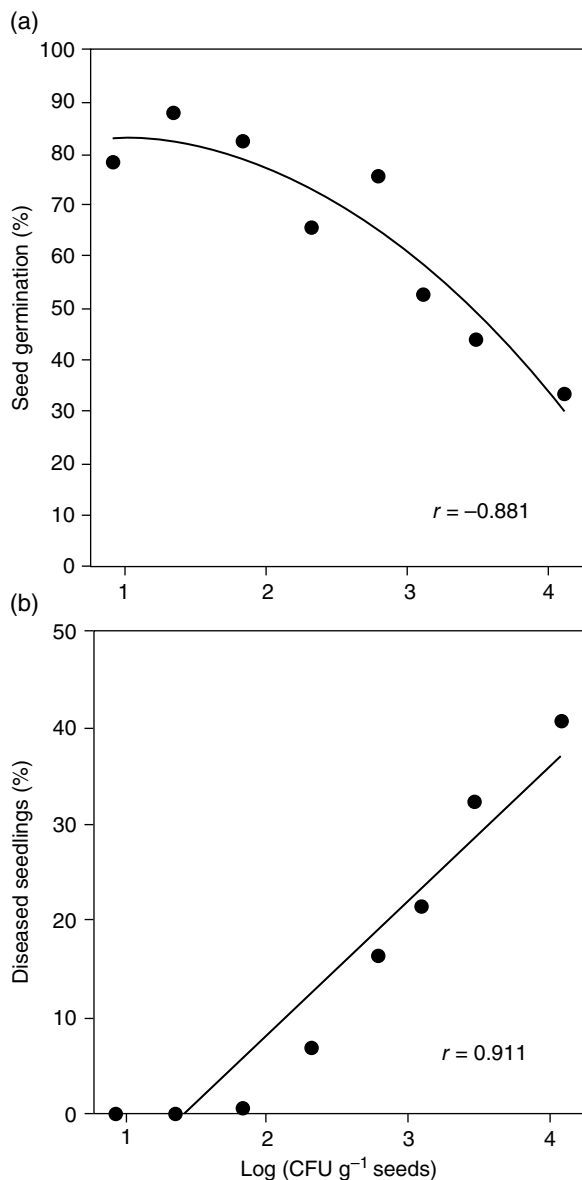


Figure 5.10 (a) Relationship between populations of pectolytic erwinias and seed germination (negative) and (b) relationship between erwinia populations and disease incidence (positive). Source: Hadas et al. (2001). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

analysis with nine primers revealed that each primer could distinguish between *Ecc* from pepper and isolates from other hosts. The isolates of *E. carotovora* subsp. *carotovora* clustered into four distinct groups and these groups correlated with different host origins (Hadas et al. 2001).

Strains of bacterial pathogens belonging to the genus *Xanthomonas* have been precisely identified and effectively differentiated by employing RAPD technique. Various

strains of *X. oryzae* pv. *oryzae* (*Xoo*), causative agent of rice bacterial leaf blight (BLB) disease, were isolated from different rice-growing regions. *Xoo* isolates (16) from different locations in India were analyzed by RAPD procedure. Seven primers OPA-03, OPA-04, OPA-10, OPA-11, OPK-7, OPK-12, and OPK-17 generated simple, specific, and reproducible fingerprints from genomic DNAs of the isolates tested. However, *Xoo* isolates could not be grouped using these primers. Primers PJEL1 and PJEL2 with an insertion element IS112 occurring in relatively high copy number (about 89 copies) were employed to generate fingerprint patterns for the same set of *Xoo* isolates. Combination of the RAPD-PCR (seven primers) and IS112-PCR (two primers) resulted in the production of distinguishable fingerprints. Five groups of *Xoo* strains were recognized based on these fingerprints. The results indicated that RAPD-PCR technique might be used for rapid identification of *Xoo* isolates and assessment of genetic variation in the populations from different locations in India (Gupta et al. 2001).

The isolates of *X. oryzae* pv. *oryzae* were classified into seven pathotypes (PbX0-1 to PbX0-7) based on their reactions on a set of near-isogenic lines in the background of IR24, international, national, and regional cultivars. Molecular profiles of the pathotypes were prepared using RAPD and IS112-based PCR which generated specific and reproducible fingerprints. The primers S1117, S1112, S1109, S1106, and PJEL were more efficient in differentiating the pathotypes. The pathotypes PbX0-1 and PbX0-2 were placed in the same group using a similarity of 0.5 as the basis. The other five pathotypes showed different lineage. RAPD-PCR and IS112-based PCR analysis provided data that were useful for generating unique DNA fingerprints specific for different pathotypes, facilitating rapid assessment of genetic variation in the pathogen population (Lore et al. 2011).

Strains of *Xanthomonas oryzae* pv. *oryzicola*, causative agent of rice bacterial leaf streak (BLS), were isolated from different rice-growing areas of Burkino Faso and Mali. *X. oryzae* pv. *oryzicola* strains were evaluated for virulence on rice and high variations in lesion length induced by the strains of the pathogen were noted. *X. oryzae* pv. *oryzicola* strains were further characterized by multilocus sequence analysis (MLSA), using six housekeeping genes. The existence of different groups among *X. oryzae* pv. *oryzicola* was indicated by inferred dendrograms. Restriction fragment length polymorphism (RFLP) analysis, using the transcriptional activator-like effector *avrXa7* as a probe, resulted in the identification of 18 haplotypes. PCR-based analysis of two conserved type III effector (T3E) genes (*xopAJ* and *xopW*) differentiated the strains into distinct groups, with *xopAJ* not detectable in most African *X. oryzae* pv. *oryzicola* strains. The functionality of *xopAJ* was confirmed by leaf infiltration on “Kitaake” rice Rxo lines. Sequence analysis of *xopW* revealed four groups among *X. oryzae* pv. *oryzicola* strains. The distribution of 43 T3E genes showed a variation in a subset of *X. oryzae* pv. *oryzicola* strains. The results indicated that African *X. oryzae* pv. *oryzicola* strains were diverse and rapidly evolving with a group endemic to Africa and another evolving from Asian strain (Wonni et al. 2014).

Amplified fragment length polymorphism (AFLP) technique is useful to assess the genetic diversity of a pathogen population existing in a location and to evaluate the genetic relatedness between the strains of a pathogen. *Xanthomonas axonopodis* pv. *allii* (*Xaa*) is pathogenic to several *Allium* spp., but disease outbreaks worldwide have been primarily recorded on onion (*A. cepa*). Among strains of *Xaa*, no host specialization was observed in strains infecting onion, leek (*A. porrum*), and garlic (*A. sativum*). Based on AFLP and restriction fragment length polymorphism (RFLP) analyses, strains

occurring in Réunion Island were found to be closely related genetically to strains isolated from diseased plant samples and contaminated seed lots in the neighboring island of Mauritius. However, these strains differed from *Xaa* strains from other countries including Barbados, Brazil, and Japan. The strains of Réunion Island were classified into two groups: Group A strains were characterized by four unique markers and contained six haplotypes, whereas Group B strains were characterized by another set of eight unique markers and enclosed nine haplotypes. All AFLP haplotypes were identified as *X. axonopodis* pv. *allii* based on PCR amplification, using specific primers, biochemical tests, and/or pathogenicity tests. Groups A and B were genetically more similar to one another than to other groups, and could not be distinguished based on virulence and rates of multiplication in onion plants. In contrast, these two groups could be clearly distinguished based on AFLP analysis, BIOLOG, and quantitative variations in xanthomonadin pigment which play a role in epiphytic survival by protecting the bacteria against photooxidative damage. *X. axonopodis* pv. *allii* was detected in seed lots imported from Mauritius, indicating the pathway of introduction of the pathogen into Réunion Island (Picard et al. 2008).

Bean bacterial wilt pathogen *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*), a Gram-positive bacterium, causes significant crop losses in Canada. The pathogen was found to be diverse phenotypically and genotypically and in the form of several different color variants. The population structure of 67 *Cff* strains collected between 1957 and 2009, including some isolated from alternative hosts, was investigated using amplified fragment length polymorphism (AFLP), repetitive extragenic palindromic polymerase chain reaction (REP-PCR), and pulsed-field gel electrophoresis (PFGE). All three typing techniques revealed a great degree of population heterogeneity, but they were not congruent in cluster analysis of *Cff* populations. Cluster analysis of a complete dataset (AFLP, PFGE, and REP-PCR) using averages of all experiments yielded two distinct groups: cluster A included strains with colonies of yellow, orange, and pink pigments; and cluster B contained strains of only yellow pigment. Strains producing purple extracellular pigment were found in both clusters. The results showed the phenotypic and genotypic diversity of the strains of *C. flaccumfaciens* pv. *flaccumfaciens* (Agarkova et al. 2012).

5.2 Detection of Bacterial Pathogens in Propagules

Cultivated plants are propagated through true seeds formed after sexual recombination and also through propagules such as tubers, corms, runners, cuttings, and setts. As for infected seeds, when infected by microbial plant pathogens, including bacterial pathogens, the propagules form the primary sources of inoculum from which the disease can spread to different locations and also to subsequent generations. Sensitive methods for the detection and identification of bacterial plant pathogens are therefore required to build a disease-free nuclear stock, forming the foundation of successful crop cultivation and a profitable approach for the growers.

5.2.1 Conventional/Biological Methods

The isolation of bacterial pathogen(s) from infected propagules in appropriate general or semiselective medium is followed for determining morphological, biochemical,

and physiological properties. The putative bacterial pathogen is subject to BIOLOG analysis to determine nutrient utilization profile with which the bacteria may be identified up to genus level. Strains (53) of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), causative agent of potato tuber ring rot disease and collected from different geographical locations, were characterized using API ZYM systems. The *Cms* strains exhibited a high level of homogeneity, both in carbohydrate utilization and in enzymatic activity. The strains of *Cms* could be differentiated from other strains examined in this investigation using API 50CH and API ZYM systems. The strains included in this study were representative strains of other subspecies of *C. michiganensis* as well as other bacterial pathogens infecting potatoes. The API 50CH and API ZYM systems were found to be alternative procedures for the conventional nutritional and physiological identification methods recommended by the European Union for detection and identification of *Cms*. The results obtained with API systems corroborated the taxonomic classification of *C. michiganensis*, clearly separating subsp. *sepedonicus* from other subspecies belonging to this species (Palomo et al. 2006).

Pathogenicity tests are necessary for the ultimate verification of the presumptive pathogen isolated from infected plants. Pathogenicity tests are carried out on sets of differential plant species or cultivars which respond in characteristic patterns to different isolates of the pathogen under investigation. Pathotypes of the bacterial pathogen are identified based on their ability to infect and induce different levels of disease severity on the differential cultivars. The procedures for isolation of bacterial pathogens from propagules are similar to those employed for isolating the bacterial pathogens from seeds, except for the need to use different culture media. Isolation of *Xanthomonas campestris* pv. *manihotis* (*Xcm*) infecting cassava from contaminated planting material was achieved by plating the infested tissues on Cefazolin trehalose agar (CTA) medium with greater success than when a starch-based semiselective medium was used. The CTA medium could also be used for reliable isolation of *Xcm* from asymptomatic plant materials, facilitating effective elimination of latently infected planting materials (Fessehaie et al. 1999). A semiselective medium MMG was consistently useful for the isolation of *X. campestris* pv. *vitians* (*Xcv*), incitant of lettuce bacterial black spot disease, paving the way for reliable identification of the bacterial pathogen (Toussaint et al. 2001).

“*Candidatus Liberibacter asiaticus*,” causing the huanglongbing (earlier known as greening) disease of citrus, is a phloem-limited bacterium and was considered as non-culturable. A new medium designated Liber A was formulated, and the HLB pathogen was successfully cultured in this medium which contained citrus vein extract and a growth factor to support the growth of “*Ca. Liberibacter*” for four single colony transfers. The identity of the isolated bacterium was confirmed by real-time PCR assay and sequencing. The colonies were irregular in shape and convex, reaching a colony diameter of 0.1–0.3 mm after 3–4 days. The presence of fimbriae-like appendages (as in bacteria) was observed under scanning electron microscope. The pathogenicity of two strains of “*Ca. L. asiaticus*” and one of “*Ca. L. americanus*” grown on Liber A medium was proved by inoculating citrus trees and seedlings, followed by reisolation of the causal agent from inoculated citrus plants. The identity of the reisolated agent was confirmed by real-time PCR assay and sequencing. The different steps of Koch’s postulates were carried out to establish the identity of the causal agent of HLB disease for the first time (Sechler et al. 2009).

5.2.2 Phage Typing

Bacteriophages (bacterial viruses) infect specific species/strains of bacterial phytopathogens and cause lysis of susceptible bacterial cells, resulting in the formation of plaques (cleared areas) in the bacterial colonies in the petridishes. Bacteriophages have been isolated from infected plant tissues. Depending on the level of sensitivity to the bacteriophage, lysotypes of bacterial species are identified. The phages CP115 and CP122 isolated from canker lesions caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) on grapefruit and sweet orange, respectively, were employed for the detection and identification of *Xac* (Wu et al. 1993). After excluding the contaminating saprophytic bacteria by passing the cultured bacteria through a membrane (pore size of 0.22 µm) and centrifuging, the effectiveness of detection of *Xac* by specific bacteriophage was significantly increased (Ebisugi et al. 1988). The citrus canker pathogen could be detected in the overwintering canker lesions by employing the phage technique, providing useful information to be transmitted to the growers for initiating necessary measures to check disease incidence and subsequent spread. Phage population detected on symptomless citrus leaves was closely related to the disease incidence (Myung et al. 2001).

Xanthomonas axonopodis pv. *citri* (*Xac*) was classified into two groups, designated lysotype I and lysotype II, based on their sensitivity to the phage. Lysotype I phages were found to be predominant in Korea, as indicated by interaction of the phage CPK with *Xac*. Comparison of the efficacy of the detection of *Xac* by phage technique and enzyme-linked immunosorbent assay (ELISA) showed that both assay methods were equally efficient in detecting the pathogen. However, the phage technique provided a distinct advantage over ELISA test because of its ability to detect and quantify only the living pathogen cells, useful for epidemiological investigations. In addition, the phage technique is rapid, simple, and inexpensive, as the phage lysis zones (plaques) could be counted after 18–24 h of incubation. Because of the effectiveness and simplicity, the phage technique has been practically applied in Korea for the detection of *X. axonopodis* pv. *citri* on Satsuma mandarin fruits meant for exports (Myung et al. 2006).

Bacteriophages (167) isolated from naturally infected citrus canker lesions from diverse locations in Florida and Argentina were characterized for host range, using a worldwide collection of *Xanthomonas citri* pv. *citri* (*Xcc*) strains. These strains showed little diversity. All phages from Argentina (30) had identical host ranges (group ARG), while 37 phages from Florida were placed in two groups (FLA and FLB). The entire ARG and 31 of FLA phages produced clear plaques and showed nearly identical host ranges to the phage CP2 of Japan in that they reacted only with typical A strains but not with the atypical A strains (A* and A^W) or other *Xanthomonas* spp. FLA phages differed in their host range and produced turbid plaques. Phage typing was performed to classify citrus-associated xanthomonads. Phage typing using 12 phages isolated from *Xcc*, *X. fuscans* subsp. *aurantifolii* (*Xfa*), *X. alfalfae* subsp. *citrumelonis* (*Xacm*), and other sources proved useful for classifying all major *Xcc* pathotypes and/or strains (A, A*, Miami (MI), Manatee (MA), and Wellington (A^W)) as susceptible. The majority of *Xcc* strains, which originated from different regions of the world and which were typical “A” pathotype strains based on pathogenicity characteristics, were sensitive to most phages (including CP2, FLA, and ARG) and had nearly identical phage sensitivity profiles (Balogh et al. 2013).

A lytic phage specific to *Xanthomonas axonopodis* pv. *allii* (*Xaa*), causing onion *Xanthomonas* leaf blight disease, was identified. Phage population persisted for about

72–96 h under field and greenhouse conditions. Weekly or biweekly application of the phage reduced the disease severity by 26–50%. Detection of *Xaa* using a specific phage has been shown to have practical utility in ecological and epidemiological investigations (Lang et al. 2007). *Ralstonia solanacearum* causes bacterial wilt disease and brown rot of potato plants and tubers. It has a wide host range, including several crop plant species. Four bacteriophages, namely ØRSL, ØRSA, ØRDM, and ØRSS, were isolated from the pathogen. These phages were able to infect a relatively large number of bacterial species and produced large clear plaques. The phage ØRSA could infect all 15 strains of *R. solanacearum* of different races of biovars tested. Three host strains contained ØRSA-related sequences in their genomic DNAs, suggesting a lysogenic cycle of ØRSA. The results suggested that the bacteriophages had the potential for use in detecting and identifying the bacterial pathogens present in propagules of several economically important crops (Yamada et al. 2007).

5.2.3 Microscopy

Histological changes induced by bacterial pathogens in infected plants have been found to be characteristic of certain host–pathogen interaction combinations. *Xylella fastidiosa* (*Xf*), the xylem-limited pathogen, causes citrus variegated chlorosis (CVC). Although severe symptoms are induced on sweet orange plants, lime and mandarin are apparently resistant to *Xf* infection. Light microscopy was employed to assess the differences in the responses of sweet orange, mandarin, and lime plants following inoculation with *Xf*. Microscopic observations on microtome sections of leaf petioles revealed clear separation of occluded xylem vessels and free xylem vessels. Occluded vessels in sweet orange were filled with an unknown substance, resulting in dark blue coloration after staining. The average percentages of positive isolations (pi) and occluded vessels (ov) were higher in sweet orange plants, compared with mandarin and lime plants. However, no obvious differences in the xylem anatomy were observed between citrus varieties, suggesting that apparent resistance of mandarin and lime to *X. fastidiosa* was not due to changes in xylem anatomy (Garcia et al. 2012).

Erwinia (= *Pectobacterium*) spp. cause soft rot diseases of tubers and aerial stem rot of potato plants. *E. carotovora* subsp. *carotovora* (*Ecc*) and *E. carotovora* subsp. *brasilensis* (*Ecb*) are more aggressive than *E. carotovora* subsp. *atroseptica* (*Eca*). Bacterial localization and movement during soft rot infection was studied using confocal laser scanning microscopy (CLSM). A derivative strain of WPP14 which contained a plasmid p19nGFP was employed. In order to differentially observe Pcb1692 strain during coinfection with PccWpp14 a similar construct was built, but GFP was replaced by a red fluorescent protein (DsRed.T39DNT). CLSM observations revealed that both strains of *Ecc* existed at the same location in symptomatic leaves. No evidence of patchy distribution of the pathogen was obtained. Both *Ecc* and *Ecb* strains were motile in symptomatic leaves and tubers. The results indicated the usefulness of CLSM method for spot-checking the presence of soft rot pathogens in seed tubers and in potato plants (Marquez-Villavicencio et al. 2011).

5.2.4 Physical Techniques

Volatile technology was employed to assess the disease potential of tubers received for storage. Volatiles from Russet Burbank potatoes inoculated with *Erwinia carotovora* subsp.

carotovora (Ecc), *E. carotovora* subsp. *atroseptica* (Eca), and fungal pathogens *Pythium ultimum*, *Phytophthora infestans*, or *Fusarium sambucinum* were monitored by sampling head space at 3, 4, and 5 days after inoculation using a solid phase microextraction (SPME) filter to trap and gas chromatography with flame ionization detection (GC-FID) to fingerprint volatiles. Noninoculated (NON) potatoes served as controls. The method involved keeping tubers in closed containers for 24 h to accumulate volatiles and discriminating disease groups on the basis of the qualitative criteria or the back-propagation networks. Volatile fingerprints varied among diseases. Within a disease, the fingerprints varied with time since inoculation among blocks. The amount of volatiles produced was generally associated with disease severity. Volatiles produced as a result of enzymatic degradation of host tissues by the pathogens. The qualitative criteria based on retention time (RT) were used to discriminate most disease groups. The relative amounts of volatiles produced by Ecc and Eca differentially varied with types of volatiles produced (in peaks at 2.0, 2.3, and 2.44 min). Ecc and Eca produced many peaks, in contrast to two unique peaks at RT by fungal pathogens *P. ultimum* and *F. sambucinum*. The volatile technology developed in this investigation has the potential for commercial application for early detection and discrimination of diseases of potato tuber samples collected after harvest (Kushalappa et al. 2002).

Complex mixtures of DNA are separated into different fragments by electrophoresis. Restriction enzymes are used to cleave the isolated DNA and fragments small enough to resolve by electrophoresis in agarose or polyacrylamide are generated. Polyacrylamide gel electrophoresis (PAGE) is very useful to identify the unique DNA fragments amplified by polymerase chain reaction (PCR) from target bacterial pathogens. A two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of acid ribosome-enriched proteins was used to detect and differentiate *Erwinia* spp. Electrophoretic profiles of acid ribosome-enriched proteins of selected strains of *E. carotovora* subsp. *carotovora* (Ecc), *E. carotovora* subsp. *atroseptica*, and *E. chrysanthemi* were compared. Seven major clusters were identified. Soft-rotting bacterial strains could be readily identified and differentiated by 2D-PAGE technique. Ecc and Eca produced protein profiles that were consistent and distinct enough to be separated into two species. Other soft-rotting isolates were also identified by this technique (Moline 1985). By measuring the conductance of the media in which the target bacterial pathogen is grown, it is possible to detect the metabolically active soft rot pathogens Ecc and Eca infecting potato tubers. The pectate medium containing pectate as the sole carbon source was used for the specific automated conductimetric detection of Ecc, Eca, and *E. chrysanthemi* in potato peel extracts. The threshold of detection of Ecc showed variations of 10^2 – 10^3 CFU mL⁻¹ of peel extracts at 20°C and 26°C, respectively. Confirmation of the conductimetric assay by additional tests such as immunoassays may be desirable (Fraaije et al. 1996).

The usefulness of the stable low-molecular-weight (LMW) RNA profiles for detection of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), the incitant of potato tuber ring rot disease, was demonstrated. Staircase electrophoresis in polyacrylamide gels was employed to analyze the LMW RNA profiles of 54 strains of Cms from different geographical origins. The profiles of several strains of other subspecies of *C. michiganensis* and other pathogens of potatoes were also analyzed and compared. All strains of Cms had the same LMW profile. A band in class 2 of tRNA was present in all Cms strain, but it was absent from other subspecies of *C. michiganensis*. In addition, the LMW RNA of Cms was different with respect of the LMW RNA profiles of other potato pathogens.

The results indicated that LMW RNA profiles might be a reliable basis for the detection and identification of *C. michiganensis* subsp. *sepedonicus* (Palomo et al. 2000).

5.2.5 Immunoassays

Both polyclonal antibodies (PABs) and monoclonal antibodies (MABs) have been used for the detection and identification of bacterial plant pathogens present in or on propagules. Among the immunoassays, enzyme-linked immunosorbent assay (ELISA) formats have been more frequently employed for detection and identification of bacterial pathogens infecting potato tubers. The popularity of immunoassays was largely due to their simplicity of operation and their applicability in large-scale indexing. One of the major drawbacks of immunoassays was found to be false positives caused by cross-reaction of antibodies with plant debris or nontarget bacterial species. The problem of cross-reaction may be greatly reduced by use of specific monoclonal antibodies (MABs), however. As infection of potato tubers by *Erwinia* spp. remains latent, visual examination of seed tubers may not be sufficient. ELISA test could detect, after an anaerobic amplification, *Erwinia chrysanthemi*, causative agent of soft rot disease in DPEM medium. This step increased the sensitivity of ELISA test and the limit of detection was improved from 10^5 to $<10^3$ bacterial cells per milliliter. The latent infection of tubers could be detected in 10% of the 133 seed lots that had been earlier certified by visual inspection as disease-free. The ELISA test was therefore recommended for large-scale adoption for seed tuber certification programs and for forecasting disease outbreaks in Switzerland (Cazelles et al. 1995). The MABs generated against the European strains of *E. chrysanthemi* reacted with a fimbrial antigen present in all strains except two strains of *E. chrysanthemi* isolated from potato, demonstrating the usefulness of the MABs for detection of this pathogen not only in potato but also in other host plant species. The specific MAB (6A6) was able to effectively detect *E. chrysanthemi* in potato tubers in the triple-antibody ELISA format. The limit of detection of this ELISA format was 10^7 CFU mL⁻¹. This MAB reacted with a fimbrial antigen and fimbrial protein of *E. chrysanthemi*, as revealed by immunogold electron microscopy and Western blot analysis, respectively. The MAB reacted with 20 out of 36 strains of *E. chrysanthemi* isolated from hosts other than potato (Singh et al. 2000).

Enzyme-linked immunosorbent assay (ELISA) was applied for the efficient and sensitive detection of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), causal agent of bacterial ring rot disease in potato tubers. The ELISA test was found to be equally sensitive to the polymerase chain reaction (PCR) assay. The effectiveness of detection of *Cms* was dependent on several factors such as inoculum dose (>10 CFU), cultivar, and interval after planting (Slack et al. 1996). The results of ELISA tests for the detection of *Cms* in potato tissue were analyzed to determine the variation that occurred when different analysts performed the tests. Sensitivity and specificity of ELISA tests were very high, as few false-positive and false-negative results were obtained. The analysis of z-scores for ELISA for positive samples in the proficiency panel sets showed that about 90% of the results were within the acceptable range around the assigned values for the samples. Rescaled sum of scores for individual analysts who had false-positive or -negative results in ELISA were generally outside the acceptable range, although most analysts with high or rescaled sums of z-scores still identified each sample correctly as being positive or negative for the ring rot pathogen, *C. michiganensis* subsp. *sepedonicus*. The extent of

variability (within analyst variation) and reproducibility (among analyst variation) were analyzed to assess the reliability of ELISA tests for detection of *Cms* in potato tissues. The standard deviation (SD) for repeatability of ELISA test was small but increased at higher absorbance values, whereas the SD for reproducibility was greater and also increased at higher absorbance values, indicating the need to adjust the bacterial concentration at optimal levels (De Boer and Hall 2000a, b).

Enzyme-linked immunosorbent assay (ELISA) has been used very widely for indexing seed potatoes for detection of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) because of its simplicity. Most commercial ELISA kits for the detection of potato ring rot bacterium are equipped with more specific monoclonal antibodies (MAbs) that react to the extracellular polysaccharides (EPS) of *Cms*. The detection efficiency depended on the amount of EPS produced by the strain to be detected. The commercially available ELISA kit could detect a mucoid strain (AS1) when populations of *Cms* were 3.91×10^5 CFU mL⁻¹ in the tuber extract and 7.62×10^4 CFU mL⁻¹ of the stem extract. The ELISA test failed to detect a nonmucoid strain of *Cms* when the population level was below 2×10^7 CFU mL⁻¹ in either tuber or stem extracts. The lack of sensitivity of ELISA might be attributed to the insufficient secretion of EPS by nonmucoid strain P45 of *Cms*. Comparison of sensitivities of ELISA and PCR showed that PCR assay was more sensitive than ELISA tests. The strain AS1 was detected by PCR (38 cycles) at a concentration as low as 260 CFU mL⁻¹ of tuber extract or 52 CFU mL⁻¹ of stem extract. Strain P45 could be detected at a concentration of 891 CFU mL⁻¹ of stem or tuber extract. The results indicated that ELISA tests have to be replaced by PCR-based assay to detect *C. michiganensis* subsp. *sepedonicus* in latently infected potato tubers to eliminate/reduce the disease incidence and subsequent spread effectively (Lee et al. 2001).

Ralstonia solanacearum (*Rs*), incitant of bacterial wilt and brown rot of potato plants and tubers, is a quarantine pathogen subject to rigorous regulations. Latent infection of potato tubers being visually unrecognizable is of much concern for the personnel of quarantine and certification programs. ELISA formats were found to be suitable for the detection of *Rs* in seed tubers. The sensitivity of ELISA was significantly increased by including an enrichment step prior to ELISA test performed on nitrocellulose membrane for detection of *Rs* (Priou et al. 1999; Elphinstone et al. 2000).

In a later investigation, a sensitive and specific ELISA format was developed for the detection of *R. solanacearum* in asymptomatic potato tubers. An efficient enrichment step was included prior to an indirect double-antibody sandwich (DAS-) ELISA, using a specific MAb 8B-1V1V. This MAb reacted positively with 168 typical strains of *Rs* and did not react with other 174 other pathogenic or unidentified bacterial isolates from potato tubers. The isolates of *Rs* were incubated in the modified Wilbrink broth for 72 h at 90°C. The detection limit of this ELISA format was 1–10 CFU mL⁻¹ of individual potato extract containing the brown rot pathogen. The results of conventional isolation procedure corroborated those obtained with the ELISA format developed in this investigation. This ELISA format has the potential for use in routine certification programs and epidemiological investigations (Caruso et al. 2002).

Potato plants infected by *Ralstonia solanacearum* (*Rs*) may not exhibit symptoms of infection in cool temperate regions. These plants may transmit *Rs* to progeny tubers as latent infection which may lead to severe disease outbreaks when the tubers are grown at warmer locations in the subsequent seasons. Detection of *R. solanacearum* (biovar 2A) in stems of symptomatic plants before harvest of potato crops, instead of tubers, therefore has immense importance; not only would it save highly valuable planting

materials, but it would also be less time-consuming and help the growers in making market decisions. The possibility of detecting *Rs* latent infection in stem pieces about 3 weeks before harvest was investigated using samples from 57 fields of the Andean highlands of Peru. Two sensitive, specific, and user-friendly immunoassays were evaluated for the comparative efficiency in detecting the pathogen in latently infected tubers and stems – double-antibody sandwich (DAS-) ELISA and indirect ELISA on nitrocellulose membrane (NCM) – after enrichment of the plant extracts in a semi-specific broth. *R. solanacearum* was detected in 17 of 27 symptomless fields (63%) and in all lots with visible wilt disease incidence, even from those with very low disease incidence. The stems and tubers sampled from four fields with 2% wilt incidence were positive with a high number of infected composite samples. NCM- and DAS-ELISA formats showed an average of 13 and 18 positive for stems and 23 and 24 positive for tubers, respectively, among the 24 sub-samples analyzed. The results of NCM- and DAS- ELISA formats were in agreement for all samples irrespective of the plant parts tested. However, the number of positive samples observed with DAS-ELISA was higher than that of NCM-ELISA for both tubers and stems. Both ELISA formats yielded results for 93% of the lots tested. A 95% probability of detection was achieved by analyzing 250 stems or 175 tubers using DAS-ELISA format, whereas the same level of probability of detection could only be reached by analyzing 400 stems or 225 tubers using NCM-ELISA format, indicating the greater reliability of DAS-ELISA method. Post-enrichment of DAS-ELISA format for a higher level of reliability might be easily applied by national plant protection services and seed tuber production programs. The NCM-ELISA procedure might however be preferred because, after dotting the membranes with enriched extracts, they might be stored or transported to laboratories to perform immunoassays (Priou et al. 2010).

Immunofluorescence (IF) assay depends on the fluorescence emitted from the labeled antibodies that may react with epitopes present in the antigen or antigenic sites. The rhodamine-labeled oligonucleotide probe in conjunction with an indirect IF procedure based on specific MAb was employed. IF procedure, using a conjugated fluorescein isothiocyanate (FITC) label, was efficient in improving the sensitivity of detection of *Clavibacter michiganensis* subsp. *sepedonicus*, causal agent of potato bacterial ring rot disease. Bacterial cells labeled simultaneously with oligonucleotide and antibody probes could be precisely identified by examination under fluorescent microscope. This technique could be useful when isolation and other methods of detection and identification of target bacterial pathogen are difficult or not possible to employ (Li et al. 1997).

IF tests were performed for the detection of *Cms* in potato tissues and the variations that occurred were analyzed when different analysts were involved in testing. Data generated in accredited laboratories from sets of proficiency panel samples were used for the analyses. The sensitivity and specificity of IF tests were very high, as very few false-positive and false-negative results were obtained. The false-negative results were not associated with aberrant rescaled sums of z-score for analysts, suggesting that perhaps an error in testing occurred rather than a problem with quantifying cell numbers (De Boer and Hall 2000a, b).

Two immunofluorescence (IF) procedures have been employed for the detection of *Xylella fastidiosa* (*Xf*), involving tissue sectioning and membrane entrapment of *Xf* from inoculated grapevine hybrid selection F8909-08. The tissue sections and bacteria-trapped polycarbonate membranes were incubated with specific polyclonal IgG and stained with fluorescein isothiocyanates (FITC-) conjugated IgG from rabbits to *Xf* cells.

The stained preparations were observed by fluorescence microscopy. Detection and identification of *Xf* in thin cross-sections of the petiole was possible at 3 weeks after inoculation to study multiplication and invasion of xylem vessels by *Xf*. Using the membrane entrapment procedure, the pathogen at low concentrations could be isolated from asymptomatic plants (Buzkan et al. 2005). Indirect immunofluorescence (IF) microscopy procedure was applied for the detection of *Xf* infecting American hybrid vineyards in the Texas Gulf Coast. The infection of grape varieties Black Spansih, Blanc Du Bois, and Cynthia by *X. fastidiosa* was detected by indirect IF format. The results of IF microscopy were in agreement with those of ELISA tests (Buzombo et al. 2006).

Immunomagnetic separation (IMS) technique involves selective separation of target bacterial pathogen species and cultivation of the separated pathogen in a selective medium that differentially favors the pathogen development. IMS procedure was applied for the detection of *Erwinia carotovora* subsp. *atroseptica* (*Eca*), incitant of soft rot disease of potato tubers. *Eca* was separated from the potato peel extract by applying a combination of advanced magnetic (AM-) protein A particles, antirabbit IgG particles, and the selective medium, crystal violet pectate medium supplemented with $100\mu\text{g mL}^{-1}$ of streptomycin. A streptomycin-tolerant strain of *Eca* was included to reveal the effect of streptomycin on the parent pathogen strain. The populations of *Eca* present in the potato tuber extracts were consistently enumerated by the IMS technique. The threshold of pathogen population that could be detected was 100 bacterial cells mL^{-1} (van der Wolf et al. 1996).

In a later investigation, the effectiveness of the combination of IMS and staining by the LIVE/DEAD BacLightTM was investigated. The Bacterial Viability Kit for the rapid detection and precise identification of living and dead cells of *Erwinia carotovora* subsp. *carotovora* (= *Pectobacterium atrosepticum*) in potato tuber tissue was assessed. Bacterial suspensions in buffer or potato peel extract were spiked with living and dead cells of *Ecc* and incubated with paramagnetic beads coated with *Ecc*-specific antibodies. Beads with *Ecc* cells were recovered from the suspensions with a magnet and the immobilized cells were stained with a mixture of Syto9 and propidium iodide. The separated cells were examined under fluorescence microscope. The assay protocol needed minimal sample preparation without even washing steps and it could be completed within 1 h, indicating its rapidity and simplicity. The limit of detection of IMS/LD assay was 10^4 and 10^5 mL^{-1} for M-280 and M-450 paramagnetic beads, respectively. The detection limit of this assay was at the same level as the ELISA test. The specificity and sensitivity of IMS/LD remained the same for both pure cultures and the pathogen in potato peel extract. The ability of the IMS/LD assay to differentiate the live and dead cells of *E. carotovora* subsp. *carotovora* was considered as a distinct advantage over the ELISA test, which has been widely used for pathogen detection in seed tubers. The conclusions derived from IMS/LD assay may be important for predicting the disease incidence and the consequent losses caused by soft rot disease (Sledz et al. 2012).

A lateral flow device (LFD) was developed for detection of *Xanthomonas campestris* pv. *musacearum* (*Xcm*), causal agent of banana *Xanthomonas* wilt (XW) disease. A polyclonal antibody (PAb) was employed in the LFD for rapid in-field detection of *Xcm*. The IgG antibodies purified from the serum were tested against a wide range of bacterial species using LFD and ELISA tests. The PAb detected all strains of *Xcm*, representing isolates from seven countries and the known genetically diverse strains. The pathogen could be detected in both naturally and experimentally infected banana plants. The limit of detection by LFD was 10^5 cells mL^{-1} . The PAb cross-reacted with

the closely related *X. axonopodis* pv. *vasculorum* (*Xav*) infecting sugarcane. Since *Xav* is not a pathogen of banana, LFD technique using the PAb might be employed as a tool for first-line screening under field conditions. LFD, a cost-effective procedure, does not require any special equipment and could be used by personnel with relatively little experience (Hodgetts et al. 2015).

5.2.6 Nucleic Acid-Based Techniques

Bacterial pathogens infecting propagules have been detected and differentiated by employing various nucleic acid-based techniques.

5.2.6.1 Nucleic Acid Hybridization Methods

Specific DNA probes have been employed to rapidly detect, identify, and quantify bacterial pathogens in propagules. *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), causing soft rot and ring rot diseases of potato tubers, were detected by employing specific DNA probes (Ward and De Boer 1984, 1990; Verreault et al. 1998). The ring rot pathogen *Cms* was detected by labeled probes obtained from three unique single-copy DNA fragments designated Cms 50, Cms 72 and Cms 85 isolated from CS3 strain by subtractive hybridization procedure. Radio-labeled probes prepared from the unique DNA fragments of *Cms* and used in Southern blot analysis revealed the absolute specificity of each fragment to all North American *C. michiganensis* subsp. *sepedonicus* strains, including plasmidless and nonmucoid strains. The probes did not show any homology with genomic DNA from related *C. michiganensis* subsp. *insidiosus* and *tesellarius*, or with DNA from 11 additional bacterial species and three unidentified strains. The three fragments with no shared homology appeared to be separated from each other by at least 20 kb in the CS3 genome. Internal primer sets only permitted amplification of each fragment by PCR from *C. michiganensis* subsp. *sepedonicus*. The detection limit of a PCR assay using a primer pair that amplified Cms 85 was 100 CFU mL^{-1} of pathogen cells in potato core fluid (Mills et al. 1997). *Ralstonia solanacearum* (*RS*), incitant of brown rot disease of potato tubers, was detected using fluorescent in situ hybridization (FISH) technique. *R. solanacearum* race 3 biovar 2 exhibited a strong fluorescent signal. Pectin methyl esterase (*Pme*) is required for pathogen development after initiation of infection. Primers designed based on the sequences of the *pme* gene coding for *Pme* amplified an expected product of 800 bp involved in the biosynthesis of *Pme* by *R. solanacearum*. By employing this sequence as a probe, PMR-coding plasmids of *Rs* genome library were identified. The results showed that *Pme* was essential for pathogen growth, but not for its pathogenicity (Wullings et al. 1998).

A tissue-blot hybridization format was employed for the detection of sugarcane ratoon stunting disease pathogen *Clavibacter xyli* subsp. *xyli* (*Cxx*) by using a 560 bp PCR-amplified product as a probe. Two universal primers were able to amplify this probe from the intergenic region of the 16S/23S rDNA of *C. xyli* subsp. *xyli*. The hybridization procedure developed in this investigation might be useful for wider application along with other diagnostic techniques (Pan et al. 1998). *Xylella fastidiosa* (*Xf*), infecting grapevine and citrus among several other economically important crops, was detected by fluorescence in situ hybridization (FISH) assay. This technique is based on the combination of both specificity and adjustability to different phylogenetic levels with suitable oligonucleotide probes and detection and direct enumeration of the bacteria

without the need for the isolation of the pathogen in pure cultures. The probes were designed based on the sequence of the 16S rRNA gene of *Xf*. The probes S-S-X.fas-0067-a-A-18 and S-S-X.fas-1439-a-A-18 hybridized only with the DNA of 12 *Xylella* isolates and 15 closely related bacterial species. *X. fastidiosa* cells present in the sap from infected plants could be detected and quantified by using the selected probes if the population of *X. fastidiosa* was at an average of $(2.9 \pm 0.3) \times 10^6$ cells mL⁻¹ (Rodrigues et al. 2006).

5.2.6.2 PCR-Based Methods

Polymerase chain reaction (PCR) assays – the standard and its various formats – have been extensively used for the detection, identification, and differentiation of bacterial plant pathogens and their variants in propagules. Using primers capable of amplifying the unique DNA fragments of the pathogen, the PCR assay detected *Erwinia carotovora* subsp. *atroseptica* (*Eca*) consistently and effectively in potato stem and tuber tissues. The PCR format was more sensitive than ELISA employing MAbs (De Boer and Ward 1995). In another investigation, the PCR primer pair SP1f and SP5r was found to specifically amplify a 215 bp fragment of genomic DNA of *Clavibacter michiganensis* subsp. *michiganensis* (*Cms*). This primer pair did not amplify DNA from phenotypically and serologically related bacteria isolated from potato plants. When PCR, ELISA, and IF tests were evaluated for their comparative sensitivities of detection of *Cms*, PCR assay was found to be more sensitive than the other two methods tested. Tubers infected by *Cms* that tested negative for IF and ELISA tests were found to be PCR-positive, indicating the higher level of sensitivity of PCR assay. Furthermore, it is difficult to detect *C. michiganensis* subsp. *sepedonicus* in potato tubers which were contaminated with other pathogenic and saprophytic bacterial species by using hybridization techniques. PCR assay may therefore provide significant help under such conditions (Li and De Boer 1995). A one-step PCR-based protocol was shown to be effective for the detection of all five species of *Erwinia* including subspecies *atroseptica* and *carotovora* and all pathovars/biovars of *E. chrysanthemi* in micropropagated potato plants. The primers SR3F1 and SR1cR, based on conserved regions of the 16S rRNA gene, amplified a DNA fragment of 119 bp from all strains (65) tested when an enrichment step was used prior to PCR amplification, and the sensitivity of detection was increased by about 200 times (Tóth et al. 1999).

In order to efficiently extract amplifiable DNA from *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum*, quarantine pathogens infecting potato, a modified NaOH alkaline boiling procedure was employed, using a mixture of lysozyme and proteases combined with minimized TRIS/HCl/BSA buffer volume. The detection limit of the PCR assay was 10^4 – 10^5 CFU mL⁻¹ extract of each pathogen species. The alkaline extraction procedure was found to be much faster and simpler with similar detection limits, in addition to being inexpensive (Niepold 1999). *C. michiganensis* subsp. *sepedonicus* (*Cms*) DNA was extracted from potato tuber or stem tissue by a modified hot alkaline DNA extraction method. The *Cms*-specific DNA sequence was amplified by digoxigenin (DIG-) labeled PCR format with primer pair CMSIF1/CMSIR1 designed on the insertion element IS1121 of *Cms*. DIG-labeled PCR products were then blotted on a nylon membrane and the signal was detected by a colorimetric assay, using alkaline phosphatase (Fig. 5.11). The DIG-labeled PCR format had a detection sensitivity of the standard ELISA test employed for testing commercial seed tuber lots. The efficacy of DIG-labeled PCR, nested PCR, and ELISA test for the detection of *Cms* in potato tubers was assessed. The results obtained with DIG-labeled PCR assay

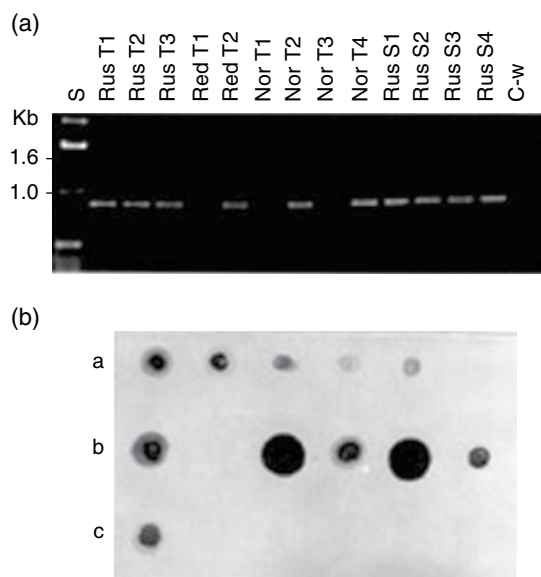


Figure 5.11 Comparative efficacies of nested PCR and DIG-labeled PCR formats for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potato cultivars. (a) Nested PCR products on agarose gel (1%); Rus: Russet Burbank; Nor: Norchip; T1–T4: tuber samples; S1–S4: stem samples; lane S: DNA standard (1 kb) and C-w: water control. (b) DIG-labeled PCR products dot-blotted on nylon membrane detected using NBT and BCIP. Row a: samples Rus T1–T3, Red T1, Red T2, and Nor T1; row b: samples Nor T2–T4 and Rus S1–S3; row c: samples Rus S4 and C-w. Source: Lee et al. (2001). Reproduced with permission of the American Phytopathological Society, MN, USA.

(with colorimetric detection of PCR products) were in agreement with that of nested PCR format. The ultra-sensitivity of DIG-labeled PCR format in detecting latent infection of potato tubers by *Cms* might be achieved by increasing the reaction cycles from 35 to 38 (Table 5.8). It could be used to detect *C. michiganensis* subsp. *sepedonicus* in asymptomatic field-grown potato tubers and mother plants (Lee et al. 2001).

A multiplex PCR assay that combines two different tests in one reaction mixture was developed for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) in potato tubers. A highly specific and sensitive detection of *Cms* and an indicator test for successful amplification (internal PCR control), which monitored potentially false-negative PCR results due to inhibition of PCR amplification, were accomplished. A mix of two different primer sets was used for the simultaneous amplification of two different targets in one reaction mixture. The pathogen-specific primer set PSA-I/PSA-R based on the intergenic region of the 16S-23S rRNA genes of *Cms* was used for the detection of potato ring rot pathogen. The plant-specific primer set NS-7-F/NS-8-R was used for the simultaneous amplification of the internal PCR control, permitting amplification of target sequence from plant DNA present in DNA extractions from potato core fluid. The effectiveness of the multiplex PCR format for the detection of *Cms* was verified by testing 3500 composite samples of 200 seed potato tubers from 143 different potato cultivars and by parallel testing, using immunofluorescence and a bioassay in eggplant seedlings. The results indicated that the multiplex PCR could be applied for the detection of *C. michiganensis* subsp. *sepedonicus* in tuber samples of various potato cultivars (Pastrik 2000).

Table 5.8 Comparative efficacy of ELISA, DIG-labeled PCR, and nested PCR assays for the detection of *C. michiganensis* ssp. *sepedonicus* in seed tubers and stem samples of potato (Lee et al. 2001).

Potato cultivar ^c	ELISA (A ₄₀₅) ^e	DIG-labeled PCR ^a		Nested PCR ^b	
		Electrophoresis	Colorimetric	Direct	Nested
Rus T1	0.019 (–)	–	+	–	+
Rus T2	0.079 (0)	–	+	–	+
Rus T3 ^d	0.023 (–)	–	+	–	+
Red T1	0.024 (–)	–	+	–	+
Red T2	0.007 (–)	–	+	–	+
Red T3	0.005 (–)	–	–	–	ND ^c
Nor T1	0.037 (–)	–	–	–	–
Nor T2	0.005 (–)	–	+	–	+
Nor T3	0.010 (–)	–	–	–	–
Nor T4	0.535 (+)	+	+	+	+
Rus S1 ^d	0.287 (+)	+	+	+	+
Rus S2 ^d	0.270 (+)	+	+	+	+
Rus S3	0.140 (+)	+	+	+	+
Rus S4	0.263 (+)	+	+	+	+

^a DIG-labeled PCR assay with 35 cycles; ^b Detection with nested PCR assay after gel electrophoresis of PCR products; ^c Cultivars: Rus: Russet Burbank; Nor: Norchip; T: tuber; S: stem; ^d Symptomatic samples and others asymptomatic; ^e ELISA threshold of A₄₀₅ = 0.1.

Erwinia carotovora subsp. *atroseptica* (*Eca*), causal agent of blackleg disease of potato, was detected by a competitive quantitative PCR format by incorporating a competitor PCR template cloned into *Escherichia coli* in the vector pGEM-T. Predetermined numbers of *Escherichia coli* cells were added to the potato peel extract, either preinoculated with *Eca* or naturally contaminated tubers. The ratio of products generated from *Eca* target DNA and competitor template DNA following PCR amplification was compared and the ratio indicated the concentration of the pathogen in relation to the competitor template DNA. Estimates of *Eca* numbers were consistent with counts obtained on crystal violet pectate medium and immunofluorescence colony staining. The assay results were not influenced by the presence of contaminants (other erwinias or saprophytic bacterial species). All serogroups of *E. carotovora* subsp. *atroseptica* could be detected by the procedure developed in this investigation, resulting in the formulation of a key relating to product ratios to the likelihood of incidence of blackleg disease. The possibility of using the competitive PCR format in quarantine and certification program was revealed by this investigation (Hyman et al. 2000).

Polymerase chain reaction (PCR) assay was found to be effective for the detection of *Xanthomonas axonopodis* pv. *citri*, causal agent of Asiatic citrus canker disease. A primer pair that amplified a 222bp DNA specific to *Xac* was employed for the detection of the pathogen and diagnosis of a field outbreak of citrus canker disease (Gillings et al. 1995). The effectiveness of plasmid-derived primers for reliable detection

of *Xac* strains (pathotypes) was indicated by Hartung et al. (1996). PCR analysis of *Xanthomonas*-like bacteria isolated from citrus was performed with a view to standardizing robust molecular diagnostic procedures for the identification of pathogens such as *X. axonopodis* pv. *citri* that were considered to be a biosecurity risk to New Zealand. Sequence analysis of the 16S and 23S ribosomal genes indicated that *Xanthomonas*-like organisms isolated from citrus were common epiphytes on citrus leaves (Taylor et al. 2002). In the attempt to target nonnuclear nucleic acids for designing primers, sets of primers based on sequence differences in the ITS and on sequence from plasmid gene *pthA* involved in pathogenicity were employed for the detection of strains obtained from various geographical locations. In addition, pathotypes of *Xac* could be identified/differentiated. A new strain of *Xac*, capable of causing canker on Key/Mexican lime (*Citrus aurantifolia*), designated *Xac*^W, was differentiated by employing plasmid-derived primers specific to *X. axonopodis* pv. *citri* (Sun et al. 2004).

As the Asiatic citrus canker disease seriously impacts citrus production worldwide, rapid and reliable identification of *Xanthomonas axonopodis* pv. *citri* and its strains was considered essential to prevent/reduce the incidence and spread of the disease. Two pathogenic variants A and A^{*}/A^W are widely distributed. Two additional pathovars with a limited geographic distribution and reduced pathogenicity, namely *X. axonopodis* pv. *aurantifoliii* and *X. axonopodis* pv. *Bilavae*, have been reported. These pathovars have been classified as quarantine pathogens in different citrus-producing countries. The specificity of nine PCR primers designed earlier for identification of *Xac* or citrus canker strains (both pvs. *citri* and *aurantifolii*) was determined on a large strain collection ($n = 87$) including two pathotypes of *Xac*, other genetically related or unrelated pathogenic xanthomonads, and saprophytic xanthomonads. When the same strains or pathovars were tested by PCR assay, similar results were obtained. New findings were however highlighted due to the use of a broad inclusivity and exclusivity panel of strains. Primers 2/3, 4/7, and KingF/R did not amplify three strains from the pathotype A^{*}/A^W. Furthermore, all pairs of primers detected at least one nontarget strain, indicating lower level of specificity of the primers. In silico analysis of the DNA sequences available from the National Center of Biotechnology also corroborated the data obtained in this investigation (Delcourt et al. 2013).

Citrus bacterial canker (CBC) is caused by two phylogenetically different groups which were renamed *Xanthomonas citri* subsp. *citri* strain A (of Asiatic origin, *X. citri*) and *X. fuscans* subsp. *aurantifolii* (*X. aurantifolii*) strain B and C (Schaad et al. 2006). *X. citri* has a worldwide distribution and wide host range, whereas *X. aurantifolii* is less virulent although it can infect the same host plant species as *X. citri* A and is restricted to South America. *Citrus aurantifolia* was infected only by *X. aurantifolii* and not by *X. citri* in South America. *Xanthomonas* isolates (42) were collected from a range of *Citrus* spp. across 17 different orchards in Argentina. Analysis of genome-specific *X. citri* markers and DNA polymorphisms based on repetitive elements-based PCR assay were performed. The well-known pathogenicity marker gene *pthA* was used. PCR amplification from total DNA of the 42 *Xanthomonas* isolates showed the presence of the *pth* marker. In addition, two genome-specific markers, one based on the ITS region of the rDNA sequence of *X. citri* and other based on the *xspD* gene, a component of the type II secretion system, were used. The expected molecular markers from the ITS and *xspD* sequences were present in all 42 *Xanthomonas* isolates and in the

X. citri reference strains tested. However, these markers were absent from *X. aurantifolii* strains B and C and *X. campestris* 8004 reference strain, indicating the absence of *X. aurantifolii* in Tucuman province of Argentina (Chiesa et al. 2013).

Repetitive PCR markers have been employed for determining genetic relationships between *Xanthomonas* spp., including those species infecting *Citrus* spp. and also the genotypic variability within the bacterial species. BOX-, ERIC-, and rep-PCR were performed using purified total DNA of the 42 strains of *X. citri* and *Xanthomonas* reference strains. The rep-PCR assay amplified fragments with complex genomic fingerprinting patterns with multiple and reproducible bands of distinct intensities. The average number of bands per strain varied from 13 to 16 for BOX- and rep-PCR and from 9 to 16 for ERIC-PCR. The molecular size of the bands was 220–1650 bp for BOX-PCR and 200–300 bp for rep- and ERIC-PCR. Easily distinguishable profiles were generated by rep-PCR from *X. citri* strains, useful for differentiating them from *X. aurantifolii* strains B and C (Fig. 5.12). Pathogenicity tests showed that one isolate, which shared >90% genetic similarity to the reference strain *X. citri* T, showed host range specificity. This new strain *X. citri* A^T, deficient in the production of xanthan, induced an atypical noncancerous chlorotic phenotype in *Citrus limon* and *C. paradisi* and weak cankerous lesions in *C. aurantifolia* and *C. clementine* leaves (Chiesa et al. 2013).

Xylella fastidiosa (*Xf*), causing citrus variegated chlorosis (CVC) and grapevine Pierce's disease, is difficult to isolate in pure culture. Nearly complete nucleotide sequences of the 16S rRNA gene and partial sequences of DNA gyrase (*gyrB*) were determined for 18 strains of *X. fastidiosa* from different hosts. The specificity of *Xf* 16S rRNA-targeted primers was confirmed by amplification of a product of 645 bp (for set B) from the *Xf* DNA only. No PCR amplification occurred for DNA of any of the 36 phylogenetically related microorganisms, endophytes, or plant pathogens tested. Specific primers targeting the *gyrB* gene were also tested with DNA isolated from all strains, as in the case of 16S rRNA-targeted primers. Amplification of the product of the expected size (429 bp) was observed only when *Xf* DNA was used for amplification. These primers were validated with 30 target and 36 nontarget pure cultures of microorganisms, confirming absolute (100%) specificity of the primers (Rodrigues et al. 2003).

The presence of *Xf* in all main sweet orange fruit vascular bundles and in seeds with no visible symptoms of infection was detected by PCR assay (Li et al. 2003). *X. fastidiosa* (*Xf*) was detected in the xylem sap of infected citrus plants by employing the PCR assay, using the primers designed based on the sequences of the 16S rRNA gene of *Xf*. The PCR amplicons (approximately 1348 bp) were partially sequenced and the data revealed a 99% identity to the 16S rRNA gene sequences, confirming the presence of *Xf* in infected plants (Rodrigues et al. 2006). A quick, simple, and sensitive one-step PCR format was applied for the detection of *X. fastidiosa* in other plant hosts such as oleander. The DNA was extracted by using DNeasy Tissue Kit and Fast DNA Kit separately, followed by PCR employing 16S set C primers. *Xf* was detected in inoculated plants showing disease symptoms when DNeasy Tissue Kit was used for DNA extraction from leaves, but not in samples extracted with Fast DNA Kit for DNA extraction. The results indicated that a proper DNA extraction procedure has to be employed for successful detection and identification of the target pathogens, since coextraction of compounds inhibitory to PCR amplification has to be avoided (Huang et al. 2006).

A PCR-based procedure involving the use of primers XF2542-L and XF2542-R was applied for the detection of *Xylella fastidiosa* in grapevine. The primers amplified a specific 412 bp product from the strains infecting grapevine. By employing appropriate

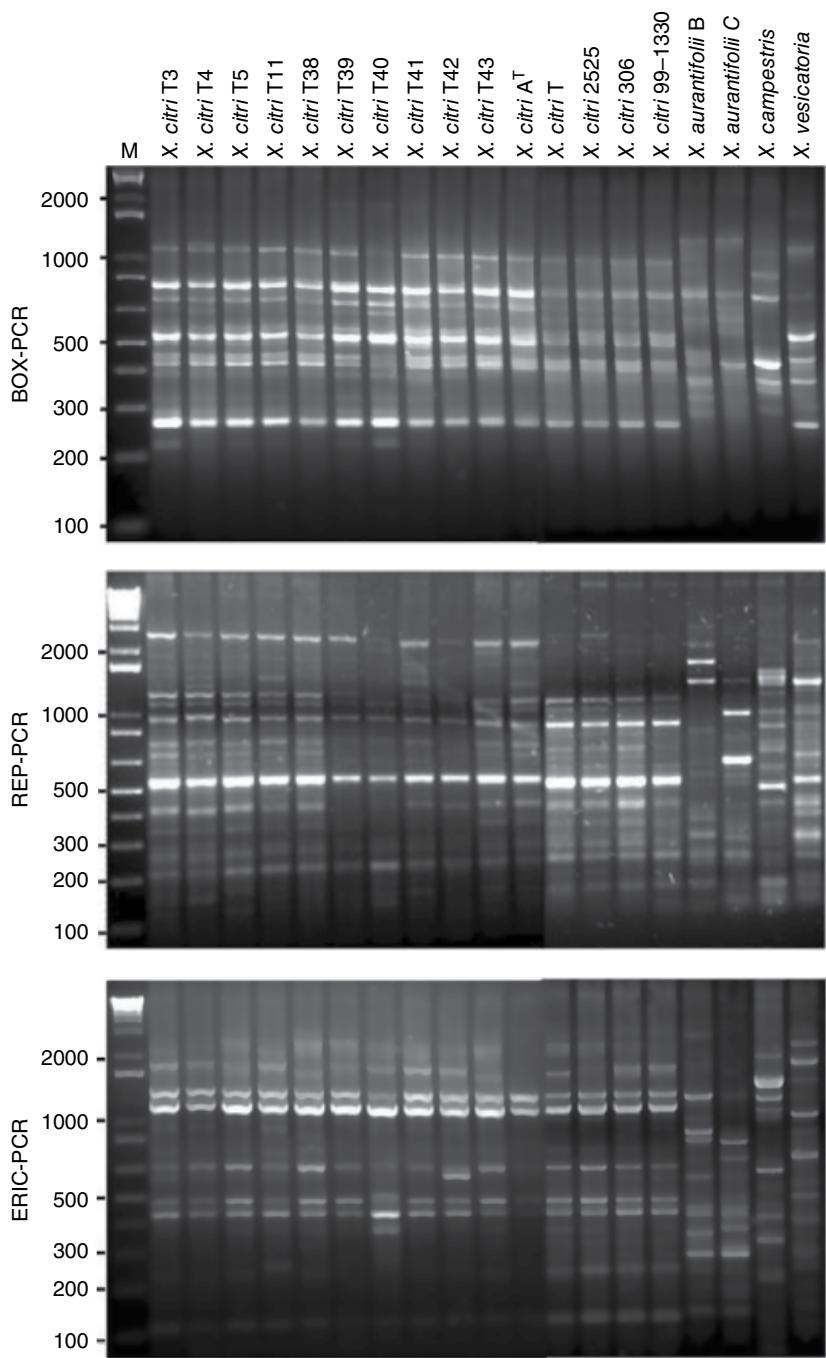


Figure 5.12 Assessment of genotypic diversity of eleven strains of *Xanthomonas citri* subsp. *citri* using BOX-, ERIC-, and rep-PCR assays. M: molecular size standards. Genomic profiles were separated on agarose gels and stained with ethidium bromide. Source: Chiesa et al. (2013). Reproduced with permission of the American Phytopathological Society, MN, USA.

primer sets, strains of *X. fastidiosa* infecting other plant hosts, oleander and almond, could also be detected by PCR assays (Hernandez-Martinez et al. 2006). A field genebank of grapevine maintained by the National Clonal Germplasm Repository (NCGR) included numerous grapevine accessions with varying levels of susceptibility/tolerance to Pierce's disease (PD), caused by *Xylella fastidiosa* (Xf). A PCR assay, using a primer pair that was genome sequence specific to Xf, was employed to detect the pathogen in grapevine accessions. The primer pair provided a high degree of sensitivity and specificity to all Xf strains prevalent in Davis, California. Basal leaf petioles were tested from 60 different accessions in the NCGR, representing a broad cross-section of taxa and period of time since collection in the region where *X. fastidiosa* was endemic. The pathogen could be detected only in Chardonnay grapevines which were inoculated with Xf. The results indicated that NCGR grape accessions collected as budwood in southern USA, where the disease and the pathogen were endemic, could not be considered as significant reservoirs of *X. fastidiosa* (Stover et al. 2008).

Sugarcane ratoon stunting disease (RSD), caused by *Leifsonia* (= *Clavibacter*) *xyli* subsp. *xyli* (*Lxx*) occurs in most sugarcane-growing countries, accounting for significant progressive crop losses. The presence of *Lxx* in sugarcane stalks was detected by employing oligonucleotide primers derived from the intergenic region between 16S and 23S rRNA genes. The primers amplified the specific DNA sequences in the pathogen. The primers failed to amplify the DNA sequences from closely related *L. xyli* subsp. *cyanodontis*, indicating the specificity of the primers and the suitability of the PCR assay for the detection of ratoon stunting pathogen (Fegan et al. 1998). In another investigation, a PCR protocol was developed for specific detection of *L. xyli* subsp. *xyli* in sugarcane stalks. Generic PCR products from the intergenic spacer (ITS) region of 16S-23S ribosomal DNA of the pathogen were cloned and sequenced. Based on a multiple sequence alignment among these two sequences and other nonredundant highly homologous sequences from the database, two *L. xyli* subsp. *xyli*-specific primers were designed. Primers Cxx1 and Cxx2 primed the amplification of the 438 bp DNA product directly from cultured *Lxx* cells and from 37 of 60 vascular sap samples from sugarcane stalks grown from *Lxx*-infected stalks, using polyvinylpyrrolidone (PVP) and ficoll-containing PCR reaction mixture (Fig. 5.13). The efficacy of PCR assay and tissue-blot immunobinding assay (TB-IBA) was compared for detection of *Lxx* in sugarcane vascular samples. PCR and TB-IBA tests detected the ratoon stunting pathogen in 34 of 37 samples. PCR assay could detect *Lxx* in three more samples which were TB-IBA negative. However, TB-IBA detected *Lxx* in seven other samples which were PCR-negative. The extraction of genomic DNA of the pathogen was not necessary for performing the PCR protocol developed in this investigation (Pan et al. 1998).

A PCR format was developed to detect *L. xyli* subsp. *xyli* in 137 sugarcane core germplasm collections from the National Nursery of Sugarcane Germplasm Resources (NNSGR, China). Under natural conditions, 21 of 137 germplasm entries tested PCR-negative whereas the bacterial pathogen could be detected in the rest of the germplasm entries (116), indicating that a relatively high ratio of the sugarcane core germplasms were susceptible to the RSD. The sequencing and analysis of 30 randomly selected PCR products showed that all 30 sequences were identical or highly homologous to the corresponding *Lxx* genome region published in GenBank (99.54–100% similarity). PCR assay was found to be a rapid, simple, and low-cost procedure to assess the levels of susceptibility/resistance of sugarcane cultivars/genotypes to ratoon stunting disease (Li et al. 2013).

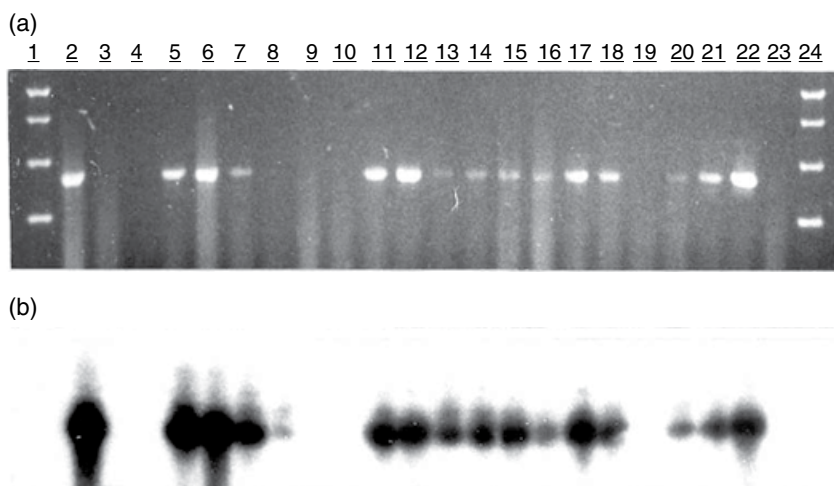


Figure 5.13 Detection of *Clavibacter xyli* subsp. *xyli* (Cxx) in sugarcane vascular samples by PCR assay and Southern blotting. (a) PCR products were stained with ethidium bromide; lane 1: DNA standards; lane 2: cultured pathogen cells; lane 3: water control; lanes 4–23: sugarcane vascular samples. (b) Autoradiograph of gels in panel (a) probed with ^{32}P labeled L1/G1, primed PCR product from Cxx genomic DNA. Source: Pan et al. (1998). Reproduced with permission of the American Phytopathological Society, MN, USA.

Banana Xanthomonas wilt (BXW) disease caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) has been reported to be a devastating disease in many African countries. The disease is spread via contaminated planting materials (suckers), indicating the importance of using disease-free planting materials. A PCR-based assay was developed using the primers BWX-1 and BWX-3, based on the conserved sequences of the *hrpB* operon of the *hrp* gene cluster of the BWX disease pathogen. All strains (50) of Xcm from Uganda, Rwanda, and Tanzania amplified a 214 bp PCR product when whole cells, bacterial ooze from the infected banana tissues, and genomic DNA purified from bacterial ooze or infected tissues were used as template. In addition, the primers amplified the 214 bp product from 50% of the strains of *X. campestris* pv. *vasculorum* isolated from sugarcane, maize, and *X. vasicola* pv. *holcicola* from sorghum. No amplicon was generated for any other strains of *Xanthomonas*, *Pseudomonas*, *Clavibacter*, or *Ralstonia* tested. The detection limit for whole cells of Xcm was 10^4 – 10^5 CFU mL $^{-1}$. ERIC-PCR genomic fingerprinting patterns consisted of 11 or more distinct bands ranging in size from 0.35 kb to c. 3.0 kb. The fingerprinting patterns of strains from Uganda, Rwanda, and Tanzania were identical, indicating that the strains of *X. campestris musacearum* from these countries were clonal. The PCR protocol developed in this investigation was practically applied to monitor spread of banana Xanthomonas wilt disease and to initiate appropriate disease management practices (Lewis Ivey et al. 2010).

Potato scab disease is due to three *Streptomyces* species – *S. scabies*, *S. turgidiscabies*, and *S. aureofaciens* – which could be detected in the contaminated tubers by employing species-specific primers. The forward (leading strand) primer was compatible with the highly variable γ -region of the rRNA gene. The reverse primer was compatible with the variable ϵ -region (*S. scabies* and *S. turgidiscabies* or β -region (*S. aureofaciens*)). Scab lesions (1245) from potato cultivars Matilda and Sabina were tested. After incubating

harvested or stored tubers at room temperature (18–21°C) under humid conditions for a few days, bacterial growth from scab lesions was collected for DNA extraction and PCR amplification. *S. scabies* and *S. turgidiscabies* were detected in the same potato fields, tubers, and scab lesions. The relative incidence of *S. scabies* was high in freshly harvested tubers and much lower than that of *S. turgidiscabies* after storage. Both pathogen species were transmitted through seed tubers in cultivars Matilda and Sabina after 24 weeks of storage at 4°C. Strain 317 earlier identified as nonpathogenic was identified as *S. aureofaciens*, pathogenic to potato. By employing species-specific primers in PCR assay, *S. aureofaciens* was also detected in potato tubers (Lehtonen et al. 2004).

The incidence and severity of potato common scab disease may vary due to the genetic variability of pathogen and host plant and environment. Streptomycetes were isolated from lesions on field-grown potatoes from six states of the USA to determine the genetic diversity of *Streptomyces* spp. associated with common scab disease. Isolates were classified into species of *Streptomyces* based on the sequences of variable regions in the 16S rRNA gene. The presence of genes associated with *S. turgidiscabies* pathogenicity island (PAI) was also determined, in addition to *S. scabies* and *S. europaeiscabiei*. The largest number of common scab-causing US isolates belonged to *S. scabies* or *S. europaeiscabiei*, based on 16S rDNA sequence. Most *S. scabies*/*S. europaeiscabiei* isolates tested had all three PAI marker genes, including *txtAB* (thaxtomin toxin) and those encoding pathogenicity factors *nec1* and tomatinase. Using the species-specific PCR primers developed in this investigation, most of the pathogenic isolates could be assigned to 16S rDNA groups identified in scab-causing streptomycetes. A substantial fraction of isolates from Idaho belonged to a new group characterized by a unique rDNA sequence and two of the three PAI marker genes. Approximately 40% of the isolates were found to be nonpathogenic, and they were more diverse in morphology than the pathogenic isolates. The results suggested that population of scab-causing species from different locations might contribute to differences in the incidence and severity of potato common scab disease in different years and the apparent regional variations in their distribution has to be taken into account (Wanner 2006).

In a further investigation, the new potato common scab-causing streptomycete was subjected to molecular analysis. The new Idaho strains contained genes encoding the synthetase for the pathogenicity determinant thaxtomin and for a second pathogenicity factor, tomatinase. However, it lacked a third gene characteristic of the *Streptomyces* PAI, the *nec1* gene. PCR analysis of the Idaho isolates revealed that the gene for a tomatinase homolog *tomA*, located approximately 300 kb away from the *txtB* genes in *S. turgidiscabies* was present in all five isolates. A primer pair amplifying the *nec1* gene located in the PAI, a few kilobases inside the *tomA* gene in *S. turgidiscabies*, did not produce the expected 700 bp fragment in any of the five Idaho isolates (Fig. 5.14). Use of the PCR primers ASE3 and scab3m separated the Idaho isolates from the four *Streptomyces* spp. The Idaho isolates represented a unique combination of 16S rDNA variable sequence regions, different from those described earlier, for pathogenic and nonpathogenic streptomycetes closely related to *S. scabies*. The new strain was pathogenic to radish and potato and more virulent than the *S. scabies* type strain, causing scab lesions on potato tubers, underground stems, and stolons (Table 5.9). The new strain therefore might add complexity in the pathogenic strains causing potato common scab disease (Wanner 2007).

Several *Streptomyces* species, namely *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies*, and other species (about 500 species) are soil inhabitants and these well-characterized

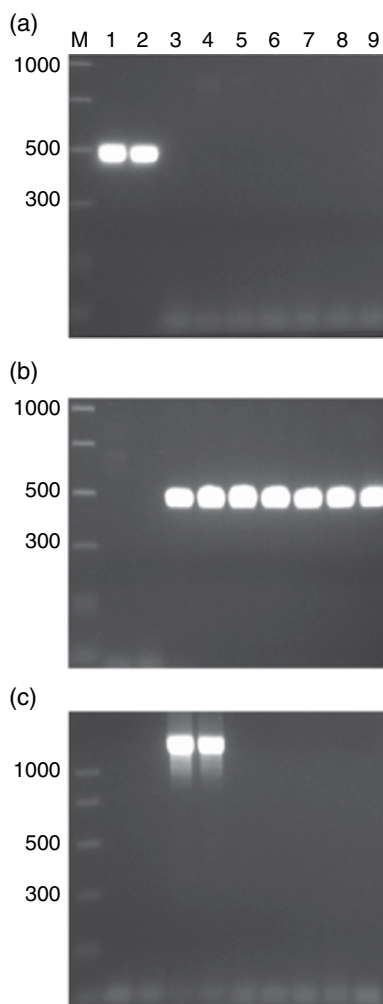


Figure 5.14 Detection and identification of Idaho *Streptomyces* isolates with three primer sets for amplification of the same set of DNA templates. (a) Primers ASE3/Scab2m producing 474 bp fragment; (b) primers ASE3/Aci2 producing 472 bp fragment; and (c) primers Aci1/Aci2 producing 1278 bp fragment. Lane M: DNA ladder; lane 1: *S. scabies* ATCC49173; lane 2: IDOI-16c (*S.europaiscabiei*); lane 3: *S. acidiscabies*^T ATCC49003; lane 4: *S. acidiscabies* ME02-6987A; lane 5: IDOI-6.2A; lane 6: IDOI-12c; lane 7: ID03-1A; lane 8: ID03-2A; lane 9: ID03-3A. Source: Wanner (2007). Reproduced with permission of the American Phytopathological Society, MN, USA.

species are associated with potato common scab disease. New pathogenic species such as *S. europaeiscabiei*, *S. stelliscabiei* (Boucheck-Mechiche et al. 2000) and *S. niveiscabiei* (Park et al. 2003) have been reported to cause common scab disease of potato tubers. All pathogenic species synthesized thaxtomins, the phytotoxin required for disease development. Purified thaxtomins induced symptoms on host tissues identical to those caused by *Streptomyces* spp. Genes encoding a peptide synthetase (*txtA* and *txtB* (*txtAB*)) and a cytochrome P450-type monooxygenase (*txtC*) are required for the thaxtomin biosynthesis (Healy et al. 2000).

Table 5.9 Virulence characteristics of Idaho isolates of *Streptomyces* on radish in comparison to type strain of *S. scabiei*^T (ATCC 49173) (Wanner 2007).

Isolate	Initial inoculum density ^a	Scab lesion score ^b	Seedling emergence (%) ^c	Plant survival after 5 weeks (%) ^c
ID01-12c	7.03	DD ^d	15	0
	6.65	4.33 (D ^e)	51.8	0
ID01.6.2a	7.47	DD	14	4
	7.06	DD	4	0
ID03-1A	7.49	4.0 (DD)	95	14
ID03-2A	7.06	4.1 (D)	95	36
ID03-3A	7.0	3.5 (DD)	90	7
<i>S. scabiei</i> type	6.75 ^f	3.48 ^f	88 ^f	82 ^f
Strain ATCC49173	14	14	12	12
independent experiments				

^a Log CFU of *Streptomyces* isolate/cc of soil; ^b Severity × area, 0–5 scale; scores are averages of 3 pots in an independent experiment or for the number of experiments given in paranthesis for ATCC 49173;

^c Percentages are averages of 3 pots in an independent experiment or for the number of experiments given in parentheses for ATCC 49173; ^d All plants dead (no radishes to score); ^e Many plants dead (few radishes to score); ^f Average in multiple independent experiments; number of experiments in parentheses.

A PCR format was developed for the direct detection of *Streptomyces* causing potato common scab from tuber lesions, employing primers designed to amplify a fragment of the *txtAB* (*txtA* and *txtB*) genes functioning as pathogenicity determinants in the principal *Streptomyces* spp. The primers Stx1a and Stxtb based on the DNA sequence of the *txtAB* genes of *S. acidiscabies* amplified a 402 bp fragment of the *txtAB* intergenic region, comprising 181 bp of the *txtA* 3'-terminal and 225 bp of the *txtB* 5'-initial DNA sequences. PCR with primers Stx1a/b allowed detection of *Streptomyces* on 40% of potato tubers with symptoms of common scab of 216 collected samples. No amplification occurred when DNA was extracted from faint superficial lesions (such as those caused by *Spongospora subterranea*). The protocol was also applied to evaluate 84 naturally infected potato samples comprising 19 potato cultivars from Netherlands, the UK, France, Germany, and Spain. Pathogenic *Streptomyces* in tuber lesions were detected by PCR in 70 samples and the pathogens were isolated successfully from all these samples that were PCR-positive. The pathogenic isolates of *Streptomyces* were phenotypically similar, although the scabbed samples showed diversity. Random fragment length polymorphism (RFLP) analysis of amplified rRNA sequences, together with carbon source utilization, allowed most isolates to be assigned to *S. europeiscabiei* which emerged as the principal causative agent of potato common scab diseases in western Europe (Flores-González et al. 2008).

Streptomyces spp., causing potato common scab (CS) disease in Norway, were investigated by isolating the bacteria from scab lesions on tubers in two consecutive years (2008 and 2009). *Streptomyces europeiscabiei* was the most abundant species isolated from common scab lesions (69%), while 31% of the isolates obtained were

S. turgidiscabiei. *S. scabiei* was not detected in any of the samples tested. The primer pair textAB1/textAB2 was used to detect and distinguish the isolates of *Streptomyces*. All of the 223 putative pathogenic isolates were PCR-positive, using the universal primers for the 16S rRNA genes of *Streptomyces* spp. Species determination was performed using the primer pair ScabI/ScabII. Of the 223 isolates, 152 (69%) produced amplicons with the ScabI/ScabII primer pair. When the primer pair TurgI/TurgII was employed, DNA of the 71 isolates could be amplified; these isolates were therefore assigned to *S. turgidiscabiei*. Distribution of the isolates was determined as *S. europaeiscabiei* 69% and *S. turgidiscabiei* 31%. Both species could be detected in the same field and even in the same scab lesion. Pathogenicity and virulence genes are clustered in a pathogenicity island (PAI). The genes associated with thaxtomin production, such as *txtA*, *txtB*, *txtC*, and *txtR*, are present in the first segment of the PAI which is called the toxigenic region, whereas the virulence-related genes *tomA* and *nec1* are located in the second segment, designated the colonization region. The Norwegian *Streptomyces* isolates showed variation in PAI marker genes and the dominant PAI genotypes differed between *S. europaeiscabiei* and *S. turgidiscabiei*. Although *nec1* and *tomA* were both present in *S. scabiei*, they were not located in the same chromosomal regions as the thaxtomin biosynthesis gene cluster and the separate presence or absence suggested that they might be independently transferable. The results showed that by using species-specific primers and restriction analysis of parts of the ITS region, the isolates could be accurately identified as *S. europaeiscabiei* or *S. turgidiscabiei*; *S. europaeiscabiei* could be mistaken for *S. scabiei*, because of the sequences of 16S rRNA were almost identical (Dees et al. 2013).

Streptomyces isolates (293) collected from field-grown potatoes showing common scab infection in 2006 and 2009 in Germany were analyzed for phenotypic characteristics, pathogenicity, and also genetic level. The isolates could not be differentiated clearly based on morphology and phenotypic characteristics. The isolates were characterized by PCR, using 16S rRNA-specific primers and PCR-RFLP of the 16S-23S internal transcribed spacer region (ITS) with Hpy991. Based on the fingerprinting data, *Streptomyces* species were differentiated genetically. The scab-causing bacterial species showed a greater level of diversity than expected. The isolates were assigned to *S. europaeiscabiei*, *S. stellscabiei*, *S. acidiscabiei*, *S. turgidiscabiei*, and *S. bottropensis* among the isolates prevalent in Germany. *S. europaeiscabiei* was the predominant species found in Germany. The distribution of other *Streptomyces* spp. was uneven. Thaxtomin biosynthetic genes were detected in most of the isolates that were pathogenic on potato tubers (Leiminger et al. 2013).

“*Candidatus Liberibacter asiaticus*,” a phloem-limited nonculturable bacterium, causes huanglongbing (HLB) (formerly known as citrus greening) disease in citrus. The HLB pathogen occurs in Asian, African, and American forms which are designated “*Ca. L. asiaticus* (Las),” “*Ca. L. africanus* (Laf),” and “*Ca. L. americanus* (Lam),” respectively. The pathogen was detected by employing primers OI1 and OI2c, designed using the sequences of 16S rDNA. Using these primers, the PCR assay detected the pathogen in 28% of the samples analyzed during the survey undertaken in Brazil (Coletta-Filho et al. 2004). In a further study, the primers LSg2f and LSg2r were developed based on the consensus sequences of 16S rDNA fragments. These primers could detect “*Ca. Liberibacter asiaticus*” in 38 samples that did not amplify the diagnostic pathogen DNA fragments when OI1 and OI2c primers were used (Coletto-Filho et al. 2005).

Infection of trees and fruits by “*Ca. L. asiaticus*” significantly reduced seed weight, seed germination, and seedling height. However, seedlings did not develop symptoms typical of huanglongbing disease throughout the experimental period. Polymerase chain reaction (PCR) assay was applied to detect “*Ca. L. asiaticus*” in the rootstock seedlings and sweet orange seedlings. The presence of huanglongbing pathogen in two of 686 rootstock seedlings and three of 431 sweet orange seedlings was initially detected by PCR assay when the seedlings were very young. Resampling and PCR analysis of these five seedlings at older ages consistently indicated that the seedlings did not have detectable concentrations of the pathogen, and none of them developed visible symptoms of HLB disease. The results suggested that “*Ca. L. asiaticus*” might have been translocated into some part of the embryo during seed development, but the pathogen was undetectable in the cells or tissues which did not allow pathogen development as the seedlings aged (Albrecht and Bowman 2009).

A quantitative PCR assay was developed to quantify “*Candidatus L. asiaticus*” genome in tissues of *Citrus* spp. The populations of the pathogen was inferred from the distribution of 16S rDNA sequences specific to “*Candidatus Liberibacter asiaticus*” genomes in tissues of six species of citrus grown under field or greenhouse conditions. The populations of the pathogen were determined in midribs, petioles, and roots of *Citrus* spp. Significant population differences were observed among these tissue types. However, these differences were not consistent across all species or trees tested. The pathogen DNA was readily detected in the roots of infected trees. The field-grown Persian lime tree roots contained 6×10^{10} pathogen genomes g^{-1} , comparable with the levels observed in the aboveground portions of trees. In symptomatic fruit tissues, “*Ca. L. asiaticus*” genomes were also readily detected and quantified. The locular membranes and septa had the highest pathogen population (10^8 genomes g^{-1}), whereas the mesocarp and pericarp of the fruits had lower levels (100-fold) of the pathogen population. The pathogen was scarcely detected at the point of graft-inoculation, but the population increased rapidly to approximately 10^6 genomes g^{-1} throughout the other regions of the trunk, both above and below the site and in root tissues. The quantitative PCR protocol was found to be useful and sensitive to detect and quantify the populations of “*Ca. L. asiaticus*” in various tissues of infected citrus trees (Fig 5.7) (Li et al. 2009b).

The use of disease-free plants is a component of a comprehensive strategy for the effective prevention of incidence and spread of huanglongbing (HLB) disease caused by “*Candidatus Liberibacter asiaticus*.” Since rootstocks are grown from seed, it is essential to test the seeds from infected trees for freedom from the bacterial pathogen. Two groups of 360 or more seedlings each of various *Citrus* species were grown from seed removed from fruits on trees that were infected with HLB disease, as indicated by PCR assay. These seedlings were tested several times during a period of up to 3 years (Fig. 5.15). No symptoms typical of HLB disease were observed in these seedlings and none of the 723 seedlings tested positive for the presence of “*Ca. L. asiaticus*,” even after repeated testing using sensitive qPCR assay, indicating the absence of seed transmission of HLB pathogen (Table 5.10) (Hartung et al. 2010).

Huanglongbing (HLB) disease of *Citrus* has been shown to be caused by three species, namely “*Candidatus Liberibacter asiaticus*,” “*Ca. L. africanus*,” and “*Ca. L. americanus*” in different geographic locations. Although real-time PCR assay was useful for detection of the bacterial species associated with HLB disease, nucleic acid purification



Figure 5.15 Detection of huanglongbing (HLB) infection by biological tests on sour orange seedlings S389, S433, and S391 from left, and healthy sour orange seedlings of the same age on the right. Source: Hartung et al. (2010). Reproduced with permission of the American Phytopathological Society, MN, USA.

Table 5.10 Detection of “*Ca. Liberibacter asiaticus*” in seedlings by qPCR assay at different time intervals between planting seed and DNA extraction and testing (Hartung et al. 2010). Total number of tests (1216) performed from January 2006 to December 2006.

Source ^b	Interval (months between planting seed and testing) ^a				
	Seedlings	6–8	20	25	35
Muraya	11	0/2	0/1	0/8	Dead
Rough lemon	106	0/106	ND	0/101	0/99
Sour orange	84	0/79	0/84	0/81	0/82
Grapefruit	15	0/14	0/13	0/14	0/15
Tangerine	18	ND	0/17	0/18	0/17
Meyer lemon	20	0/19	0/20	0/19	0/18
Valencia	109	0/106	0/83	0/106	0/104
Total ^c	363	326	208	347	335

^a No. of positive qPCR tests/no. of tests performed; ^b All source trees were highly symptomatic and PCR-positive; ^c Total number of seedlings and tests performed at each interval.

steps limit the number of samples that could be processed by PCR assay. Universal detection of “*Ca. Liberibacter*” species could be achieved by direct tissue-printing and spotting of plant leaf petiole extracts, or squashing of individual psyllids onto paper or nylon membranes. Primers were designed and used with TaqMan chemistry for precise detection of HLB pathogen in immobilized targets (prints of ten overlapping leaf petioles/tree or squashed single psyllid vector insect) by extraction with water and direct use for real-time PCR. This simple procedure could detect HLB liberibacters in 100% of leaves with symptoms and 59% of symptomless leaves collected from HLB-infected trees. The direct assays, as template, showed good agreement with those obtained with purified DNA. The squash assay allowed detection of HLB pathogen in 40% of adult psyllids (*Diaphorina citri*) that fed on leaves of HLB-infected trees with or without symptoms. A commercial ready-made kit based on this technology showed 96% accuracy in intra-laboratory performance studies. The protocol developed in this investigation might be useful for certification programs and epidemiological investigations (Bertolini et al. 2014).

“*Candidatus Liberibacter solanacearum*,” the incitant of zebra chip disease of potato tubers, is a major economic threat to potato production and marketability because of the tuber discoloration in the form a light striped pattern that darkens on frying for chip production. The primers CL-16S-F and CL-16S-R designed from 16S rRNA sequences of the pathogen were designed and a 1695 bp product, including the 1418 bp full-length 16S rRNA region, was amplified from multiple samples of ZC symptomatic tubers from field-collected potato psyllids (*Bactericera* (= *Paratrioza*) *cockerelli*) exposed to ZC-affected potato plants. No product of expected size was amplified, using DNA template from psyllid nymphs, healthy potato, clean psyllids, or PCR-negative controls. Further, the 16S rRNA gene sequences were amplified from DNA samples of all three HLB-associated “*Ca. Liberibacter*” spp. The 16S rRNA sequences amplified from ZC-diseased potato and potato samples were entirely (100%) identical. The ZC-associated 16S rRNA gene sequences were compared with those from “*Ca. L. americanus*” (Lam), “*Ca. Liberibacter asiaticus*” (Las), and “*Ca. L. africanus*” (Laf), and were 93.7–94% identical to sequences from “*Ca. L. americanus*” (Lam), associated with the huanglongbing (HLB) disease infecting citrus. The results indicated the extent of relatedness among the bacterial pathogens infecting potato and citrus (Lin et al. 2009).

The identity of the causative agent of a new disease of glasshouse-grown tomato and pepper occurring in New Zealand was investigated. The presence of phloem-limited bacterium-like cells was observed in the infected plants under transmission electron microscope (TEM). Specific prokaryotae PCR primers were employed in combination with universal 16S rRNA. Sequence analysis of the 16S rRNA gene, the 16S/23S rRNA spacer region, and the *rplKAJL-rpoBC* operon revealed that the new bacterial pathogen shared high homogeneity with “*Candidatus Liberibacter*” species. The bacterium infecting tomato and pepper had characteristics distinct from the three *Liberibacter* species infecting citrus species. The new bacterial pathogen was named “*Candidatus Liberibacter solanacearum*,” based on the sequence similarity to the pathogen causing zebra chip (ZC) disease of potato. A specific PCR primer was developed for the reliable detection of the new bacterial pathogen infecting tomato and pepper (Liefting et al. 2009).

In another investigation, samples of ZC-affected potato tubers from different locations in the USA, Mexico, Guatemala, and Australia were tested. The PCR-amplified 16S rDNA sequences were almost identical to the 16S rDNA of “*Ca. L. solanacearum*” which was reported earlier from solanaceous plants in New Zealand and the USA.

"*Ca. L. solanacearum*" was detected by a rapid PCR protocol developed in this investigation; results could be obtained in less than 90 min. The association of "*Ca. L. solanacearum*" with ZC disease of potato tuber was confirmed (Secor et al. 2009).

"*Candidatus Liberibacter*" strains occurring in North America associated with potato zebra chip disease complex were studied for their genetic diversity. By applying conventional PCR and using pathogen-specific primers and probes and real-time PCR formats, "*Ca. L. solanacearum*" and "*Ca. L. psyllauros*" were detected in potato. "*Ca. Liberibacter*" species was divided into two clusters, showing a similarity of 99.8% in their partial 16S rRNA gene sequences and 99.3% in their partial intergenic spacer region ISR-23S rRNA gene sequences. Genetic variation in the 16S rDNA consistently matched that of the ISR-23S rDNA region. In this partial 16S-ISR-23S rDNA region, eight single nucleotide polymorphisms were detected among "*Ca. L. psyllauros*" and "*Ca. L. solanacearum*" strains examined. These two species of "*Ca. Liberibacter*" were very closely related. The results provided the basis for fixing the current taxonomic status of "*Ca. L. solanacearum*" and "*Ca. L. psyllauros*" associated with the zebra chip disease complex affecting potatoes (Wen et al. 2009).

"*Candidatus Liberibacter solanacearum*," the incitant of zebra chip (ZC) of potato tubers, was detected and quantified in different parts of potato plant and tuber by employing quantitative PCR assay. Potato plants were inoculated by allowing psyllids (two insects/leaf) access for 7 days. The pathogen was detected by employing 16S rDNA specific primers, LsoF and HLBr, and cytochrome oxidase primers, COXf and COXr, for host plant (positive internal control). "*Ca. L. solanacearum*" was detected in leaves and noninfected stem when stems remained joined via tuber. Quantitative PCR assay showed that bacterial populations were frequently low in potato samples (<genomic units per nanogram of DNA). The conventional PCR format was somewhat unreliable relative to qPCR assay in detecting the pathogen in potato, even in symptomatic plants. The results indicated that unless an appropriate sampling procedure was applied, the pathogen might not be detectable by conventional or qPCR assays until 3 weeks after infestation, particularly if insect pressure was low (Levy et al. 2011).

Detection of "*Candidatus Liberibacter solanacearum*" using the primers designed based on the sequences of 16S rRNA was found to be variable and less consistent. In order to improve reliability and consistency, new primers that target a conserved intergenic region between the 16S and 23S rDNA genes and a conserved bacterial housekeeping gene, adenylate kinase (*adk*), of "*Ca. L. solanacearum*" were developed. The two newly designed primer pairs Lso Tx16/23 F/R and Lso *adk* F/R were able to amplify a 383bp region of "*Ca. L. solanacearum*," 16S/23S intergenic spacer, and a 770bp product from the pathogen genomic region containing the *adk* housekeeping gene, respectively. Both primer pairs detected "*Ca. L. solanacearum*" in different plant parts, producing single bands of the expected size. Overall, detection rates were 49.1% for the LsoF/OI2c primer pair (employed earlier) compared with 69.5% for the LsoTx 16/23 F/R and Lso *adk* F/R primer pairs. No amplification occurred in the extracts of greenhouse-grown psyllid-free plants in PCR reactions with both primer pairs. Both LsoTx 16/23 F/R and Lso *adk* F/R primer pairs were more sensitive than the LsoF/OI2 primer pair. The low detection limit for the new primers was four times lower (0.65 ng) than the limit (2.5 ng) for the LsoF/OI2 primer pairs employed in earlier investigations. The results indicated that use of the new primer pairs might improve the sensitivity and reliability of detection of "*Ca. L. solanacearum*" in the field as well as in the laboratory, contributing to more effective management of ZC disease of potato (Ravindran et al. 2011, 2012).

Table 5.11 Influence of temperature range on zebra chip disease development in inoculated potato plants (Munyanenza et al. 2012).

Effect on ZC symptom	Temperature range (°C)		
	12–17	25–30	30–37
Total number plants exposed	12	12	12
No. of plants with ZC symptoms	12	12	4
Plants PCR-positive for <i>Liberibacter</i>	12	12	4
Time to onset of symptoms ^a	21.5 ± 0.9	18.0 ± 0.0	24.0 ± 0.0
Time to plant death ^b	27.5 ± 0.6	24.0 ± 0.0	30.0 ± 0.0

^a Time (days) required for expression of ZC disease symptoms in potato plants exposed to different temperature range following inoculation using infective psyllids; ^b Time (days) taken for ultimate death of inoculated plants; control senesced at about 90 days after transfer to growth chamber maintained at different temperature range.

A PCR-based assay was applied to investigate the influence of temperature on the development of potato zebra chip (ZC) disease caused by “*Candidatus Liberibacter solanacearum*.” Potato plants were exposed to *Liberibacter*-infective psyllids at various temperatures under laboratory conditions. The inoculated potato plants were maintained at selected temperature regimes in growth chambers observed for expression of ZC disease symptoms and tested by PCR assay to confirm the infection by the pathogen. All inoculated potato plants maintained at 12–17°C, 20–25°C, and 27–32°C developed ZC symptoms and tested positive for the presence of “*Ca. Liberibacter solanacearum*” by the PCR format. None of the plants maintained at 32–35°C or 35–40°C developed symptoms of ZC disease or tested positive for *Liberibacter* by PCR assay (Table 5.11). The results revealed the adverse effects of temperatures above 32°C on the development of ZC pathogen and consequently the symptom expression. The sensitivity of the bacterial pathogen and its insect vector might be partially responsible for variations in disease incidence, severity, and distribution of ZC disease of potato in different locations (Munyanenza et al. 2012; Section 5.4.11).

A high-fidelity PCR format was developed for detecting “*Candidatus Liberibacter solanacearum*” (*Lso*) using a proof-reading enzyme with 3′–5′ exonuclease activity. This PCR procedure could detect *Lso* at low concentrations and when large amounts of competitor DNA or other host cellular contents capable of inhibiting PCR amplification were present. Assessment of the comparative efficacy of high-fidelity PCR and standard PCR procedures showed that the high-fidelity PCR was able to detect *Lso* in higher percentages of symptomatic tubers (30–40%) than the standard PCR format (Cating et al. 2015).

Modifications in the standard PCR assay have been introduced to improve the effectiveness, sensitivity, and reliability of the detection of bacterial pathogens whose concentration may be variable or below the detection limit of the standard PCR format. Repetitive-sequence (rep-) PCR genomic fingerprinting is based on PCR-mediated amplification of DNA sequences in prokaryotic genomes. Isolates of *Ralstonia solanacearum*, causative agent of bacterial wilt diseases in ginger, curcuma, and moiga, were subjected to REP and BOX-PCR analysis using selected strains infecting these

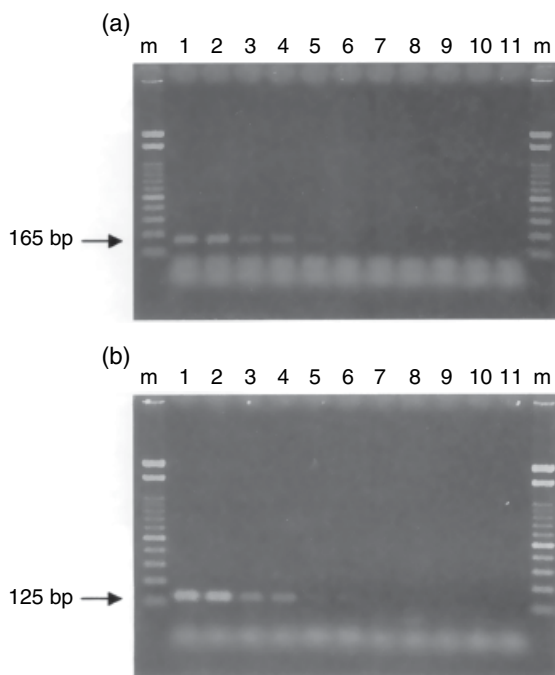


Figure 5.16 Detection of two strains of *Ralstonia solanacearum* by PCR using two primer sets AKIF-AKIR and 21F-21R: (a) MAFF 211490 and (b) MAFF 211471. Lanes 1 and 2: 2.2×10^4 CFU; lanes 3 and 4: 2×10^3 CFU; lanes 5 and 6: 6.2×10^2 CFU; lanes 9 and 10: 2 CFU; lane 11: control without template; lane m: DNA ladder marker (100 bp). Source: Horita et al. (2004). Reproduced with permission of Springer Science, Heidelberg, Germany.

host plant species in Japan, Thailand, Indonesia, Australia, and China. Two primer sets, AKIF-AKIR and 21F-21R, designed from the sequences that had no homology with the known bacterial genes, were employed. The AKIF-AKIR primer set amplified a single band (165 bp) from genomic DNA obtained from moiga, curcuma, and ginger isolates that represented type I rep-PCR fingerprints. The 21F-21R primer set amplified one band (125 bp) from ginger isolates that represented type II fingerprints. Only genomic DNA from *R. solanacearum* race 4 yielded the expected amplified products. The specificity of the primers was revealed by the absence of amplification of DNA from other *Ralstonia* spp. The protocol developed in this investigation was efficient in identifying race 4 isolates of *R. solanacearum* (Fig. 5.16; Horita et al. 2004). Representative Japanese *R. solanacearum* race 1 and 3 biovar 2, 3, and 4 strains from other hosts were examined. The DNA profiles obtained with BOX-PCR were highly reproducible. Two distinct types of DNA fingerprints based on the presence or absence of a few bands were recognized among the Japanese strains from plants belonging to Zingiberaceae family. Both DNA patterns were similar to the strains from hosts belonging to families other than Zingiberaceae (Tsuchiya et al. 2005).

Large quantities of ornamental and food plant propagative stocks are imported into North America and several other countries. *Ralstonia solanacearum* (*Rs*) was detected in these plant materials, which were frequently asymptomatic. *R. solanacearum* strains (107) were collected over a period of 10 years from imported propagative

stock and compared with 32 strains characterized earlier, using repetitive (rep-) PCR element (BOX, ERIC, and REP) primers. Additional strain comparisons were taken up by sequencing the endoglucanase and the cytochrome *b561* genes. Populations of *R. solanacearum* were distinguished by biovar using rep-PCR primers and, to a limited extent, country of origin and original host. Similarity coefficients among rep-PCR clusters within biovars were relatively low in many cases, indicating that the disease outbreaks over time might have been induced by different clonal populations. Similar population differentiations of *Rs* were accomplished by comparing strains sequences, using either the endoglucanase or cytochrome *b561* genes. Most of the new biovar 1 strains of *Rs* entering the USA were genetically distinct from biovar 1, already present in the vegetable crops. The new biovar 1 strains showed extended host range, being able to infect anthurium in addition to potato, tomato, and tobacco. Further, pothos and banana were also infected by new biovar 1 strains. All introductions into North America of race 3, biovar 2 strains were found to be linked to geranium production and appeared to be clonal. Rep-PCR assay could distinguish biovar 3 from biovars 1 and 2 of *R. solanacearum* (Norman et al. 2009).

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), causative agent of tomato bacterial canker disease, is one of the most important pathogens capable of reducing yields in greenhouse-grown crops. Strains of *Cmm* could be analyzed by rep-PCR by employing either ERIC or BOX primers. The strains of *Cmm* were placed in four groups: A, B, C, and D. Results obtained by using pulsed-field gel electrophoresis (PFGE) analysis were similar in grouping the strains of *Cmm*. The *Cmm* strains from Israel were highly homogenous (>92% similarity) within each of the four groups. PCR-based detection of three genes located on different sites of the pathogenicity island (PAI) revealed that all tested pathogenic strains of *Cmm* were positive for the presence of *ppaA*, *chpC*, and *tomA*, whereas nonpathogenic strains lacked *ppaA* and *chpC* but not *tomA*, indicating the requirement of *ppaA* and *chpC* for virulence of *C. michiganensis* subsp. *michiganensis* (Kleitman et al. 2008). Disbudding and defoliation contribute substantially to the secondary spread of the disease in commercial greenhouse. Strains of *Cmm* (180) collected during 2005–2008 in different locations in Okayama Prefecture, Japan were fingerprinted by repetitive-sequence (rep-) PCR assay with ERIC and BOX primers. The strains in each greenhouse and location belonged to the same haplotype with no recognizable effect due to location or cultivar of tomato grown. The results suggested that the strains originated from the previous greenhouse population of *C. michiganensis* subsp. *michiganensis*, existing on residual plant debris, rather than the introduction of *Cmm* via infected seeds (Kawaguchi et al. 2010).

The occurrence of false negatives in samples of planting materials may limit the use of PCR assays. In order to overcome this situation, a competitive-PCR technique may be useful to eliminate the risk of false negatives for the detection of bacterial pathogens. A competitive PCR format was employed to detect and quantify *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) present in potato tubers. An internal standard DNA template was generated by the amplification of *Arabidopsis* (model plant) genomic DNA under low annealing temperature with primers specific for *Cms* to serve as control for all PCR assays. The 450 bp product amplified from the internal standard DNA template was distinct from the 250 bp product, characteristic of *Cms*. The ratio of the PCR amplicons in the presence of a constant amount of internal DNA template increased with increasing amount of *Cms* DNA. The PCR product ratios obtained from bacterial cells in cultures and in tissues of inoculated potato plantlets were corroborated by the cell

numbers estimated by immunofluorescence antibody staining (IFAS) technique. The competitive PCR format was more sensitive (10-fold) than IFAS procedure and this PCR format could detect as few as 100 pathogen cells (Hu et al. 1995).

A competitive PCR assay was employed for the detection and quantification of "*Candidatus Liberibacter asiaticus*," incitant of huanglongbing (HLB) disease of citrus. Competitive DNA was generated from a DNA fragment amplified by rep-PCR procedure with DNA extracted from HLB-infected citrus plants. The PCR primer sequence for primer pair OI1/OI2c was designed for the specific amplification of 16S rRNA gene sequence of "*Ca. Liberibacter* (CL)." With an increase in the number of amplification cycles to 45, the sensitivity of detection was increased. As the amplification of target DNA and competitor DNA occurred in the same tube, the inhibitory activity of compounds coextracted with host DNA was reduced to the maximum extent. The amounts of CL varied between citrus varieties, with cultivar Cam Sanh having a greater pathogen concentration than cultivar Nam Roi. A lower risk of error resulting from differences in amplification efficiencies due to inhibitors and requirement of less expensive equipment and chemicals are the advantages of comp-Rep over real-time PCR assay (Kawabe et al. 2006).

The comparative efficiency of single and duplex conventional PCR formats was assessed for the rapid and accurate detection of "*Candidatus Liberibacter asiaticus*" (*Las*), causative agent of citrus huanglongbing (HLB) disease in plant materials. Symptomatic and asymptomatic leaves of *Citrus* spp. from commercial citrus groves and private gardens in Thailand were tested by extracting total DNA of citrus leaf mid veins following standard CTAB procedure. Single conventional PCR assay was performed, using outer membrane protein (*omp*) gene with forward primer OMP/TH1 and reverse primer OMP/TH2, and DNA polymerase (*phpol2*) gene with forward primer PhPol2F and reverse primer PhPol2R, resulting in a 981 bp and 710 bp product, respectively. Duplex conventional PCR gave two bands of 981 bp and 710 bp at the same size as single conventional PCR assay. Detection accuracy of *omp* and *phpol2* genes by single and conventional PCR techniques was 100% and 85.7%, respectively, when compared with *rplJ* gene as a standard method. Detection of the pathogen in 61 citrus samples by single conventional PCR of *omp* and *phpol2* genes by single and duplex conventional PCR format provided *Las*-positive results for 72.13%, 80.33%, and 78.69%, respectively. As duplex conventional PCR assay could detect *Las* in tests using both genes or each gene separately, it was considered to be more effective. The detection limit of total DNA with *omp* and *phpol2* genes by single conventional PCR was 10^{-2} ($1.6 \times 10 \mu\text{g}$) dilution and 10^{-6} ($2 \times 10^6 \mu\text{g}$) dilution, respectively. The use of two genes in a duplex conventional PCR was more reliable and was less costly than two separate single conventional PCR reactions, and has the potential for use in certification programs (Donnua et al. 2012).

Geranium plants are infected by leaf spot pathogen *Xanthomonas campestris* pv. *pelargonii* (*Xcp*) and wilt pathogen *Ralstonia solanacearum* (*Rs*). Asymptomatic infections by these bacterial pathogens were found to be important reservoirs of inoculum in commercial growing facilities. A multiplex PCR assay capable of detecting infection by either or both of these pathogens was developed. The primer pair for *Xcp* amplified a region of 200 bp, whereas the primer pair for *Rs* amplified a region of 822 bp. By employing the two primer pairs, either *Xcp* or *Rs* or both pathogens could be detected. As geranium plants contained inhibitors of PCR amplification, "amplification competence" primers targeting a portion of the geranium 18S rRNA gene were designed; these primers

amplified a 494bp product and validated the negative assay result. The triple primer pair multiplex PCR format could be employed to screen the geranium plants for infections by the two most important bacterial pathogens of geranium and to confirm amplification competence for each geranium sample (Glick et al. 2002).

The sensitivity and specificity of detection of bacterial pathogens in propagules may be enhanced by integrating PCR assay with other diagnostic methods. Based on a sequential nested amplification by PCR of a region of plasmid DNA that was found to be highly conserved in *Xanthomonas axonopodis* pv. *citri* (*Xac*), a highly sensitive nested PCR format was developed. The amplified PCR products were detected colorimetrically by detection of immobilized amplified nucleic acid (DIANA) procedure which uses labeled primers to allow amplification product capture and detection in a microtitre plate. Amplification of predicted PCR products occurred from the DNAs of all strains of *Xac* and also from six strains of *X. axonopodis* pv. *aurantifolii* (*Xaa*), but not from *X. axonopodis* pv. *citrumello*, incitant of citrus bacterial spot disease and other xanthomonads. This procedure was 50–100 times more sensitive than the standard PCR format. Immunomagnetic separation (IMS) of target bacterial pathogen prior to amplification by PCR was employed to concentrate and recover *Xac* from the extracts of infected plant tissues. The sensitivity of the assay was further improved, by over 100-fold, by immunocapture (IC-) PCR combination by concentrating *X. axonopodis* pv. *citri* from dilute plant extracts over nested PCR format. The integration of different techniques was shown to be more effective in detecting the canker pathogen, even when the concentration was low (Hartung et al. 1996).

Xylella fastidiosa (*Xf*) causes grapevine Pierce's disease and also infects citrus, almond, and coffee plants. The bacterial pathogen is limited to xylem tissues. A pressure chamber was used to obtain xylem fluids to reduce the effects of inhibitors and consequently to enhance sensitivity of detection of *Xf*. A purification procedure involving the use of immunocapture (IC) was applied prior to PCR amplification. The *Xf* suspension (mixture of pellets of *Xf* and specific antibodies) were used as templates. Two target fragments of 511 and 733bp were amplified from various xylem exudates samples. The pathogen-specific 733bp fragment could be detected in weakly infected grapevine plants. The IC-PCR assay might be used to detect the pathogen in xylem exudates even before visible symptoms were expressed (Guo and Lu 2004). In another investigation, *X. fastidiosa* was detected by IC-PCR procedure. The *Xf* cells were captured by antibody coated magnetic immunocapture beads, followed by amplification of *Xf*-specific DNA fragments by PCR assay. IC-PCR format and ELISA test were equally effective in detecting *Xf* from naturally infected grapevine plants. The ELISA test was therefore recommended for use under field conditions for detection of *X. fastidiosa* on grapevines, being easier, less expensive, and faster than the IC-PCR technique (Costa et al. 2004).

The presence of inhibitory compounds often affects the sensitivity of detection of *Xylella fastidiosa* (*Xf*), causing Pierce's disease, in ground grape tissue by standard PCR assays. The effect of immunocapture (IC) and nested primers (N) on the sensitivity of PCR assay was assessed by using a 10-fold dilution series of aliquots of standardized *Xf* suspensions in healthy grapevine tissue extracts. Standard and nested PCR formats, not preceded by immunocapture (IC) step, did not detect the pathogen in the suspension serially diluted. In contrast, a combination of the immunocapture (IC) procedure and standard primers resulted in a successful amplification of pathogen-specific DNA fragment, with a sensitivity of 10^4 cells mL⁻¹ of grape extract. N-PCR combination further increased the sensitivity of the pathogen detection by 10,000-fold over standard PCR

primers. The combined IC-N-PCR procedure achieved a maximum sensitivity of 2 CFU mL^{-1} of bacterial cells in grape leaf extract. Another method was developed for specific amplification of genomic fragments of *Xf* with nucleic acid deposition on small pieces of charged nylon membrane, by spotting crude infected grapevine sap (spot-PCR) in combination with the nested primers. Spot-nested (Sp-N-) PCR format was as sensitive as IC-N-PCR procedure when different dilutions of grapevine tissue extracts were used for testing (Buzkan et al. 2003).

Another grapevine disease caused by bacterial pathogen *Xanthomonas campestris* pv. *viticola* (*Xcv*) was detected by including a pre-enrichment step prior to PCR amplification, employing the primer pairs *Xcv*1F/*Xcv*3R and *RSt*2/*Xcv*3R based on the partial sequences of *hrpB* gene. The *Xcv* was detected in petioles of artificially inoculated plants. Detection of *Xcv* was not possible when the suspensions prepared from macerated tissues were added directly to the PCR reaction mixture. In contrast, with a pre-enrichment step prior to PCR amplification, only DNA from viable bacterial cells was used for amplification. It was only possible to detect *Xcv* by BIO-PCR assay in samples from symptomatic petioles of inoculated grapevine plants. PCR was performed on aliquots of plate wash obtained from symptomatic tissues and cell suspensions from a single suspect colony. Amplification occurred on both plate washes and cell suspensions. The identity of the bacterium was established by *Hae*III digestion of PCR products. The restriction fragment length polymorphism (RFLP) analysis revealed the similarity of the restriction profiles of the samples to that of *X. campestris* pv. *viticola*. As the BIO-PCR procedure provided the results much earlier than the isolation method, the BIO-PCR format could be employed as an effective tool for monitoring *X. campestris* pv. *viticola*-infected or -contaminated plant materials (Trindade et al. 2007).

Polymerase chain reaction (PCR) may be applied in tandem with an immunoassay to enhance the sensitivity, rapidity, and effectiveness of detection of bacterial plant pathogens in propagules. The immunoenzymatic detection of PCR-amplified products, such as in the PCR-ELISA technique, is useful for circumventing the need to perform electrophoresis, image capture, and the other steps required. A multiplex PCR-ELISA procedure was developed for detection of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), causative agent of potato ring rot disease. The assay is based on primers for amplification of three single-copy unique DNA sequences: *Cms*50, *Cms*72, and *Cms*85. These three sequences were simultaneously amplified from the genomes of all 42 strains of *Cms* that were tested, including variant mucoid forms, but not from strains representing five related subspecies and other bacteria tested. The lowest detection limit by electrophoresis was estimated to be approximately 300 CFU mL^{-1} when bacterial cells were spiked into potato core fluid, but sensitivity increased by approximately 10-fold using PCR-ELISA. An engineered sea anemone DNA fragment was included so that it could be amplified from the *Cms*72 primer set to provide the simultaneous signal, indicating proper functioning of the assay system when the control sample (free of *Cms*) was used. The addition of hydrolyzed casein to the reaction mix markedly reduced or eliminated inhibition of PCR by inhibitors. The multiplex PCR-ELISA procedure could be reliably used for the detection of *C. michiganensis* subsp. *sepedonicus* in both stem and tubers because of the amplification of multiple sites in its genome, providing absolute specificity and a higher level of sensitivity (Mills and Russell 2003).

Strains (20) of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) from different geographical locations and reference strains of the same or different species were analyzed by applying the two primers random amplified polymorphic

DNA (TP-RAPD) procedure. Two primers were used to amplify the 16S rDNA gene of *Cms*. At 45°C of annealing, the PCR product electrophoresed in agarose gels produced a band pattern that was different in all bacterial species and subspecies of *C. michiganensis* investigated. TP-RAPD produced fingerprints characteristic of the strain/species/subspecies. All strains of *Cms* displayed the same number of TP-RAPD patterns. The Gram-positive bacteria such as *Clavibacter* with high G + C content produced low bands in TP-RAPD analysis. By using a different set of two primers also based on the 16S rDNA sequence from *Escherichia coli*, a more adequate amplification of Gram-positives of high G + C including a greater number of bands was observed. The results indicated that strains of *C. michiganensis* subsp. *sepedonicus* could be reliably and rapidly detected and identified by TP-RAPD patterns using set of two primers for PCR amplification (Rivas et al. 2002).

By subjecting the PCR amplicons to random fragment length polymorphism (RFLP) analysis, some of the bacterial pathogens may be detected and identified precisely. *Erwinia carotovora* (EC) subspecies could be detected by PCR-RFLP analysis based on a pectate lyase-encoding gene (*pel*), since pectate lyases play an important role in the development of soft rot diseases. The sensitivity of the PCR-RFLP analysis could be significantly enhanced by including a 48 h enrichment step prior to PCR amplification. The presence of *E. carotovora* subsp. *atroseptica* (*Eca*) could be detected reliably and efficiently in wash water, leaves, stem, and tuber peel extract (Helias et al. 1998). PCR-RFLP analysis was applied for the detection and differentiation of strains of *E. carotovora* subsp. *carotovora* based on the *pel* gene sequence, using *Sau3AI* restriction enzyme. Three patterns (1, 2, and 3) were generated by the strains of *E. carotovora* subsp. *carotovora* (Seo et al. 2003). Three strains of *E. carotovora* from mulberry with RFLP pattern 3 of the *pel* gene produced the expected size of c. 430 bp product. The PCR amplification of these strains allowed them to be assigned to *E. carotovora* (Seo et al. 2004).

PCR-based techniques either alone or in combination with other detection methods have been shown to be useful for detecting bacterial pathogens for which the isolation method and immunoassays were not effective or unavailable. Enzyme-linked oligonucleotide assay (ELOSA) was applied for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), causing potato ring rot disease. PCR amplification was achieved by employing primer sets *Cms50* and *Cms72* designed through subtractive hybridization procedure. The sensitivity of ELOSA technique seemed to be equally or more sensitive in detecting all strains of *Cms* when compared to gel electrophoresis for evaluation PCR amplicons, using either primer. In bacterial suspension, fewer than 3 cells/10 μL^{-1} of reaction mix were detected after PCR amplification and hybridization with specific-digoxigenin (DIG-) labeled DNA probes in ELOSA test. The diagnostic sensitivity for detecting *Cms* in naturally infected tubers of three potato cultivars was 96%, while the specificity of detection was more than 99%. Colony morphology of *Cms*, primer sets, or potato cultivar did not have any influence on the detection of *C. michiganensis* subsp. *sepedonicus* in naturally infected potato tubers from commercial production areas. As a high-throughput method, PCR-ELOSA protocol utilizing two specific DNA sequences of the ring rot pathogen was shown to provide a higher level of reliability than standard PCR format and ELISA tests (Baer et al. 2001).

Compared with standard PCR format, real-time PCR is simpler to perform, less labor-intensive, and much faster at providing results (in about 60 min). Real-time PCR with TaqMan probes that utilize the 5' exonuclease activity of *Taq* polymerase to

separate quencher and fluorophore moieties on labeled probes permits measurement of the amplified product as the reaction proceeds. By adding a DNA-binding dye such as SYBR Green, postreaction melt analysis of amplicons may also be conducted to confirm amplicon identity directly in the reaction tube. A quantitative real-time (QRT-) PCR protocol involves the use of the point in time when amplification of the target is first detected to estimate bacterial concentration. Fluorescence can be generated by nonspecific dyes such as SYBR Green or by fluorogenic probes. During amplification, a specific DNA probe conjugated with a reporter and quencher dye hybridizes with target sequence and is degraded by *Taq* polymerase. After degradation, reporter and quencher are separated, resulting in the emission of a fluorescent signal of which the accumulation is monitored during the consecutive PCR cycles. The cycle at which the fluorescence signal exceeds a certain background fluorescence level, known as the threshold cycle (*Ct*), is directly proportional to the quantity of target DNA present in the sample (Gibson et al. 1996).

A fluorogenic (TaqMan) PCR assay was developed for the detection of *Ralstonia solanacearum* (*Rs*), causing brown rot disease of potato tubers and bacterial wilt diseases of several crops. Asymptomatic potato tubers carrying the pathogen form potential sources of inoculum for the spread of the disease to different locations and also subsequent generations. Two fluorogenic probes were utilized in a multiplex reaction: one broad range probe (*Rs*) detected all biovars of *Rs*; and a second more specific probe (B2) detected only biovar 2. Amplification of the target was measured by the 5' nuclease activity of TaqDNA polymerase on each probe, resulting in the emission of fluorescence. Detection limit of the assay was $\geq 10^2$ cells mL⁻¹ in pure cultures. When inoculated potato tissue extract was tested, the sensitivity of the assay was reduced. The fluorogenic probe (COX) designed with potato cytochrome oxidase gene sequence was used as an internal control and detected potato DNA in an *Rs*-COX multiplex TaqMan PCR assay with infected potato tissues. The specificity and sensitivity of the TaqMan assay showed that this robust test could yield results rapidly and reliably. A multiplex TaqMan method specific for the detection of all strains of *R. solanacearum*, as well as race 3 biovar 2, permitted analysis in real-time within a closed tube system, reducing the risk of cross-contamination between samples. This protocol provides advantages in routine indexing of potato tubers and other plant materials in detecting the presence of *R. solanacearum* (Weller et al. 2000).

A real-time PCR primer and probe and a highly sensitive BIO-PCR format were developed for the efficient detection of *Rs* in tubers with latent infection by *Rs* strains of race 3, biovar (bv) 2. The biovar-specific primers and probe reacted with all 17 strains of bv 2 of *Rs* tested, including 12 from potato and 5 strains from geranium. None of the other strains of *Rs* reacted, including four strains of bv 1 from potato or the closely related blood disease bacterium infecting banana. None of the 13 other bacteria including *Erwinia* sp. and *Clavibacter* spp. associated with potato diseases showed positive reaction. Using undiluted potato tuber extract inoculated with *Rs* bv 2, as few as 30 cells mL⁻¹ of extract could be detected. The real-time BIO-PCR assay detected the pathogen effectively in 2 of 14 naturally infected potato tubers without any visible symptoms. In contrast, standard real-time PCR format did not detect *R. solanacearum* in the same samples. The real-time BIO-PCR assay was found to be simpler, faster, and more reliable compared to standard PCR format. Real-time BIO-PCR assay may therefore be preferable when accuracy and reliability of results are demanded (Ozakman and Schaad 2003).

Ralstonia solanacearum (Rs), causing ginger bacterial wilt disease, adversely affects ginger production in Thailand and other Asian countries. The comparative efficacy of real-time PCR and real-time BIO-PCR formats for detecting the pathogen was assessed. A unique 329bp DNA fragment from Rs biovar 4 isolated from ginger rhizomes was identified using amplified fragment length polymorphisms (AFLPs), and the nucleotide sequence was determined. PCR primer and probe sequences were designed for real-time PCR assays and screened against strains of Rs. The primers RSAF1 and RSAR1 and probe RSP1 reacted with all strains of Rs race 1 biovars 3 and 4, but not with biovars 1 and 2. Real-time TaqMan® PCR protocols were developed for two real-time PCR platforms: the ABI7700 sequence detection system (Applied Biosystems, CA); and portable Smart Cycler (Cepheid, CA). Detection efficiency of classical real-time PCR and real-time BIO-PCR formats were determined using 15 asymptomatic rhizomes from fields/markets. The classical real-time PCR and real-time BIO-PCR assays detected *R. solanacearum* in 13 and 9 of 15 rhizomes tested, respectively. The results indicated that asymptomatic ginger rhizomes were marketed, posing a significant risk of spreading the disease to locations where the disease might be absent (Thammakijawat et al. 2006).

Several strains of *Ralstonia solanacearum* (Rs) have been isolated from many crops including potato and geranium. The strains of Rs were detected by PCR assay and quantified by employing the real-time PCR format. Using universal primer pair 759/760, the PCR amplified the expected 280bp fragment from all 15 strains tested. The race 3 was, however, not amplified by the primer set, requiring the race-specific primer set 630/631 for its amplification, indicating that none of the Florida isolates belonged to the quarantine subgroup R3B2. A quantitative real-time PCR assay using the universal Rs primer/probe set amplified the expected Rs target DNA sequences from the strains tested. Real-time PCR format, using the biovar 12-specific B2 primer/probe, also amplified the expected target sequence from the biovar 2 reference strains as well as biovar/strain (potato isolate from Peru). Since a “zero tolerance” level for infection of potato and geranium by *R. solanacearum* is adopted, it is essential to accurately detect and identify both race 1 and race 3 strains so that production of pathogen-free stocks may be achieved successfully (Ji et al. 2007).

The usefulness of diagnostic techniques depends on their ability to offer adequate provision for validating negative results as well as confirming positive results when applied by regulatory agencies. For the detection of *Ralstonia solanacearum* in potato tubers the negative results could be validated through the use of reaction control plasmid, designated pRB2C2, which was designed to generate a 94 bp product using the same amplicons targeting primary diagnostic 68bp sequence in *R. solanacearum* race 3 bv 2 DNA. SYBR Green dye was included in the reaction mix to facilitate the identification of postreaction products, using a melt peak analysis. The reaction control (94 bp) and diagnostic target (68 bp) amplicons had melt peak temperatures of about 90°C and 83°C, respectively. The positive results of real-time PCR assay could be confirmed and distinguished from reaction control product. The modified TaqMan assay protocol developed in this investigation reliably and consistently detected *R. solanacearum* race 3 bv 2 in infected asymptomatic potato tubers and stems, and also in stems and leaves of tomato plants (Smith and De Boer 2009).

Strains of *Ralstonia solanacearum* (Rs) show extensive genetic diversity. Based on multilocus DNA sequence typing, *R. solanacearum* is considered to be a species complex divided into four groups known as phylotypes which are subdivided into sequevars, based on the DNA sequence of the endoglucanase gene *egl*. Using the host

range/differential host responses as the basis, strains of *Rs* are classified into five races or six biovars loosely based on their ability to produce acid from several carbohydrate substrates. There is no general correlation between races and biovars, except that race 3 and race 5 strains are usually grouped as biovar 2 and biovar 5, respectively. The strains of race 3 biovar 2 (R3BV2), although generally limited to the tropics and subtropics, could cause disease in cooler tropical highlands and temperate regions. To prevent/reduce the incidence of R3BV2 in the USA and for quarantine screening for R3BV2 strains, a rapid and robust assay capable of detecting small populations of the pathogen in plant tissues or soil was considered essential. A real-time PCR assay was developed for accurate detection and quantification of R3BV2 populations of *R. solanacearum*. This assay was able to detect *Rs* populations of >1000 cells mL^{-1} . Since the inhibitory compounds were present in plant and soil samples, immunomagnetic separation (IMS) or magnetic capture hybridization (MCH) method was used to concentrate the populations of *R. solanacearum* before application of real-time PCR format. The primer set specific to R3BV2, targeting the RRSL_2403 gene of strains UW551, was able to detect as few as five R3BV2 cells in 2 h. This format was as sensitive as plating and approximately 25 times faster. By concentrating *R. solanacearum* cells, IMS increased the sensitivity of R3BV2 detection by RT-PCR 8 by 64-fold (depending on the target population size) and reduced the time required by a factor of 10.

Magnetic capture hybridization (MCH) using a bacterium- or strain-specific oligonucleotide (capture probe) coupled to magnetic beads could be used to capture, via hybridization, a diagnostic DNA sequence that may be detected by RT-PCR assay. A 60 bp oligonucleotide (complementary to an RRSL_2403 sequence adjacent to the R3BV2-diagnostic amplicon) was coupled to magnetic beads. These beads consistently allowed RT-PCR detection of *Rs* R3BV2 at a concentration as low as 10^3 CFU mL^{-1} , whereas direct RT-PCR detection at this concentration occurred only in about 50% of the samples. Although MCH functioned equally efficiently as IMS at cell concentrations $>10^5$ CFU mL^{-1} , IMS showed better sensitivity at all concentrations $<10^5$ CFU mL^{-1} . The results indicated the effectiveness of IMS and MCH methods coupled with real-time PCR assay for sensitive and specific detection of the strain R3BV2 of *R. solanacearum* in plant and soil samples (Ha et al. 2009).

Clavibacter michiganensis subsp. *sepedonicus* (*Cms*) was effectively detected by using a combination of BIO-PCR and an automated real-time fluorescence assay. PCR primers and a fluorescent probe for use in Perkin Elmer 700 automated real-time PCR detection system (TaqMan®) were designed from a *Cms*-specific generic DNA fragment. The comparative effectiveness of the plating method, standard PCR format, and BIO-PCR assay was assessed. All the three techniques detected *Cms*, but to different levels. The number of samples in which the pathogen was detected was 4, 8, and 26, respectively, indicating the greater efficiency of BIO-PCR combined with automated real-time PCR assay. The BIO-PCR combined with TaqMan® automation-based detection protocol was demonstrated to be rapid and reliable when large samples of seed tuber extracts had to be tested, particularly when the samples contained low pathogen population (Schaad et al. 1999).

In a later investigation, an internal reaction control was integrated into the TaqMan PCR assay for the detection of *C. michiganensis* subsp. *sepedonicus*. The reaction control, cloned into the plasmid pCmsC4, consisted of a sequence unrelated to *Cms* flanked by the primer sequences used in the TaqMan PCR, thus eliminating the need for multiplexing. The presence of a reaction control did not affect the specificity or

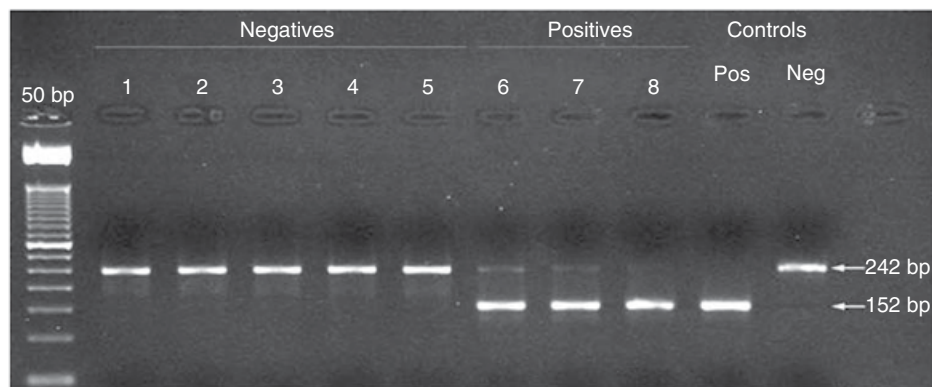


Figure 5.17 Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by PCR assay performed at two melting temperatures of 85.5°C (positives) and 94.5°C (negatives). Left: DNA size standards; lanes 1–5: negative samples; lanes 6–8: positive samples generating a 152 bp amplicon. Source: Smith et al. (2008). Reproduced with permission of the American Phytopathological Society, MN, USA.

threshold of detection of *Cms*. The addition of SYBR Green allowed melt analysis of PCR products. The 242 bp reaction control amplicon, with a melt temperature of c. 94.5°C, could be easily differentiated from the 152 bp primary diagnostic target amplicon which had a melt temperature of c. 85.5°C. A set of 234 diagnostic samples was analyzed using the TaqMan assay. Of these, 71 were used to compare fluorescence signal in real-time PCR with visualization of amplicon by agarose electrophoresis as in conventional PCR format. Of the 28 samples that generated a Cy5 signal, all but two generated an 85.8°C melt peak and all but one generated a 152 bp band in agarose gel electrophoresis. Overall, the fluorescence signal plus melt peak analysis of the PCR results correlated very well with the result obtained by agarose electrophoresis (Fig. 5.17). The remaining 43 samples of this group tested negative in PCR and did not produce 152 bp amplicon in agarose electrophoresis. The results of immunoassay for all 234 samples were compared with those obtained from TaqMan PCR assay. The ELISA-negative samples (25) tested positive for *Cms* in TaqMan PCR assays. However, unless TaqMan PCR assay becomes economically feasible, it is unlikely that PCR will replace ELISA test as the primary screening method for *C. michiganensis* subsp. *sepedonicus* infection in seed potato tubers. The PCR protocol may be used as a confirmatory method for a limited number of samples or supplement immunofluorescence microscopy (IMF) (Smith et al. 2008).

A real-time PCR protocol was developed using the primers based on the sequences of cellulase gene A (*CelA*) which was responsible for the virulence of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), causative agent of bacterial ring rot (BRR) disease of potato. The pathogen is a globally important pathogen of quarantine importance that has to be managed in North America using “zero tolerance” regulations in the certified seed industry. Symptomless infection by *Cms* contributed to its persistence in certified seed stocks. *CelA* primers were specific to *C. michiganensis* subsp. *sepedonicus* grown in culture and did not amplify the DNA of any other coryneform bacteria or other bacteria pathogenic to potato. In *CelA* real-time PCR assays, the primers were more sensitive than immunofluorescence assay (IFA) and *Cms* 50/72 PCR assay in detecting *Cms*-infected cores that were blended with healthy tuber cores (2:198). The limit of

detection of IFA and *Cms* 50/72a primers was 10 infected cores blended with 190 healthy tuber cores, whereas *CelA* primers detected *Cms* in samples containing 1 tuber core and 99 healthy cores (1/100) in a simulated ring rot infected seed lot experiments. On the other hand, the efficiency of pathogen detection by the standard PCR format with *Cms*50/72a primers was much less. In naturally infected seed lots, the *CelA* real-time PCR assay was more sensitive than other diagnostic methods in detecting symptomless infection in seed tubers prior to planting. The ELISA test was ineffective in detecting symptomless infections of *Cms*, despite ring rot disease levels of >4.0%. The ELISA test detected the pathogen in 4 of 11 ring rot infected seed lots, while IFA detected symptomless infections in 7 of 11 seed lots. In contrast, real-time PCR format, using the *Cms* 50/72a and *CelA* primers, could detect the ring rot pathogen in 10 of 11 seed lots. *CelA* real-time PCR proved to be more sensitive by detecting *C. michiganensis* subsp. *sepedonicus* in a greater percentage of naturally infected seed lots than other detection procedures tested in this investigation, and has potential as a robust tool for the detection of this important bacterial pathogen in asymptomatic seed tubers (Gudmestad et al. 2009).

Citrus bacterial canker disease caused by *Xanthomonas citri* strains spreads long distances through the movement of infected plant materials; enforcement of quarantine regulations does not eliminate this problem, but possibly reduces it. This situation may be partly due to ineffective methods of detection for indexing plant materials. Two phylogenetically distinct groups of *Xanthomonas* strains, one originating from Asia (*X. citri* pv. *citri* groups) and the other from South America (*X. citri* pv. *aurantifolii* groups), have been recognized. Identical symptoms induced by phylogenetically distinct groups of strains indicate the presence of a common pathogenicity factor in strains. In real-time PCR assay, the total SYBR Green fluorescence detected after each amplification cycle provided a measure of PCR product formation. The process is monitored in real-time and graphically represented as a semilog curve of the accumulating SYBR Green fluorescence over the number of cycles. The crossing point (Cp) of each graphical curve was defined as the cycle in which the level of fluorescence of the particular sample increases above the background. The Cp was used as one criterion to determine the target presence or absence and also indicated the concentration of the target. A higher target concentration resulted in lower Cp values, thus allowing reliable quantification of purified DNA from the sample. A melting curve analysis was used as the second criterion to determine the presence or absence of the target, detecting false-positive PCR product formation. Each PCR product exhibited a characteristic peak at its melting temperature T_m . The melting curve analysis was used to identify nontarget PCR product and primer-dimer formation. Results based on both Cp values and melting curve analysis might complement each other (Fig. 5.18; Mavrodieva et al. 2004).

A fast, sensitive, and reliable real-time PCR assay was developed using a portable, field-hardened RAPID machine and primer pair VM3 and 4 based on the most highly conserved *pthA* gene sequence of *X. citri*. The primer pair exhibited high specificity and reacted with representative strains of all known canker groups. Single-lesion sampling methods, requiring minimal handling, allowed real-time PCR detection of the canker pathogen in a total time of 4 h. The assay could detect a population of less than 10 CFU/target cells from diseased lesions, indicating the high level of sensitivity of the primers employed in the real-time PCR assay. The high sensitivity of the real-time PCR protocol developed in this investigation allowed molecular detection of *X. citri* in a herbarium sample from a 1912 canker outbreak. Bands of expected size were observed on agarose

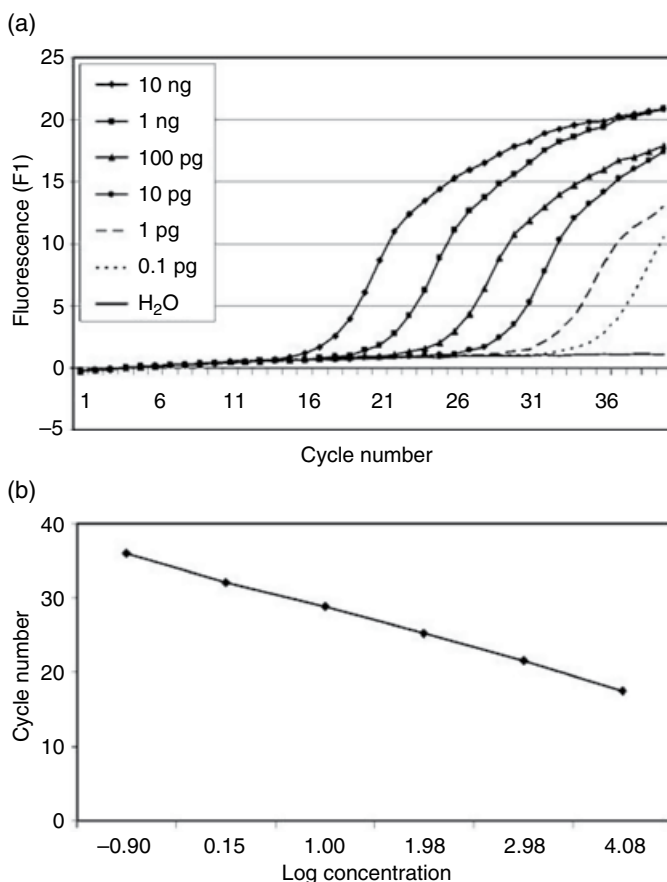


Figure 5.18 Optimization of real-time PCR assay with primer pair VM3/4 to illustrate sensitivity and linearity of the assay using 10-fold serial dilutions of *Xanthomonas citri* pv. *aurantifolii* B DNA. (a) Sensitivity of the real-time PCR assay; and (b) standard curve showing linear relationship between cycle number and pathogen concentration. Source: Mavrodieva et al. (2004). Reproduced with permission of the American Phytopathological Society, MN, USA.

gels following electrophoresis of real-time PCR products (Fig. 5.19). The PCR products were cloned and sequenced, revealing the expected amplification product sequences. The sensitivity of detection could be enhanced by using CaCO₃ and Silwet L-77 and by either minimizing the amount of citrus lesion tissue sampled or by soaking or swiping, but not grinding the lesions (Mavrodieva et al. 2004).

Citrus bacterial canker (CBC) caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) is regulated by quarantine requirements, whereas citrus bacterial spot (CBS) caused by *X. axonopodis* pv. *citrumelo* is not. A quantitative real-time (QRT-) PCR format was applied for detection, identification, and enumeration of pathogen population in citrus plant samples infected by both bacterial canker and spot pathogens. Three sets of primers based on the pathogenicity gene *pth* in *Xac*, a ribosomal gene in *X. axonopodis* pv. *citrumelo*, and the leucine-responsive regulatory protein (*lrp*) gene in both pathogens were combined with TaqMan probes and applied for specific strain detection and quantification. Calibration curves were generated for *Xac* and pv. *citrumelo* based on

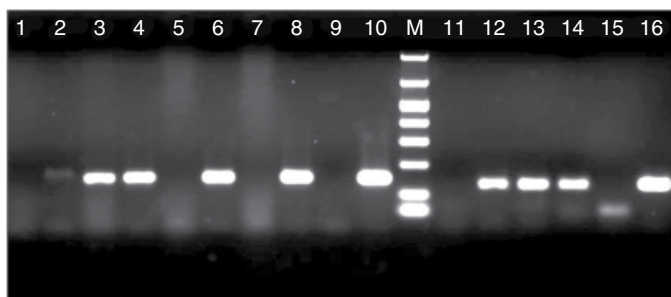


Figure 5.19 Detection of *Xanthomonas citri* (Xc) 3213 by real-time PCR assay in herbarium samples collected in 1912. Lanes 1 and 2: A1 extract and DNA; lanes 3 and 4: A2 extract and DNA; lanes 5 and 6: F3 extract and DNA; lanes 7 and 8: F4 extract and DNA; lane 9: water control; lane 10: 10 ng of Xc 3213 DNA; lanes 11–14: Kingsley's primer pair PCR products; lane 11: A1 DNA; lane 12: A2 DNA; lane 13: F3 DNA; lane 14: F4 DNA; lane 15: water control; lane 16: 10 ng of *X. citri* pv. *citri* A DNA; lane M: DNA marker standards. Source: Mavrodieva et al. (2004). Reproduced with permission of the American Phytopathological Society, MN, USA.

the assumption that the population of bacteria was directly related to DNA concentration. Specificity for CBC detection was ensured because the gene *pth* was present only in *Xac*. The sensitivity of the QRT-PCR was improved as evidenced by the apparent overestimation of bacterial cells compared with number of colonies growing on a semiselective medium. However, in most of the samples colony counts coincided with the results of QRT-PCR assay. Although QRT-PCR assay efficiently quantified the pathogen populations, the assessment by QRT-PCR did not reflect the viable bacterial cells. Nevertheless, QRT-PCR assay reduced the risk of false positives and enhanced the reliability of detection and differentiation of strains of *Xanthomonas* infecting citrus by allelic differentiation (Cubero and Graham 2005).

The detection system for *Xanthomonas axonopodis* pv. *citri* has to be rapid and highly sensitive as fruit infection seriously reduces the quality and marketability due to the unacceptable appearance of the fruits. An integrated approach including isolation, pathogenicity tests, and molecular technique was applied for the detection of *Xac*. The efficiency of pathogen isolation, three conventional PCR formats, and real-time PCR with SYBR Green or TaqMan probe was compared. The pathogen was isolated from 16 of 130 lesions and its pathogenicity was confirmed by inoculation on grapefruit. With PCR assays, *Xac* was detected in 11 of 15 fruit samples. On the other hand, real-time PCR SYBR Green or TaqMan probe detected canker pathogen in 58 and 80 lesions respectively, whereas the presence of *Xac* was detected in 39–52 lesions, depending on the protocols of conventional PCR applied. Real-time PCR with TaqMan probe was the most efficient in detecting *X. axonopodis* pv. *citri* in naturally infected samples (Fig. 5.20; Golmohammadi et al. 2007).

Xylella fastidiosa (Xf), causative agent of citrus variegated chlorosis (CVC), has posed a problem for detection in asymptomatic grapevine plants because of its presence only in xylem tissue in low concentrations and its irregular distribution in infected plants. A real-time quantitative PCR (qPCR) assay for the detection of *X. fastidiosa* was developed, using the 5'-nuclease PCR in xylem vessels. The number of Xf cells in citrus tissue samples was calculated by extrapolating the mean Ct values for the test samples into a standard curve generated with Xf cells. The mean number of *X. fastidiosa* cells detected in the leaf xylem ranged over 0–32 cells mg⁻¹ of tissue. Pathogen population increased

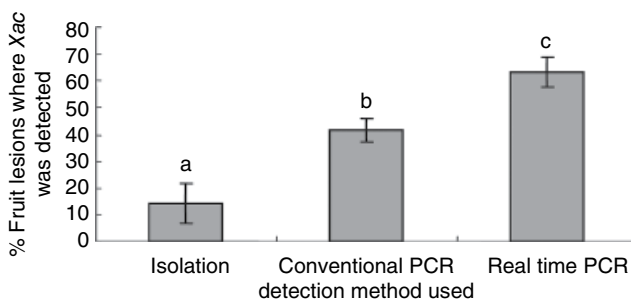


Figure 5.20 Detection of *Xanthomonas axonopodis* pv. *citri* (Xac) by isolation, standard PCR, and real-time PCR assays in citrus fruit lesions. Bars represent percentages of positive detections by different detection methods. Source: Golmohammadi et al. (2007). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

with the age of leaves and susceptibility of citrus cultivar. Pera sweet orange, highly susceptible cultivar to CVC disease, had 109.2 and 487 *X. fastidiosa* cells mg^{-1} of tissue when inoculated by the needle inoculation and natural infection. The real-time qPCR protocol developed in this investigation was found to be a straightforward, sensitive, and reproducible tool for quantification of *X. fastidiosa* in different tissues of citrus plants (Oliveira et al. 2002).

Grapevine Pierce's-disease-causing *Xylella fastidiosa* (Xf) is limited to xylem vessels of infected symptomatic grapevine plants, and the population of Xf is low in asymptomatic planting materials. It is unevenly distributed in infected grapevine plants. A direct real-time PCR assay was employed for the detection of Xf in plant sap and samples of macerated chips of secondary xylem from trunks of vines without extraction of DNA. *X. fastidiosa* could be detected using two different sets of primers and probes in 7 of 27 vines (26%) from 4 of 6 vineyards sampled at 10–12 days after outbreaks of disease in four counties of California. Standard PCR assay results confirmed the results of direct real-time PCR format. The protocol developed in this investigation consistently detected *X. fastidiosa* in asymptomatic vines just breaking their dormancy, indicating its potential for large-scale application to eliminate the infected vines to arrest the subsequent spread of Pierce's disease from infected sources of inoculum (Schaad et al. 2002).

Xylella fastidiosa (Xf) is actively spread by the glassy-winged sharpshooter (GWSS) *Homalodisca coagulata*. A reliable detection method was considered to be vital for monitoring the spread of the disease, especially in asymptomatic plants. A quantitative PCR (qPCR) with TaqMan system, including improved DNA extraction and genome-wide PCR primers and probes, was developed. A dual purpose conventional PCR (C-PCR) and qPCR system was useful for the generic detection of Xf strains. Primers HL5 and HL6 designed to amplify a unique region common to the sequenced genomes of four *Xylella* strains amplified a 221 bp fragment from strains associated with grapevine PD and from an Xf strain associated with citrus variegated chlorosis (CVC) disease. No amplification occurred in the DNA extracts from healthy grapevine. Of the 28 grape and 38 almond samples assayed, 14 grape (50%) and 27 almond (71%) samples showed the presence of *X. fastidiosa* when primer set HL5/HL6 was employed in C-PCR and qPCR formats. The primers HL5 and HL6 were highly specific and sensitive for Xf detection by both C-PCR and qPCR, because they amplified a sequence that was

common to the completely sequenced genomes of four *Xf* strains. The size of the HL5/HL6 amplicon (221 bp) provided greater specificity and sensitivity in detection and identification of *Xf* by C-PCR than was achieved with the primer set RST31/RST33 employed earlier. All the isolates of *Xf* (42) from PD-infected grapes and other hosts, as well as genomic DNA from 10 strains of *Xf* associated with CVC, amplified the expected DNA fragment. The DNA extraction and *Xf* detection in protocols developed in this investigation allowed the recovery of the entire sample in one tube; it was therefore able to detect low levels of pathogen cells (5–10 cells/reaction). Using these protocols, *X. fastidiosa* was detected in the vectors as well as in other plant hosts such as citrus and almond in San Joaquin Valley of Central California in the USA (Francis et al. 2006).

Eradication of grapevine plants infected by *Xylella fastidiosa* (*Xf*), which may serve as sources of inoculum for nearby healthy vines, is the major approach for the management of Pierce's disease (PD) caused by this pathogen. This approach can only be effective if a sensitive and reliable method is employed for the detection of *Xf* in the asymptomatic plants in which the pathogen reaches high titer before visible symptoms appear. A biomarker system that simultaneously monitored *Xylella*-induced plant transcripts as well as *Xylella* rRNA was therefore applied. A diagnosis system that may be able to monitor the presence of the pathogen rDNA and of pathogen-induced host gene transcripts could potentially be more robust than one focusing on a single factor. Plant biomarker genes were derived from a combination of in silico analysis of grape expressed sequence tags (ESTs) and validation by means of a reverse-transcription polymerase chain reaction (RT-PCR) assay. Four genes upregulated upon PD infection were individually multiplexed with an *X. fastidiosa* marker rRNA and scored using either real-time or gel-based conventional RT-PCR techniques. Both host gene transcripts and pathogen rRNA in asymptotically infected plants could be effectively detected. Further, these plant biomarker genes were not induced by water deficit which was commonly associated with PD development. The results showed that host-derived biomarkers might be reliable tools for early detection of infection by *X. fastidiosa*. Host transcriptional pathway occurring prior to the onset of visual disease symptoms, which might be in conjunction with pathogen biomarkers, could form the basis for detection of infection by *X. fastidiosa* at early stages (Choi et al. 2010).

Grapevine crown gall disease caused by *Agrobacterium vitis* (= *A. tumefaciens* biovar 2) is considered to be a limiting factor in grape production. *A. vitis* persists in systemically infected vines and the need to use clean planting materials and rootstocks has to be realized to avoid crown gall disease incidence in new locations. Indexing of grape propagation material using sensitive, reliable, and rapid methods is regarded as an important strategy for effective disease management. Several detection methods based on isolation of pathogen, immunoassays, and conventional PCR assay were tested and found to be unsuitable because they were time-consuming, less sensitive, and/or inconsistent. In the process of developing an efficient and sensitive method for detecting *A. vitis* in grape, a real-time PCR procedure with primers specific for diverse tumorigenic strains of *A. vitis* was initially developed using the *virD2* gene sequences governing pathogenicity of the strains. The vines used were initially propagated from tissue culture and had no visible crown gall symptoms. Although real-time PCR assay provides sensitive and rapid detection, it is still limited by the presence of inhibitory compounds in plant tissues and by the requirement of a threshold level of target nucleic acid. DNA extraction methods that included magnetic capture hybridization (MCH), immunomagnetic separation (IMS), and extraction with the MoBio Powerfood Kit were therefore employed

prior to real-time PCR procedure. IMS real-time PCR format was found to be more rapid in plants artificially inoculated with *A. vitis* at 10CFU mL^{-1} at 1 day postinoculation (dpi). The overall frequency of detection by IMS improved with higher inoculum densities (10^2 – 10^7CFU mL^{-1}) and longer incubation times of 2 and 3 days. On the other hand, MCH real-time PCR assay only detected *A. vitis* in plants inoculated at 10CFU mL^{-1} at 2 and 3 days dpi, but not at 1 dpi as in IMS real-time PCR format. All three methods detected the crown gall pathogen in affected gall tissues taken from naturally infected grapevine after incubation for all days tested. In all, 18.75% of vines tested with Powerfood DNA extraction or IMS real-time PCR were positive, whereas 68.75% of the samples tested positive by MCH real-time PCR assay. The assays incorporating MCH or IMS followed by real-time PCR were 10,000-fold more sensitive than direct real-time PCR assay (Johnson et al. 2013).

Xylophilus ampelinus (*Xa*), causative agent of grapevine bacterial leaf blight disease, is primarily spread through infected propagules. For effective management of the bacterial leaf blight disease, early and reliable detection of this pathogen in infected plants could be expected to facilitate elimination of the infected plants to prevent the spread of the disease to other grapevines. A real-time PCR assay was developed based on a 5'-nuclease and minor groove binding (MGB) probe to provide a rapid, sensitive, and reliable tool for the detection of *Xa*. With the MGB probe chemistry and real-time PCR product detection provided by this technique, it was possible to avoid laborious post-PCR handling and the associated high risk of cross-contamination. The real-time PCR assay could amplify all isolates of *X. ampelinus* from diverse geographical locations, as the target sequence seemed to be highly conserved in all *Xa* isolates tested. The specificity of the assay was revealed by the absence of specific signal with other bacterial pathogens of grapevine or microflora associated with grapevine tissues. The limit of detection of the procedure was about 100 cells of *Xa* from grapevine tissue extracts, which was 10-fold lower than that of nested PCR format employed in earlier investigations. A high correlation was observed between real-time PCR cycle threshold (Ct) values obtained and the pathogen isolation on artificial media. The real-time PCR procedure developed in this investigation was found to be reliable and sensitive for the detection of *X. ampelinus* in asymptomatic grapevine plants and suitable for the quantitative estimation of pathogen population in different grapevine cultivars with different levels of susceptibility/resistance to *X. ampelinus* (Dreo et al. 2007).

Sugarcane ratoon stunting disease (RSD) caused by *Leifsonia xyli* subsp. *xyli* (*Lxx*) (= *Clavibacter xyli* subsp. *xyli*) does not induce any reliable external symptoms and infection may be recognized only by splitting the stalk to expose the internal tissues. The pathogen is transmitted through infected stalks or setts (cuttings) used to raise new sugarcane crops and harvesting operations. Techniques to detect the pathogen in seed cane nurseries were therefore considered vital to reduce the incidence of RSD. A real-time PCR assay was developed for sensitive detection of *Lxx* in the youngest fully expanded leaf and five leaves basal to it. The primers Lxx202F/Lxx33R produced a single melting peak, a single band of expected size on electrophoresis gel, and the expected nucleotide sequence. Conventional PCR formerly tested positive for *Lxx* infection, and the results were in agreement with that of real-time PCR format in the sugarcane cultivar L62-96. Assays conducted on older, more rapidly growing plants indicated that leaf position affected the assay efficiency. Conventional PCR assay was less efficient than real-time format in detecting *L. xyli* subsp. *xyli* in leaf tissues. Sugarcane cultivars were ranked for their level of susceptibility based on the relative titer of *Lxx* in leaves of

Table 5.12 Comparative efficacy of diagnostic techniques in detecting *L. xyl*i ssp. *xyl*i infection in sugarcane leaf 5 (fourth leaf basal to leaf 1; fully expanded youngest leaf) of sugarcane stalks (7 month old) (Grisham et al. 2007).

Cultivar/source*	Number of plants showing positive reaction		
	Real-time PCR	Conventional PCR	TB-EIA
L62-96			
RSD	4	0	4
HWT	1	0	1
CP70-321			
RSD	3	0	4
HWT	1	0	0
CP52-68			
RSD	2	0	0
HWT	0	0	0

* Nursery source: RSD: samples from *Lxx*-inoculated nursery plot; HWT: samples from hot-water-treated nursery plot.

inoculated 3 and 4 month old greenhouse-grown plants. The results of real-time PCR assay were comparable with the ranking determined by using tissue-blot enzyme immunoassay performed in tissues from 7–9 month old stalks of sugarcane (Table 5.12; Grisham et al. 2007).

Citrus huanglongbing (HLB) pathogen “*Candidatus Liberibacter asiaticus*” (CLA) is able to survive in the plant host *Citrus* spp. and the Asian citrus psyllid *Diaphorina citri*. Assessment of CLA population in the plants and insects may be useful for investigations on virulence mechanisms and disease management. A procedure was developed to detect and quantify live bacterial populations using a novel DNA-binding dye, propidium monoazide, in conjunction with real-time PCR (PMA-qPCR) format. Chinese box orange (*Severinia boxifolia*), an ornamental plant, was found to be a good host for both the bacterial pathogen and psyllid vectors. The changes in the live “*Ca. L. asiaticus*” population in graft- and psyllid-inoculated Valencia sweet orange (*Citrus sinensis* cultivar Valencia) and *S. boxifolia* plants were monitored for 20 months using PMA-qPCR assay. The results showed that the live pathogen population was significantly lower in the months of December, January, and February than the rest of the year in plant and the vector insects. No significant pattern in the total bacterial population was observed in plants and vectors. The results showed that “*Ca. Liberibacter asiaticus*” had a seasonal growth pattern along with the growth of both host species (Hu et al. 2014).

In a later investigation, the real-time quantitative PCR (qPCR) format was applied to detect the HLB pathogen DNA in the nucleic acid extracts of fruit peduncles, seed coats, seeds, and in germinated seedlings from Sanguenelli sweet orange and Conners grapefruit sampled from the infected trees. “*Ca. Liberibacter asiaticus*” was detected in fruit peduncles of Sanguenelli (36%) and Conners (100%). Seed coats (37–98%), seeds (1.6–4%), and Sanguenelli seedlings (10%) contained detectable populations of HLB

pathogen. Although colonization of seeds of Sanguenelli and Conners by the pathogen was indicated by the qPCR assay, no pathogen DNA could be detected in Conners seedlings grown from the seed colonized by the pathogen. HLB pathogen DNA was detected initially in the Sanguenelli seedlings. No relationship could be established between the presence of pathogen DNA in fruit peduncles and seed coats and in the seedlings. The results suggested that the presence of “*Ca. Liberibacter asiaticus*” in seed tissues may not necessarily result in vertical transmission of the pathogen to citrus seedlings (Hilf 2011).

“*Candidatus Liberibacter solanacearum*” (*Lso*), the incitant of potato zebra chip (ZC) disease in tubers, is a phloem-limited bacterial pathogen transmitted by natural biological vector potato psyllid *Bactericera cockerelli*. Long-distance dispersal is through infected tubers. Control strategies relied primarily on early detection of *Lso* in seed tubers using sensitive, rapid, and reliable techniques. Two assay procedures were developed to detect the pathogen in New Zealand field samples. A single-tube seminested gel-based-PCR assay was employed for the genus-specific detection of *Liberibacter* species and provided improved sensitivity over standard and nested PCR formats. Another single-tube seminested SYBR real-time PCR (qPCR) assay was applied for specific detection of *Lso* in field samples, with a detection limit of five copies of target gene per reaction. Seminested qPCR was equally sensitive as the TaqMan qPCR format with the primer-probe combination *LsoF*-*HLBpr*, and the assay was 10- to 50-fold more sensitive than conventional PCR format. Quantification of the pathogen population in *Lso*-affected tubers by SYBR Green qPCR assay showed a positive relationship between pathogen titer and the discoloration of fried tuber slices characteristic of ZC pathogen infection. Quantification of *Lso* in field samples of potato and tomato indicated the low pathogen population that was below the detection limit of conventional PCR format. The results indicated the effectiveness of SYBR Green qPCR assay in detecting the pathogen in samples with low titer and confirmation of qPCR results by identifying false-positive results (Beard et al. 2013).

Detection of *Spiroplasma citri*, causal agent of citrus stubborn disease (CSD), is a phloem-limited bacterial species; its isolation in pure culture is very difficult because of its uneven distribution and low titer in infected citrus plants. PCR assay with primers developed from sequences of *S. citri* housekeeping genes was used earlier. Genome sequencing of *S. citri* revealed that the bacterium harbors multiple copies of prophage genes. The targeting of multicopy prophage genes was therefore considered to improve the sensitivity of detection of *S. citri* by PCR assay. Two primer sets, namely *Php*-orf1 and *Php*-orf3, were developed from conserved prophage sequences in the pathogen genome. These primer sets were employed to evaluate detection sensitivity in SYBR-Green-based quantitative PCR (qPCR) assays with 18 *S. citri* cultures isolated from different hosts and locations. Prophage primer set *Php*-orf1 enhanced the sensitivity of detection by 49.1 and 3.65 cycle threshold (Cq) units, compared with housekeeping gene primers for spiralin and P58 putative adhesion gene, respectively. Detection sensitivity with *Php*-orf3 primer set was slightly less at 3.02 and 1.75 Cq units respectively, over the same housekeeping gene primers. The prophage primer sets were validated for qPCR detection with field samples from three citrus orchards in San Joaquin Valley, CA, USA collected from 2007 to 2013. The results showed that *S. citri* prophage sequences could be employed for improving the sensitivity of qPCR detection of *S. citri* in infected citrus trees by at least 10-fold and for reducing the number of false-negative results. No false-positive results were obtained with any

of the prophage sequence-based primer sets. The higher copy number of prophage genes in *S. citri* genome resulted in enhanced sensitivity and also improved the level of reliability of the qPCR assay procedure developed in this investigation (Wang et al. 2015).

5.2.6.3 Loop-Mediated Isothermal Amplification Technique

The loop-mediated isothermal amplification (LAMP), a DNA amplification procedure, is based on autocleaved strand displacement DNA synthesis by a DNA polymerase which has high strand displacement activity and a set of specially designed inner and outer primers. The LAMP reaction is initiated by hybridization of inner primers to specific sites on the target DNA, followed by strand extension at an elevated temperature (c. 65°C) in the presence of a strand displacement DNA polymerase (Kubota et al. 2008). Outer primers, complementary to specific priming sites just outside the inner primer binding sites, indicate synthesis of new complementary strands that displace DNA strands initiated from the inner primers, resulting in the release of those strands. The first amplification products from the stem-loop structures at each end as a result of the unique design of the inner primers are autoprimered, driving the amplification of massive amounts of DNA. The amplified end product is a mixture of large amounts of stem-loop DNA strands with manifold inverted repeats of the target, exhibiting a cauliflower-like structure with multiple loops. LAMP-amplified DNA forms a ladder of multiple bands in gel electrophoresis and can also be visualized directly in the amplification tube by adding SYBR Green I, a fluorescent DNA-binding dye, and observing under UV-light (Li et al. 2009c). A LAMP reaction may be performed in a heat block or water bath, thereby removing the need for specialized equipment. Furthermore, positive amplification can be observed by colorimetric or fluorescent dyes, eliminating the need to run gels. Both of these factors contribute to transferability of the technology to the field (Harper et al. 2010).

Infection of citrus by "*Candidatus Liberibacter*" (CL), inducing huanglongbing (HLB) known earlier as greening disease, was detected by the LAMP technique. The 16S rDNA and 16S/23S intergenic regions of CL were not suitable because of their homology with plant genomic cDNA. The *nusG-rplKAJL-rpoB* gene cluster was therefore selected because of its uniqueness and consequent specificity. Thermal asymmetric interlaced (TAIL-) PCR assay was performed to amplify the uncharacterized regions adjacent to the *nusG-rplKAJL-rpoB* gene cluster of CL isolates from different locations in Japan and Indonesia. The internal *nusG-rplKAJL-rpoB* gene cluster of CL was amplified by standard PCR format for determination of the complete sequence of the 6.1 kb fragment. A LAMP assay based on the conserved sequence of the *nusG-rplKAJL-rpoB* gene cluster was developed for the detection of CL. The LAMP product was detected on nylon membranes by staining with Azur B with a detection limit of about 300 copies of the target fragment. Neither the thermocycler nor the electrophoresis equipment is required for LAMP assay as in the standard PCR assay for pathogen detection. In addition, the detection of colored products on nylon membrane is not only simple but also very rapid and amenable for wide application, facilitating the elimination of infected plants or planting materials (Okuda et al. 2005).

Citrus huanglongbing (HLB) disease caused by "*Candidatus Liberibacter*" forms has assumed great importance because of its worldwide distribution and significant yield losses caused by them. The conventional PCR format, loop-mediated isothermal amplification (LAMP), and three TaqMan real-time PCR protocols were compared for their

accuracy, sensitivity, specificity, and reliability of detection of HLB pathogen in tissues of greenhouse- and field-grown citrus plants. “*Ca. Liberibacter asiaticus*” (*Las*), “*Ca. L. africanus*” (*Laf*), and “*Ca. L. americanus*” (*Lam*) forms have been recognized. The conventional PCR assays employing primer sets OI1/OI2c or OAI/OI2c detected “*Ca. Liberibacter*” spp. in 2–10 μ L of 100 μ L of the original DNA extracts obtained from mixed samples, consisting of 1 g of healthy midribs with a minimum of 20 mg of infected midribs. The limit of detection of the protocol developed in this investigation was equivalent to 0.01 mg of infected midribs per reaction. The TaqMan real-time PCR format efficiently detected “*Ca. Liberibacter*” spp. from the equivalent of 20 mg of midribs of symptomatic leaves, demonstrating an enhancement of sensitivity of detection by 100-fold. The higher sensitivity of TaqMan real-time PCR assay might be due to increased efficiency of amplification and its lower level of vulnerability to PCR inhibitors present in citrus plants. Real-time PCR assays provided reproducible detection of “*Ca. Liberibacter*” spp. strains in the 10^{-2} – 10^{-5} dilutions of DNA extracts obtained from plants infected by *Las*, *Laf*, and *Lam* forms. All 63 asymptomatic greenhouse-grown plants tested negative for *Las* by conventional PCR format, but real-time PCR assay detected HLB pathogen in one plant. LAMP assay yielded positive results for six of these asymptomatic plants. However, detection in a greater number of plants by LAMP assay was attributed to possible contamination of the reactions, when *Bst* polymerase was added to each tube. The validated conventional and newly developed real-time PCR formats were found to be reliable for confirmatory testing for “*Ca. Liberibacter*” spp. in suspect HLB symptomatic citrus samples. The real-time PCR system was more efficient in detecting the HLB pathogenic forms in both symptomatic mature and asymptomatic young tissues of infected citrus plants before detection of the pathogens was possible by the conventional PCR formats. The results indicated the usefulness of real-time PCR assays for regulatory response, effective management of infected citrus trees, and the development of an HLB-disease-free nursery system (Li et al. 2007).

Loop-mediated isothermal amplification (LAMP) was developed for the rapid and sensitive detection of “*Candidatus Liberibacter asiaticus*” (CLAs), causing citrus huanglongbing or greening disease in the psyllid vector *Diaphorina citri*. Using LAMP technology, a field detection kit was developed for testing psyllids for the presence of HLB pathogen. An amplicon of 137 bp and multimers of this product were synthesized in the LAMP reaction. The efficiency of LAMP method was compared with standard qPCR assay. Six samples with pools of 1–10 psyllids plus a positive and negative control could be tested at a time in about 30 min (10 min for crude DNA extraction and 20 min for target amplification). The LAMP assays were conducted in a Smart-DARTTM detection unit which was operated from an android device. The LAMP detection method was about 100 times more sensitive than the conventional real-time PCR procedure. The LAMP reaction products could be visualized after electrophoresis on 2% agarose gels. A typical ladder pattern was observed. The *Liberibacter* genomic region selected in this investigation for LAMP test was specific to CLAs. Psyllids are known to harbor several symbiotic bacteria and the primers did not amplify DNA from symbionts populations. The LAMP protocol was validated by testing psyllids from Florida, Brazil, India, and Pakistan and from different citrus varieties, and has the potential for large-scale application by growers and extension personnel to monitor psyllid movement and subsequent initiation of proactive management strategies, soon after the detection of infective vectors (Keremane et al. 2015).

Xylella fastidiosa (*Xf*), a xylem-limited bacterial pathogen, infects grapevine, citrus, and several other economically important crop plants. A loop-mediated isothermal amplification (LAMP) and real-time PCR assay were developed based on the sequences of the rim gene of *Xf* and their levels of sensitivity and specificity of detection of the pathogen in infected grapevine plants were assessed. The real-time PCR assay targeting the rim PRF was robust over a range of annealing temperatures of 58–62°C, the highest reaction efficiency (94.7%) being attained at annealing or extension stage of 62°C. Addition of BSA (bovine serum albumin) was necessary to reduce inhibitory effects of compounds from grapevine leaf samples. The detection limit was c. 10 copies/reaction, but a concentration of 125 copies was required for consistent amplification. Both LAMP and rim-targeting real-time assay amplified the expected DNA fragment from all 20 isolates of *X. fastidiosa*. Amplification of *Xf* DNA was successfully accomplished from total DNA extracted from a range of infected plant hosts, infective insect vectors, and cultured pathogen cells. The limit of detection of LAMP assay (in the absence of hydroxynaphthol blue dye) was c. 250 copies of template per reaction, compared with 10 copies for both real-time PCR assays and 500 copies for conventional PCR assays. Both LAMP and real-time PCR assays were found to be rapid, being able to detect *X. fastidiosa* extracted from infected tissues using a simple magnetic-based method in 1 h. The cost of the tests could be a deciding factor for its widescale applicability. The LAMP assay, requiring specialized enzymes and reagents, costs US\$ 5.30 compared with US\$ 1.00 for conventional and real-time PCR formats. However, the cost of specialized equipment for real-time PCR format may limit its applicability, and the time-saving advantage of LAMP assay might appeal over other assays when results are required rapidly (Harper et al. 2010).

A loop-mediated isothermal amplification (LAMP) assay was developed for the rapid detection of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) (renamed *Pectobacterium atrosepticum*), causing potato blackleg disease, and its differentiation from other species of pectolytic bacterial pathogens of potato. Selected genomic regions, especially the region containing a gene cluster encoding a phytotoxin coronafacic acid-like (*Cfa*) compound which played an important role in bacterial pathogenicity, virulence, and pathogen–host interaction, were used to develop LAMP primer sets. The gene cluster, comprising nine putative protein-encoding genes that were not present in any other *Erwinia carotovora* subspecies, was identified. Primers based on *Cfa* 6 of the gene cluster (IPKS genes) were selected for the LAMP assay due to its close association with pathogenicity of *Eca*. The selected primer sets amplified DNA from *Eca* at 65°C, while DNA from other pectolytic bacteria were not amplified. DNA templates from 15 other plant pathogenic bacteria, some of which also had a *Cfa* gene cluster in their genomes, were also not amplified by these primers, confirming the high specificity of the LAMP assay for *Eca*. Addition of the *Bst* DNA polymerase derived from *Bacillus stearothermophilus* to the reaction mix increased the efficiency of detection, probably because of its greater resistance to inhibitors. By adding SYBR Green dye to the reaction mix, the results of LAMP assay could be visualized under UV-light and the positive reaction could be differentiated from negative reactions with other pectolytic bacterial pathogens tested. The sensitivity of LAMP assay, combined with the simplified DNA extraction procedure using a dilution series of suspensions of *E. carotovora* subsp. *atroseptica*, was 2.5×10^2 CFU mL⁻¹ when evaluated by gel electrophoresis. The LAMP assay could be employed for effective detection of *Eca* in 16 field-grown blackleg-infected plants and 2 greenhouse-grown potato plants inoculated with *E. carotovora* subsp. *atroseptica*.

Potato plants inoculated with *E. carotovora* subsp. *carotovora* or *P. carotovora* subsp. *brasiliensis* tested negative for LAMP assay, however. The high specificity of LAMP assay, due to the use of two or three different primer pairs, and isothermal reaction conditions at a moderately high reaction chamber, as well as visualization of results colorimetrically, are the advantages of LAMP assay over standard PCR formats. The latter are less likely to be acceptable for large-scale application for rapid diagnosis of potato blackleg disease (Li et al. 2011; Section 5.4.12).

“*Candidatus* Liberibacter solanacearum,” causal agent of potato zebra chip (ZC) disease, was detected by employing the PCR-based loop-mediated isothermal amplification procedure (LAMP). The 16S rDNA gene of “*Ca. L. solanacearum*” was used to design a set of six primers for LAMP-PCR detection of the bacterial pathogen in potato plants and the psyllid vector. The LAMP procedure reliably detected the ZC disease pathogen and the closely related species “*Ca. L. asiaticus*,” the causal agent of huanglongbing (HLB) disease infecting citrus. Although the LAMP method was not as sensitive and quantitative as real-time PCR assay, it was equivalent to conventional PCR in tests for detection of ZC pathogen in field-grown potato plants. The distinct advantage of using the LAMP method is the nonrequirement of a thermocycler for amplification or agarose gel electrophoresis for resolution. Positive results of LAMP assay could be visualized directly from the formation precipitate. The LAMP diagnostic procedure holds promise as a reliable, rapid, and cost-effective tool for detecting “*Ca. Liberibacter*” pathogens in field-grown potato plants and tubers, as well as in psyllid vectors (Ravindran et al. 2011, 2012).

5.2.6.4 Cycleave Isothermal and Chimeric Primer-Initiated Amplification of Nucleic Acids

Spread of citrus huanglongbing (HLB) disease caused by “*Candidatus* Liberibacter asiaticus” is primarily restricted by removal of infected trees. Infected citrus plants therefore have to be identified by detecting the pathogen, using a sensitive method that does not require expensive equipment such as a high-throughput thermocycler. Isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) by employing BCS BEST DNA polymerase, TliRNaseH, and two DNA-RNA chimeric primers has been shown to be rapid and effective. ICAN may also be combined with luminescence detection by probe hybridization for detection of human bacterial pathogen, *Mycobacterium tuberculosis* (Shimada et al. 2002; Mukai et al. 2007).

The detection system for “*Candidatus* Liberibacter asiaticus” involved the amplification of 16S rDNA by ICAN and detection of the amplified products with cycling probe (cycleave) technology. ICAN technique, requiring two DNA-RNA chimeric primers, was found to be suitable for bacterial pathogens such as “*Ca. L. asiaticus*” with limited information on the genomic DNA. The cycling probe is a chimeric DNA-RNA probe that hybridizes to an amplified target sequence, but not to nonspecific primer dimers. After hybridization with probes, the RNA part of the probe is cleaved by TliRNaseH. The fluorescer ROX and quencher Eclipse on each side of the probe are therefore separated and red fluorescence is emitted. With this cycling probe technology, red fluorescence was obtained rapidly from “*Ca. L. asiaticus*”-positive samples and the occurrence of false-positives could be prevented (Urasaki et al. 2008). The comparative efficacy of Cycleave ICAN assay and standard PCR format was assessed for the detection of “*Ca. Liberibacter asiaticus*” (CLA), using the primers specific to CLA 16S rDNA. The standard PCR format detected a 1160bp rDNA fragment in positive templates

from 40 ng to 12.8 pg, but not in the samples containing 2.56 to 0.102 pg of pathogen DNA. In the Cycleave ICAN, red fluorescence was recorded from 40 ng to 0.512 pg. The fluorescence intensity was observed in samples containing 0.102 pg and above. No fluorescence was observed in healthy samples. The sensitivity of Cycleave ICAN was found to be at least 25 times higher than that of the conventional PCR assay. Further, the reaction could be carried out in a single tube in 1 h without the need for electrophoresis, as in the case of standard PCR format. Of the 95 samples tested, standard PCR format and Cycleave ICAN assay detected CLA in 35 and 39 samples respectively, indicating the ability of Cycleave ICAN assay to detect CLA in samples containing populations of the pathogen below the limit of detection by standard PCR format and revealing the reliability of the Cycleave ICAN assay for early detection of CLA in citrus plants that have to be eradicated (Urasaki et al. 2008).

5.2.6.5 DNA Array Technology

The identification of DNA fragments specific for target microorganisms may be accomplished by DNA array technology which is essentially a reverse dot-blot technique. This emerging technique may be useful for rapid identification and detection of microbial plant pathogens, including bacterial pathogens present in propagules/plants. An array of species-specific oligonucleotide probes representing the different pathogens, built on a solid support such as nylon membrane or microscope slide, could be probed readily with labeled PCR products amplified from the test sample. By using conserved primers to amplify common pathogen genome fragments in plant tissue extracts that might contain target pathogen(s), the presence of DNA sequences indicative of pathogenic species would be revealed by hybridization to species-specific probes within the array.

The possibility of employing DNA array technology for the detection, identification, and differentiation of five bacterial pathogens infecting potato was examined. Oligonucleotides (16–24 bp long) were selected from the 3' end of the 16S gene and 16S–23S intergenic spacer regions of *Clavibacter michiganensis* subsp. *sepedonicus*, *Ralstonia solanacearum*, and the pectolytic erwinias including *Erwinia carotovora* subsp. *atroseptica*, *E. c. carotovora*, and *E. chrysanthemi*. Oligonucleotides were designed and formatted into an array by pin-spotting on nylon membranes. Seventeen oligonucleotides were designed with specificity to *E. carotovora* subspecies and *E. chrysanthemi*, six with specificity to *R. solanacearum* and to *C. michiganensis* subsp. *sepedonicus*. Genomic DNA from bacterial cultures was amplified by PCR assay, using conserved ribosomal primers. Probes labeled simultaneously with digoxigenin (DIG-) label revealed different hybridization patterns that were distinct for each species and subspecies tested. Hybridization of amplicons was generally restricted to appropriate homologous oligonucleotides and cross-hybridization with heterologous oligonucleotides was rare. Hybridization patterns were recorded as separate gray values for each hybridized spot and revealed a consistent pattern for multiple strains of each species or subspecies isolated from diverse geographical regions. Hybridization signal strength expressed as gray values ranged from 0 to 65,000 among the nucleotide spots on the arrays. Gray values of >500 were arbitrarily considered as positive hybridization signal, with lower values not visible without computer enhancement considered to be background signals. Several mixtures of bacterial strains were amplified by PCR with the conserved primers and hybridized to the oligonucleotide array. The amplicons in the mixture did not interfere with one another and the expected pattern for each species was obtained. An array probed with amplicons from a mixture of *E. carotovora* subsp.

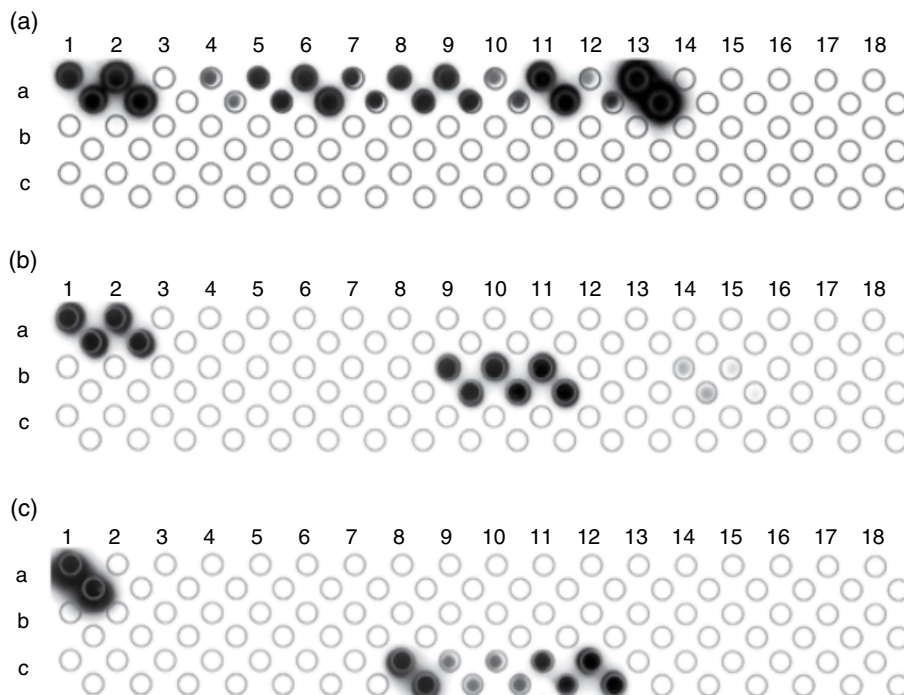


Figure 5.21 Patterns of hybridization of DIG-labeled PCR amplicons from *Erwinia carotovora* subsp. *atroseptica* (Eca), *E. chrysanthemi* (Ec), and *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) on the oligonucleotides array. a: Eca strain 31; b: Ec strain 340; and c: Cms strain R3. Positive hybridization signals are seen as dark spots within template circles. Source: Fessehaie et al. (2003). Reproduced with permission of the American Phytopathological Society, MN, USA.

atroseptica strain 31 and *E. chrysanthemi* strain 340 showed the consistent hybridization pattern expected for the two *Erwinia* spp. (Fig. 5.21). Bacterial pathogens in potato tissue could be directly detected and identified by using the oligonucleotide array. PCR amplicons generated from potato tubers inoculated with Eca strain 31 produced the same hybridization pattern on the array as pure cultures. Bacteria-free potato tuber tissue from microplantlets did not hybridize with any of the oligonucleotides designed for bacterial pathogens of potato tubers (Fig. 5.22; Fessehaie et al. 2003).

Erwinia carotovora subsp. *carotovora* (Ecc) (= *Pectobacterium carotovorum*) and *E. carotovora* subsp. *atroseptica* (Eca) (= *P. atrosepticum*), causative agents of blackleg and soft rot diseases affecting potatoes, could be detected and distinguished by employing the microarray technique. Gene-specific probes were designed for all genes of Eca, about 50% genes of *Streptomyces scabies* (causing common scab disease), and 30% of genes of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) (causing ring rot disease), utilizing whole genome sequence information available. In the case of *S. turgidiscabies* (causing common scab disease), 226 probes were designed based on the sequences of the pathogenic island containing important virulence genes. In addition, probes were designed for the virulence-associated *nip* (necrosis-inducing protein) genes of Eca and Ecc and for intergenic spacer (IGS) sequences of the 16S-23S rRNA gene region. The probes contained about 40 target specific nucleotides and were synthesized on the

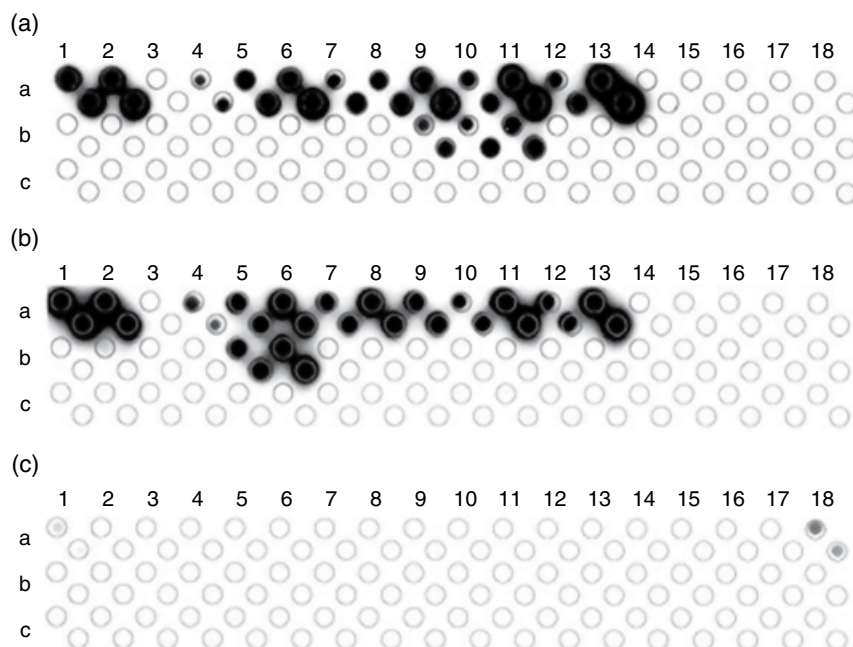


Figure 5.22 Patterns of hybridization of DIG-labeled PCR amplicons from a mixture of *E. carotovora* subsp. *atroseptica* (*Eca*) and *E. chrysanthemi* (*Ec*) on the oligonucleotides array. (a) Mixture of *Eca* strain 31 and *Ec* strain 340; (b) DNA from *Eca*-inoculated potato tuber tissues; and (c) DNA from potato tissue culture. Positive hybridization signals are observed as dark spots within template circles. Source: Fessehaie et al. (2003). Reproduced with permission of the American Phytopathological Society, MN, USA.

array in situ, organized as eight subarrays with an identical set of probes which could be used for hybridization with different samples. All bacterial species were readily distinguished using a single channel system for signal detection. Nearly all of the c. 1000 probes specific for *Cms*, about 50% and 40% of c. 4000 probes specific to genes of *S. scabiei* and *Eca*, respectively, and over 100 probes for *S. turgidiscabiei*, showed significant signals only with respective pathogen species tested. *Eca* and *Ecc* could be detected simultaneously with 110 common probes. However, the strains of *Eca* and *Ecc* showed variations in their signal profiles. The probes targeting IGS region and *nip* genes were useful for differentiating strains with differences in virulence levels. The custom-designed genome-wide microarrays may be useful as robust tools for differentiating bacterial pathogens infecting potatoes (Aittamaa et al. 2008).

The diversity of bacterial species in leaf midrib of citrus plants infected by huanglongbing (HLB) pathogen “*Candidatus Liberibacter asiaticus*” (CLA) was investigated by applying a PhyloChip high-density 16S rRNA gene microarray and 16S rRNA clone library procedure. PhyloChip analysis revealed the presence of 47 orders of bacteria in 15 phyla in citrus leaf midribs. Cloning and sequence procedure showed that 20 orders in 8 phyla were also present in the leaf midribs. PhyloChip arrays indicated that nine taxa were significantly more abundant in symptomatic midribs than in asymptomatic midribs. In asymptomatic plants, CLA was present in low populations. On the other hand, the pathogen was over 200 times more abundant in symptomatic plants. PhyloChip

analysis confirmed the dominance of CLA in symptomatic plants based on the data of sequencing 16S rRNA gene clone libraries. The results of the PhyloChip established the identity of the causative agent of citrus huanglongbing disease (Sagaram et al. 2009).

5.2.6.6 Nucleic Acid-Based Amplification Assay

Polymerase chain reaction (PCR-) based assays have been extensively employed for the detection of numerous microbial plant pathogens, including bacterial pathogens infecting a wide range of crops. However, PCR amplification has been found to be less suitable for epidemiological investigations for studying pathogen population dynamics and disease management strategies based on the eradication and chemical suppression of pathogen population, where determination of viability of pathogen propagules is vital. As PCR-based assays are unable to differentiate the dead from living bacterial cells, detection in the sample of RNA, which is rapidly degraded upon cell death, could be achieved by reverse-transcription (RT-) PCR assay. However, in a mixture of genomic DNA and RNA, RT-PCR format may amplify the DNA present in the sample, thus rendering RT-PCR format also unsuitable for viability studies. Nucleic acid sequence-based amplification (NASBA) assay was therefore developed to isothermally amplify a specific RNA sequence. NASBA is based on the differential activity of avian myeloblastosis virus (AMV) reverse-transcriptase RNase H, T7RNA polymerase and two sequence-specific primers for amplification of nucleic acids of target bacterial pathogen. The reaction is carried out at 41°C constant so that the use of thermocycler can be dispensed with. The desired RNA sequence is amplified in one step and antisense RNA is produced as a major amplification product.

The NASBA assay was applied for the detection of specific 16S rRNA sequences of *Ralstonia solanacearum* (Rs), causing bacterial wilt and brown rot of potato plants and tubers. Detection of RNA coincided with cell viability, so the NASBA assay enabled specific detection of living cells of target pathogen. The sensitivity of the NASBA/Northern blot procedure was determined by testing a 10-fold serial dilution of purified *R. solanacearum* rRNA in concentrations from 10^8 to 10^4 molecules of rRNA per reaction, and a concentration of 10^4 molecules could be detected by NASBA. RNA of *R. solanacearum* cells was exclusively detected, but not DNA, following treatment with RNase. The suitability of NASBA to assess cell viability was demonstrated. Viable and chlorine-killed cells of Rs were added to a potato tuber extract and tested by NASBA and PCR assays. In the NASBA test, only extracts spiked with viable cells of Rs resulted in a specific signal after Northern blot analysis, whereas PCR assay detected the DNA from both viable and chlorine-killed cells of Rs. In another experiment conducted on metal strips, a positive correlation was established between NASBA and dilution plate methods which detected/favored the development of viable cells of *R. solanacearum*. In contrast, PCR amplification occurred positively in living and dead cells of the pathogen. Loss of NASBA signal coincided with a loss of culturability of Rs present in different samples. The limit of detection of *R. solanacearum* by NASBA assay was 10^4 CFU mL⁻¹ of potato tuber extract, equivalent to 100 CFU/reaction which corresponded to less than one bacterial cell (based on the assumption that a metabolically active cell may contain c. 10^5 copies of RNA). The NASBA assay may be useful for determining the extent of cell viability in extracts of plant tissues (Bentsink et al. 2002).

Improvement of the NASBA technique for the detection of *Ralstonia solanacearum* (Rs) in potato tubers was required because Northern blotting was not suitable for routine large-scale application for the detection of pathogens in planting materials.

Northern blotting was found to be laborious and carried substantial risk of cross-contamination. AmpliDet RNA technique was therefore developed, based on a combination of NASBA amplification and homogenous real-time detection of amplicons with molecular beacons. AmpliDet RNA procedure was applied for the detection of *R. solanacearum* in potato peel extract and surface water, based on the amplification of specific 16S rRNA sequences by NASBA and monitoring of NASBA amplicons with a molecular beacon. This procedure was carried out in sealed tubes to reduce the risks of carryover contamination. Real-time detection of amplicons reduced labor input and contamination risks drastically. AmpliDet RNA technique was evaluated for the detection of *R. solanacearum* in 18 extracts of composite samples of 200 tubers from which *R. solanacearum* had been previously isolated. In addition, 91 extracts free of *R. solanacearum* on the basis of dilution plating on SMSA medium and pathogenicity assay were also tested. All 18 samples contaminated with *Rs* were consistently positive in all tests. Likewise, all but 1 of 91 pathogen-free samples tested negative. AmpliDet RNA procedure provided reliable detection of rRNA sequences of *R. solanacearum* in race 3, biovar 2 in total RNA extracts from potato tubers in 90 min at a level of 10 cells per reaction, equivalent to c. 10^4 cells mL⁻¹. All strains of *R. solanacearum* could be detected by NASBA technique (van der Wolf et al. 2004).

5.3 Detection of Phytoplasmal Pathogens

The causal agents of the yellows-type of plant diseases were assumed to be viruses because of the similarity of symptoms and insect vectors involved in their spread from infected plant to healthy plants. In certain cases, as in the case of rice tungro and yellow dwarf diseases, the same leafhopper species may transmit the diseases caused by viruses and the agent now known to be a phytoplasma. The possibility of wall-less prokaryotes being the causal agents of yellows diseases was first indicated by Japanese researchers (Doi et al. 1967). The investigation conducted subsequently established that pleomorphic wall-less prokaryotes occurring in the phloem tissues of plants affected by yellows diseases were the causal agents of such diseases. The term “mycoplasma-like” organism (MLO) was first applied to this different group of disease-causing agents, since morphological and ultrastructural similarity to mycoplasmas infecting human beings were observed. However, the primary difference in the culturability of the cell-wall-less organisms included in the class Mollicutes grouped the MLOs as distinctive microorganisms. The mycoplasmas causing human diseases could be obtained in auxenic cultures, whereas the MLOs defied all efforts to culture them in cell-free media; they are therefore obligate pathogens of plants differing from the mycoplasmas in this respect (Lee and Davis 1986).

Morphological characteristics of phytoplasmas have to be determined by taking ultrathin sections of infected plant tissues and observing them under transmission electron microscope (TEM). Localization of wall-less pleomorphic bodies in the phloem cells is revealed when stained/labeled sections are viewed. Symptoms similar to those induced by Australian papaya yellow crinkle (PYC) affecting papaya were observed in Mexico. Scanning electron microscopy (SEM) procedure was used to visualize the phytoplasma in infected samples from symptomatic and asymptomatic papaya plants. The samples were fixed in 2.5% glutaraldehyde dissolved in 0.2M sodium cacodylate

buffer (pH 7.2–7.4) for 24 h at 4°C. The samples were dehydrated, after rinsing the samples in the same buffer, using increasing grades of ethanol (30–100%), followed by treatment with acetone (100%). The samples were then dried in carbon dioxide followed by attachment to SEM stages by double-sided adhesive tape. The samples were coated with palladium in an ion sputter and examined under the scanning electron microscope at different accelerating voltages (5–20 kV). The phytoplasma bodies were detected in stems, leaf stalk, roots, axillary leaflets, leaf veins, and flowers. In addition, the presence of phytoplasma was detected in dry and germinated seeds within the fruit, suggesting possible seed transmission of the pathogen (Lebsky et al. 2010). Scanning electron microscopy was used to localize the phytoplasmas (16Sr III- X-disease group) in tomato plants. The field and greenhouse indexed samples revealed the presence of phytoplasma cells with size range of 400–1500 nm in the sieve tubes of leaf stalks, leaf midribs, stems, floral parts, and roots. Some asymptomatic field samples also had low phytoplasma titer in the phloem tissues (Lebsky et al. 2011).

As the phytoplasmas do not exhibit differentiating morphological characteristics, different groups of phytoplasmas cannot be distinguished from observations made under transmission electron microscope or scanning electron microscope. Electron microscopy in conjunction with assays based on immunological and nucleic acid properties therefore has to be employed for reliable detection and identification of phytoplasmas. The application of molecular techniques revealed the differences in the genetic structure of plant pathogenic mollicutes, resulting in the creation of a trivial name “phytoplasma” to embrace all plant pathogenic cell-wall-less pleomorphic microorganisms. The designation of a new taxon for these microorganisms was proposed as “*Candidatus* phytoplasma” (IRPCM 2004). Studies on phytoplasma phylogeny and taxonomy have provided a reliable basis for their classification based on the sequence analysis of 16S rDNA. Phytoplasmas constitute a coherent, genus-level taxon. In the monophyletic phytoplasma clade groups and subgroups have been delineated, many of which are considered as species under the provisional status “*Candidatus*” for incompletely described prokaryotes. The IRPCM have defined the characteristics of provisional species and also rules for putative species delineation to be described in future (IRPCM 2004).

Diagnostic methods based on morphological and serological properties and nucleic acid characteristics of phytoplasma have been the primary tools applied for the detection, identification, differentiation, and quantification of phytoplasma pathogens infecting various plant hosts and their insect vectors. Polyclonal and monoclonal antibodies (PABs and MABs), cloned phytoplasma DNA probes, and especially phytoplasma-specific universal (generic) or phytoplasma group-specific PCR primers designed based on the sequences of highly conserved 16S rRNA gene, have been employed for detection and identification of a large number of phytoplasmas that are presumably involved in the causation of disease with characteristic symptoms such as dwarfing, chlorosis, and virescence. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA sequences, using several restriction endonucleases such as *AluI*, *HaeIII*, or *RsaI*, was employed to identify and differentiate phytoplasmas, resulting in characteristic RFLP patterns for different strains. This simple, reliable, and practical procedure was found to be useful for identifying phytoplasma groups based on comprehensive RFLP analyses. Based on the extensive RFLP analyses of phytoplasma 16S rDNA sequences, phytoplasmas have been classified into 14 groups and 38 subgroups (Lee et al. 1998). However, this method did not provide clear resolution of all 16S rDNA groups and usually does not resolve subgroups clearly without the use of a wide range

of restriction endonucleases (Wei et al. 2007). Other techniques, including heteroduplex mobility assay (HMA) based on the 16S-23S ISR, have therefore been used to differentiate the 16Sr I subgroups (Wang and Hiruki 2005). Terminal-RFLP (T-RFLP) technique, based on the 23S rRNA gene sequences which distinguished the different groups of 16Sr, has been employed for identification of phytoplasmas (Hodgetts et al. 2007). In silico RFLP analysis was also shown to be useful for differentiation of phytoplasma groups (Lee et al. 2007). The classification of strains of propagule-borne phytoplasmas based on in silico RFLP analysis, referred to as the 16Sr RNA groups of phytoplasmas, is presented in the following.

- 16Sr III: X-disease group subgroup: III-A; strain designation: Western X-disease.
- 16Sr IV: Coconut lethal subgroup: IV-A; strain designation: Coconut lethal yellowing Yellows group.
- 16Sr X: Apple proliferation subgroup: X-A; strain designation: *Candidatus Phytoplasma group mali*.
- 16Sr XII: Stolbur group subgroup: XII-A; strain designation: Stolbur.
- 16Sr XVI: Sugarcane yellow subgroup: XVI-A; strain designation: *Ca. P. graminis* leaf syndrome.
- 16Sr XVIII: American potato subgroup: XVIII-A; strain designation: *Ca. P. americanum* purple top.
- 16Sr XXVI: Mauritius sugarcane subgroup: XXVI-A; strain designation: Sugarcane yellows D3T1 group phytoplasma D3T1.
- 16Sr XXVII: Mauritius sugarcane Subgroup: XXVII-A; strain designation: Sugarcane Yellows D3T2 group phytoplasma D3T2.

Phytoplasmal pathogens cause certain symptoms characteristic of this group, which include general stunting or dwarfing, chlorosis, smalling of leaves, proliferation of axillary buds, and antholysis of floral parts, resulting in partial or total sterility of the affected plants. No consistent association was observed between symptom type and phytoplasma group identity based on molecular characteristics. Various methods based on immunological and nucleic acid properties have been developed for the detection and identification of several phytoplasmas infecting different crop plants.

5.3.1 Immunoassays

In the absence of information on cultural and morphological properties of the phytoplasmas in cell-free media, development of detection methods based on the serological properties of phytoplasmas was found to be essential. This approach indicated the presence of a single reasonably abundant surface membrane protein that was immunodominant (Clark et al. 1984). Polyclonal and monoclonal antibodies (PAb and MAbs) have been demonstrated to be useful for reliable and sensitive detection of phytoplasmas affecting many plants. Among the immunoassays, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence (IF) methods have been used more frequently for the detection of phytoplasmal pathogens in plant tissues.

Pathogen-specific polyclonal antibodies were generated against *Spiroplasma citri*, causing citrus stubborn disease. The presence of *S. citri* in infected citrus plants and leafhopper vector *Circulifer haemotoceps* was detected by ELISA tests (Najar et al. 1998). Corn stunt disease appeared to be due to a complex caused by mollicutes. The involvement of *Spiroplasma citri* as one of the causative agents was demonstrated by employing

specific F(ab')₂ in protein A-ELISA format (Henriquez et al. 1999). A surface protein p89 in *S. citri* was detected by electrophoresis and Western blotting. By using ELISA, *S. citri* was detected in the vector *Circulifer tenellus*. The results indicated that the surface protein might have a role in the adhesion of *S. citri* to vector cells (Yu et al. 2000). Citrus stubborn disease (CSD) caused by *Spiroplasma citri* is a widespread bacterial disease prevalent in most arid citrus-growing regions in the USA and Mediterranean region. The major commercial, economically important hosts include grapefruit, lemon, mandarin, orange, and sour orange. Detection of *S. citri* by PCR-based methods provided erratic results because of the uneven distribution of pathogen population in infected trees. A novel diagnostic method was therefore developed based on the presence of *S. citri*-secreted protein as a detection marker. *S. citri* secreted specific proteins in the phloem during infection. The phloem extract from infected plants contained six secreted proteins with relatively high abundance. The protein CAK98563 was selected as a CSD detection marker. Using mass spectrometry analysis, the unique protein secreted by *S. citri* was identified. The antibody ScCCPP1 was generated by expressing the cloned gene in *Escherichia coli*. Western blots developed with this antibody confirmed that CAK98563 was induced by the phloem extract and secreted into the medium by *S. citri* in liquid culture. Direct tissue-print immunoblot technique was applied to detect *S. citri* in young branches and petioles. The antibody ScCCPP1 gave clear positive signals from citrus graft-inoculated with both S616 and C189 strains of *S. citri*, whereas no signal was seen in healthy tissues. ScCCPP1-based imprint method was compared with a newly developed TaqMan PCR assay, using the same citrus samples. All six naturally infected trees from the field tested positive in the imprint assay, but one of six trees tested negative by qPCR assay. The results indicated that ScCCPP1-based direct tissue-print protocol could overcome the uneven distribution or low titers of *S. citri* in infected plants, resulting in the possibility of avoiding false-negative diagnosis. The investigation introduces a novel concept of using proteins secreted by *S. citri* as markers to detect pathogens residing in plant vascular tissues (Shi et al. 2014).

Sugarcane grassy shoot disease (GSD) is transmitted via planting materials (setts) obtained from infected mother plants. Indirect ELISA format was found to be more sensitive in detecting the phytoplasmal pathogen in sugarcane tissues. The antiserum raised against sugarcane white leaf disease (WLD) occurring in Taiwan cross-reacted with the extracts of GSD-infected sugarcane plants (Viswanathan 1997). Sugarcane yellow leaf syndrome was shown to be due to combined infection by sugarcane yellow leaf phytoplasma and *Sugarcane yellow leaf virus* (ScYLV), by performing DAS-ELISA, PCR, and RT-PCR techniques (Aljanabi et al. 2001). Immunofluorescence (IF) technique has been shown to be efficient in detecting the phytoplasmas in situ. Aster yellows (AY) phytoplasma infecting lettuce plants was detected by examining acetone-fixed sections of midribs of AY-infected leaves stained with fluorescein isothiocyanates (FITC) conjugated antimouse IgG. Use of monoclonal antibodies (MAbs) specific to AY phytoplasma resulted in specific binding of MAbs with AY phytoplasma bodies in the sieve tubes, whereas application of PABs led to fluorescence throughout the sections in both healthy and diseased plants due to nonspecific binding of PABs to cell-wall membranes. The IF tests revealed the specificity of MAbs in detecting the AY-phytoplasma in plant tissues (Lin and Chen 1986). Immunofluorescence staining procedure was effective for the detection, identification and assessment of genetic relatedness of phytoplasmas associated with the occurrence of grapevine yellows disease in several countries (Chen et al. 1994). The presence of sugarcane grassy shoot disease (GSD)

phytoplasma in different tissues of sugarcane such as leaf lamina, midrib, leaf sheath, nodes, and roots was revealed by the characteristic apple green fluorescence in indirect immunofluorescence test (Viswanathan 2000).

5.3.2 Nucleic Acid-Based Techniques

Although the immunoassays such as ELISA tests are simple, cost-effective, and suitable for large-scale utilization, their lower level of sensitivity compared to nucleic acid-based assays may lead to inaccurate diagnosis if the phytoplasma titer is low in the plant tissues sampled (Loi et al. 2002). Further, cross-reaction with other strains of a phytoplasma and inconsistency in the presence of phytoplasma-specific membrane epitopes at all stages of the life cycle of the target phytoplasma have been the major limitations of immunoassays, restricting their applicability for routine detection of phytoplasmas. On the other hand, nucleic acid-based techniques possess potential for rapid, specific, sensitive, and reliable detection of various phytoplasmas infecting a wide range of plants and insects that are involved in their transmission from infected plant to healthy plants. Specific tissues such as phloem sieve tube elements or haemolymph or salivary glands of insect vectors may be used for extraction of phytoplasmal DNA, since these tissues contain higher concentrations of the target phytoplasmal pathogen. Two types of tests, based on hybridization and polymerase chain reaction procedures, have been employed with varying degrees of success.

5.3.2.1 Hybridization Techniques

Taxonomic classification of phytoplasmas is primarily based on the sequences of the 16S rDNA which has been sequenced for nearly all known types of phytoplasmas. DNA probes specific to phytoplasma groups and strain-specific oligonucleotides for PCR amplification have been constructed. Phytoplasma-specific DNA probes have also been prepared from chromosomal or plasmid (extra-chromosomal) DNA of the phytoplasmal pathogens. These probes may be labeled with either radioactive ^{32}P or nonradioactive biotin or digoxigenin. Labeled oligonucleotide probes have been effectively employed as strain-specific probes in the dot-blot hybridization of PCR-amplified phytoplasma DNA fragment, and in localization of phytoplasmas in plant cells using electron microscopy. Digoxigenin-labeled DNA probes were effective for the detection of sweet potato witches' broom phytoplasma in infected sweet potato plants. Most of the probes positively hybridized with serologically related peanut witches' broom phytoplasma DNA at 10 and 0.39 ng of DNA from periwinkle and sweet potato, respectively, indicating that this phytoplasma might reach higher concentrations in sweet potato than in periwinkle (Ko and Lin 1994). The phytoplasma causing decline syndrome in coconut was detected by dot-blot hybridization by using two probes from palm lethal yellowing (PLY) phytoplasma (Tymon et al. 1997).

5.3.2.2 Polymerase Chain Reaction Techniques

High-quality phytoplasma DNA without PCR inhibitors of plant or insect origin is the basic requirement for performing PCR assays. General and specific primers located in the 16S rDNA intergenic spacer (IGS) and the 23S rDNA regions of the phytoplasma genomes have been frequently used. A cloned fragment of a plasmid from the phytoplasma is sequenced to identify oligonucleotide primers for PCR amplification. Amplified DNA fragments of the predicted size are then obtained from the DNA

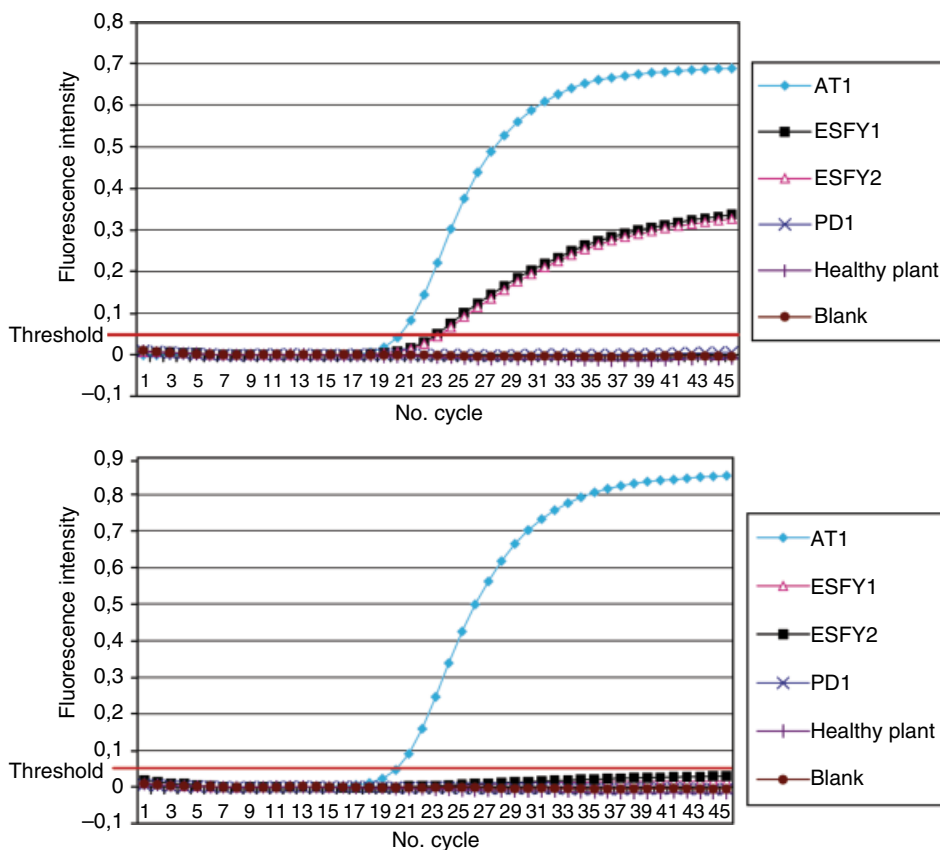


Figure 5.23 Amplification curves of fluorescent signals from different isolates of "*Candidatus Phytoplasma mali*" (AT1), "*Ca. P. prunorum*" (ESFY1 and ESFY2), and "*Ca. P. pyri*" (PD1) following real-time PCR. Top: qAP-16S-F/R primers; bottom: AP-MGB probes at 64°C as hybridization temperature. Source: Aldaghi et al. (2007). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

extracted from plants infected by the target phytoplasma, and no amplification occurs in the extract from healthy plants. The PCR-based methods have been shown to be more sensitive than hybridization-based assays in general (Narayanasamy 2011).

An effective procedure was developed for extracting the DNA from tissues of apple trees infected by "*Candidatus Phytoplasma mali*." Endpoint and real-time PCR detection procedures were applied for the detection of "*Ca. P. mali*." From the crude extract, the pathogen was detected in 86 and 35 samples from inoculated and naturally infected apple trees. Different TaqMan MGB-SYBR Green-based real-time protocols efficiently detected "*Ca. P. mali*" in DNA extracted from infected apple trees (Fig. 5.23) using the improved method developed by Aldaghi et al. (2009). Apple proliferation (AP) and pear decline (PD) caused by phytoplasmas could be detected by PCR assays using primers designed based on the sequences of ribosomal and nonribosomal DNA. A ribosomal primer pair fU5/rU3 was employed to initiate amplification of the target DNA from all 42 samples from PD-infected pear trees and 36 samples from AP-infected trees. A pair of group-specific primers fo1/ro1 derived from the 16S rRNA gene was used for detection of closely related phytoplasmas associated with AP and PD diseases, in addition to

European stone fruit yellows. Restriction enzyme analysis of the PCR amplicons obtained with primer pairs fO1/rO1 was useful for differentiation of AP and PD phytoplasmas. However, the pathogen-specific primers failed to detect all strains of these two phytoplasmas (Lorenz et al. 1995).

A PCR-based assay developed for the detection of European stone fruit yellows phytoplasma involved the amplification of a 237bp DNA fragment from total DNA extracts derived from over 300 stone fruit samples. A high correlation (97%) was observed between the results obtained with specific and universal primers employed for PCR amplification (Jarausch et al. 1998).

Primers capable of functioning at high annealing temperatures were designed for detection of the Australian isolate of European stone fruit yellows (ESFY) phytoplasma. The specificity of detection was enhanced in addition to lowering of the risk of false positives. The new primers PA2F/R employed in direct PCR assay could detect phytoplasmas in tissue cultures of apple, pear, and apricot infected by apple proliferation (AP), pear decline (PD), and European stone fruit yellows (ESFY). A nested PCR format was found to be necessary for the detection of the phytoplasmas in field samples, because of low concentration and uneven distribution of phytoplasmas. A concentration of 1:2000 dilution was found to be optimal because of the presence of PCR inhibitors in the fruit plant extract. By using an appropriate restriction enzyme to cleave at specific sites, it was possible to distinguish the phytoplasmas. When *TaiI* enzyme was used ESFY could be distinguished from AP/PD phytoplasmas, whereas *Tsp509* was useful for differentiating AP/ESFY and PD phytoplasmas. *TaqI* was employed to distinguish AP from ESFY and PD phytoplasmas. In addition, phytoplasma strains could be identified by direct PCR amplification by specific primers followed by RFLP analysis. This procedure was found to be efficient in detecting the phytoplasmas in micropropagated fruit-tree-planting materials (Heinrich et al. 2001).

Lethal yellowing (LY) disease infecting palms was diagnosed by employing universal rRNA primer pair P1/P7, nested LY-group-specific rRNA primer pair 503f/LY16Sr, or LY phytoplasma-specific nonribosomal primer pair LYF1/R1. The phytoplasma could be detected in the embryos from fruits of diseased Atlantic coconut palms. Distribution of LY phytoplasma in sectioned tissues from embryos was traced by applying in situ PCR and digoxigenin-11-deoxy-UTP (DIG) labeling of amplified products. Colorimetric assay was used to detect DIG-labeled DNA products (Cordova et al. 2003). Coconut tissue samples collected from 50 sites with coconuts showing varying levels of mortality due to lethal yellowing (LY) disease were examined by PCR assay. A 1.8kb rDNA fragment was amplified from all samples, using the primer pair P1/P7 in conventional PCR format. As the next step, amplification of the products of the P1/P7 was carried out using the nested PCR format with the 16Sr IV group-specific primer pair LY16Sf/LY16S-23Sr. A product of the expected size of 1.7 kb was generated from all samples. Evaluation of samples by PCR with pathogen-specific nonribosomal primer pair LYR1/LYF1 amplified a 1.0kb product providing specific detection of LY phytoplasma in the coconut tissues from all sites in Jamaica (Myrie et al. 2007).

Grapevine yellows (GY) are associated with three molecularly distinguishable phytoplasmas. Flavescence dorée (FD) is due to the phytoplasma belonging to the subgroup 16SrV-C (elm yellows group), while Bois noir (BN) disease is associated with the phytoplasma belonging to the subgroup 16SrVII-A (stolbur (STD)) phytoplasma. A mixed infection of European stone fruit yellows (apple proliferation group, 16SrX-B subgroup) has also been observed. Flavescence dorée is considered to be the most severe threat to

grape production among the phytoplasmas infecting grapevine. FD has been included in several quarantine and sanitary programs for eradicating or excluding the introduction and further spread of the disease via planting materials.

For the detection of the FD phytoplasma, probes surrounding the polymorphic sites specific to phytoplasma groups were designed to distinguish between FD phytoplasma (16SrV-C and 16SrV-D) from that associated with Bois noir (BN, 16SrXII-A). The discriminating and common probes were chosen carrying the SNP in their 3' end and an optimal length was fixed to obtain T_m of 60°C of the six probes. Two specific sequences for the phytoplasma group 16SrXII were attached to zips 1 and 3, while other specific probes for group 16SrV were attached to zips 5, 11, and 13. A universal probe, matching with all known phytoplasma 16S sequences, was designed and linked to zip 15. The primer pair 16SrF2n/16SrR2 designed for universal amplification of 16S rDNA gene from all known phytoplasmas was used for a first round of PCR tests. A second round of PCR test (nested PCR) was separately performed for grapevine samples, using primer pairs designed for specific amplification of stolbur (R16(I)F1/R) and elm yellows (R16(V)F1/R1) phytoplasma groups. Ligase detection reaction (LDR) assay was applied for detection of phytoplasmas in selected DNA samples from grapevine plants showing typical symptoms of grapevine yellows (GY). Two samples (343 and 355) reacted with stolbur-specific probes (zip 1 and zip 3), but not with those designed for group V hybridization (zip 11 and zip 13). All grapevine samples reacted with the phytoplasma universal probe (zip 15), while no signal was recorded for DNA amplicon of 16SrXII-A-infected grapevine amplified with 16SrF1/R1(V) primer pair. The results indicated that the LDR technique might be an alternative rapid procedure for detection of phytoplasmas and the phytoplasmas causing FD and BN diseases could be differentiated by this technique (Frosini et al. 2002).

Molecular characterization of RFLP analysis of *rpS3* and *SecY* genes of flavescence dorée (FD) phytoplasma in Serbia was useful to distinguish their strains occurring in Italy. RFLP profiles obtained after treatment with *TaqI* enzyme were indistinguishable from each other and reference strains of FD. A further collective RFLP characterization of nonribosomal FD9 and rpVF2/rpR1 amplicons with the restriction enzymes *TruI* allowed finer differentiation among phytoplasmas of group 16SrV and, in particular, indicated that the Serbian grapevine FD showed the highest identity to the phytoplasma in grapevine from the Italian Treviso province (TV46) (Fig. 5.24) (Duduk et al. 2004). Typical symptoms of grapevine yellows (GY) were observed in grapevine red cultivar "Merlot." By using generic primers for phytoplasmas, positive amplification of expected pathogen DNA fragment was accomplished. In the second round amplification using specific primers for FD and BN, the presence of BN (stolbur) was detected in three samples. Other 17 samples as well as the healthy grapevine control samples tested negative for BN. Infection of grapevines by stolbur phytoplasma was detected using a multiple nested PCR format, in addition to FD phytoplasma. This protocol provided reliable results based on which the testing of mother plants during growing season was organized, resulting in the eradication of infected vines as a preventive measure to restrict disease spread (Avramov et al. 2008).

Electron microscopic observation and histochemical tests using a fluorescent DNA-binding dye 4,6-diamidino-2-phenylindole-2HCl (DAPI) indicated the involvement of a phytoplasma in the citrus huanglongbing (HLB) disease for which "*Candidatus Liberibacter asiaticus*" (CLA) has been established as a causal agent. The PCR assay was applied using the phytoplasma-specific primer sets fU5/rU3 nested with primer set

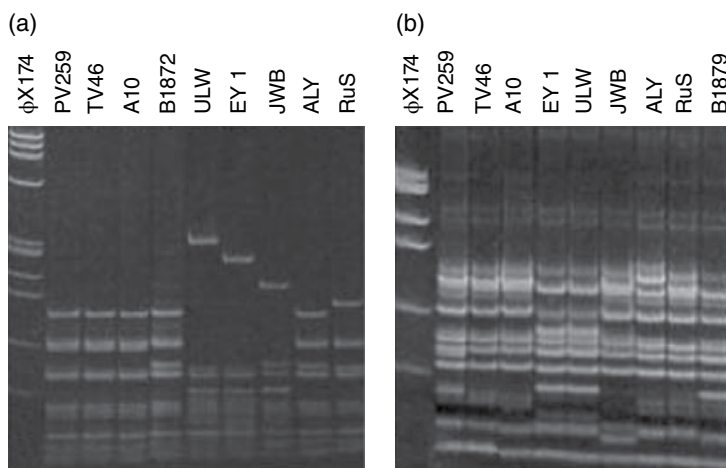


Figure 5.24 Differentiation of strains of Flavescence dorée (FD) phytoplasma in Serbia using restriction fragment length polymorphism analysis. (a) FD0f3/r2 amplicons; (b) rp(V)F2/rpR1 amplicons of FD-infected Serbian sample (A10). Fragment sizes from top to bottom: 310, 281, 271, 234, 194, 118, and 72. Source: Duduk et al. (2004). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

P1/P7 to re-examine the nature of causal agent of HLB disease. This primer set detected the phytoplasma in 110 (78%) samples. A 1785 bp amplicon was obtained with primer set P1/P7. Sequence analysis revealed a 100% identity of the sequence in the region of 16S rDNA and 16S-23S rRNA intergenic transcribed spacer (ITS) to three strains of “*Candidatus Phytoplasma asteris*” (onion yellows). Of the 141 samples, 89 (63.1%) samples were positive for CLA. Observations under electron microscope showed the presence of both walled and wall-less bodies in the phloem sieve-tubes of HLB-affected citrus plants. The results indicated that both CLA and the phytoplasma related to “*Ca. P. asteris*” might have a role in the induction of symptoms similar to HLB, which might be difficult to differentiate by visual examination (Chen et al. 2009).

Sugarcane grassy shoot disease (GSD) phytoplasma is spread primarily through infected planting materials (setts) and the leafhopper (*Deltocephalus vulgaris*). The universal phytoplasma-specific primer pairs P1 and P7 derived from the highly conserved ribosomal sequence primers within the 16S and 23S rRNA gene sequences and intergenic spacer regions were used in direct PCR format, whereas P4 and P7 primers were employed for nested PCR format for the detection of GSD phytoplasma in plant and insect tissues. The phytoplasmal DNA was detected in sugarcane cultivar COLK 8102 and the leafhoppers fed on infected plants. There was no amplification of phytoplasma DNA from healthy sugarcane or leafhoppers in direct PCR assays, using the primer pair P1/P7. A product of about 1600 bp was amplified from all samples of infected sugarcane leaves. Nesting of P1/P7 amplified product with primer pair P4/P7 primers resulted in the amplification of c. 500 bp products both in infected leaves and insects exposed to infected sugarcane plants. Nested PCR protocol developed in this investigation was found to be a reliable molecular tool for detection of sugarcane grassy shoot phytoplasma in infected sugarcane plants and infective leafhoppers (Srivastava et al. 2006).

“*Candidatus Phytoplasma aurantifolia*,” the causal agent of witches’ broom disease of lime (WBDL), occurs in a devastating form in southern Iran. Natural spread of the

disease occurs through infective leafhopper (*Hishimonus phycitis*) and infected rootstocks. As the possibility of transmission of "*C. P. aurantifolia*" via seeds was suggested by earlier studies, seed transmission of the phytoplasma was examined by testing seeds, leaf, stem, and root samples by employing direct and nested PCR assays. None of the leaf, stem, and root tissues of 600 samples showed the presence of any phytoplasma by both PCR formats. Likewise, in-vitro-grown plants from the seed of infected lime trees revealed no phytoplasmal DNA by PCR amplification. However, direct and nested PCR formats detected WBDL phytoplasma in 20 and 34% of seed samples from infected trees, respectively. The excised embryos, seedlings, and in-vitro-grown seedlings did not contain any phytoplasmal DNA, as indicated by direct and nested PCR assays. "*Ca. P. aurantifolia*" was detected in the seed coats of some seeds collected from infected lime trees. Although WBDL phytoplasma was detected in seeds harvested from infected trees, no transmission of the pathogen to seedlings occurred. In all cases of positive PCR results, the RFLP patterns were similar to that of "*Ca. P. aurantifolia*," establishing the identity of the causal agent of witches' broom disease of lime in Iran (Faghihi et al. 2011).

Cooperational (Co-) PCR assay performed in a single reaction minimizes the risks of contamination (associated with nested PCR format) and also provides similar levels of sensitivity as nested PCR and real-time PCR assays. In addition, it is also possible to couple Co-PCR procedure with dot-blot hybridization, making it possible to characterize the nucleotide sequence of the desired DNA fragment/amplicon. A triprimer reaction coupled with hybridization using general and specific probes were employed for the detection of "*Candidatus Phytoplasma mali*," "*Ca. P. prunorum*," and "*Ca. P. pyri*." The comparative sensitivities of Co-PCR coupled with dot-blot hybridization, single round and nested PCR, and real-time PCR formats were assessed. The single round (standard) PCR format could only detect the phytoplasma DNA in the undiluted, purified sample. However, Co-PCR, nested PCR, and real-time PCR formats were more sensitive (by a factor of 1000) and detected the pathogen DNA at a dilution of 1:1000. Specific hybridization and color precipitation was observed for all phytoplasma isolates when the general probe was used. Specific detection of two isolates belonging to 16SrX group was achieved by employing group-specific probe. The specificity and sensitivity of Co-PCR were demonstrated by testing field samples collected with seven phytoplasmas maintained in the indicator plant *Catharanthus roseus*. The Co-PCR protocol developed in this investigation represented a high-throughput detection procedure for simultaneous detection and characterization of members of the 16SrX phytoplasma group placed under quarantine regulations. Because of its relative simplicity and high sensitivity, the Co-PCR assay has the potential for large-scale use in routine detection of phytoplasmas, particularly for laboratories lacking a real-time PCR facility (Bertolini et al. 2007).

Potatoes are infected by many fungal, bacterial, phytoplasmal, viral, and viroid diseases. The infection of potato seed tubers by phytoplasmas has been assessed less frequently compared to other microbial pathogens. Eight batches of seed potatoes collected from different locations in Italy were planted in a greenhouse. Sprouts growing from the selected tubers were tested for the presence of phytoplasmas by employing universal primers P1/P7 in direct PCR, followed by nested PCR with primer pairs R16F2/R2 and R16(1)F1/R1. The primer pair R16F2/R2 was also used in a direct PCR assay. RFLP analyses were performed with *TruI* and *TaqI* for phytoplasma identification. Small shoots (12–15 cm long) collected from either single potatoes or from batches of three

potatoes each were used for DNA extraction and tested for the presence of phytoplasmas. The assays showed that phytoplasmas belonging to diverse ribosomal groups were present in the potato tubers. Based on RFLP analyses on 16S rDNA gene, phytoplasma belonging to ribosomal subgroups 16SrI-B (related to "*Candidatus Phytoplasma asteris*"), 16SrI-C (related to clover phyllody), 16SrII-D (related to tomato big bud from Australia), 16SrX-A (related to "*Ca. P. mali*"), and 16SrXII-A (related to stolbur) were identified. The protocol employed in this investigation provided reliable results rapidly (Paltrinieri and Bertaccini 2007).

When a large number of samples has to be tested, standard PCR format may not be suitable for the detection of phytoplasmas. A real-time PCR assay is desirable under such conditions to provide more sensitive and rapid detection of the phytoplasmal pathogens in planting materials. Real-time PCR assays for the specific diagnosis of flavescence dorée (FD), bois noir (BN), and apple proliferation (AP) phytoplasmas, and a universal pair for the detection of phytoplasma belonging to groups 16SrV, 16SrX, and 16SrXII, were developed. The expected fragments from the DNAs of respective phytoplasmas in field-collected samples infected with FD and BN diseases were amplified by the primers constructed in this investigation. The group-specific assays provided highly efficient detection comparable to nested PCR assay (Galetto et al. 2005). TaqMan minor groove binder probes were used in the real-time PCR format for the detection of grapevine yellows (GY) phytoplasma. Two amplicons for group-specific detection of FD and BN phytoplasmas, plus a universal phytoplasma amplicon, were also included. The FD and BN amplicons were designed to amplify species-specific genomic DNA fragments, and the universal amplicon to amplify the 16S rDNA region. Specific reactions devoid of cross-reactivity with other phytoplasma strains, plant, or insect vector DNA were indicative of the specificity of the primers employed in the real-time PCR assay. The real-time PCR procedure developed in this investigation detected the phytoplasmas in several PCR-negative and in all PCR-positive samples, revealing its higher level of reliability, sensitivity, and rapidity, and its potential for large-scale use for the detection of grapevine yellows phytoplasmas (Hren et al. 2007).

Diagnosis of flavescence dorée (FD) disease of grapevine was routinely detected by different PCR-based assays, but the low pathogen titer and presence of PCR inhibitors were found to be important limitations of PCR formats. Reverse transcription has the advantage of the high ribosomal RNA copy number in the pathogen cell, resulting in an increased amount of initial template for the amplification, even though only two copies of the 16S rRNA gene are present in the phytoplasma genome and available for detection by nested PCR format. In addition, a positive RT-PCR result indicates the active metabolism of the pathogen, whereas nested PCR format relies on the mere presence of phytoplasma DNA in the samples which could be derived from both live and dead bacterial cells. A simple, quick, sensitive, and reliable method for mass screening of FD in grapevine was developed based on reverse-transcription (RT-) PCR amplification of the 16S rRNA of FD phytoplasma. Of the 232 samples tested, 135 were positive for FD and 97 samples were FD-negative by nested PCR format, whereas 112 samples tested positive and 110 tested negative for FD by RT-PCR assay. Positive results for 115 samples were obtained for both nested PCR and RT-PCR assays. However, negative results obtained with nested PCR (97) were greater than that of RT-PCR (90) assay. The rapidity, sensitivity, and reliability of the RT-PCR protocol developed in this investigation has the potential to detect FD phytoplasma infection, in addition to grapevine viruses for which RT-PCR assay was already in use (Margaria et al. 2007; Section 5.4.13).

A multiplex real-time PCR format was developed for simultaneous amplification of a fragment of apple proliferation (AP) phytoplasma 16S rRNA gene and the host *Malus domestica* chloroplast gene coding for tRNA leucine, which was used as the internal analytical control to validate the false-negative results due to PCR inhibition. The pathogen was detected based on the highly conserved 16S rRNA gene to ensure amplification of different strains of AP phytoplasma. The primer/probe set allowed specific detection of all AP strains tested, since the primers and probe matched the sequences in the variable region of the 16S rRNA gene starting at position 82 and ending at position 157. No variation in the sequences was detectable within this region of all four AP strains collected from Spain, France, Italy, and Germany. This real-time protocol provided accurate, reproducible, and positive results even for samples with low pathogen titer that was not detectable by conventional PCR format. By simultaneous amplification and individual detection of pathogen and host DNA, it was possible to detect the AP phytoplasma and to distinguish uninfected plant material and PCR inhibitors. The results indicated the usefulness of the real-time PCR procedure for early detection of AP phytoplasma in the infected apple plants (Baric and Dalla-Via 2004).

Flavescence dorée (FD) and bois noir (BN) are the phytoplasma components of grapevine yellows (GY) disease complex in the European vineyards. Although they are genetically distinct pathogens, they cause similar symptoms. In addition, *Grapevine leafroll-associated virus-1* and *-3* (GLRaV-1 and *-3*) and *Grapevine virus A* (GVA) are also associated with grapevine yellows. Methods for multiple detection of the phytoplasmas and viruses associated with GY were developed. Real-time reverse-transcription (RT-) PCR assay was applied for the detection of FD phytoplasma (FDp) and real-time nested PCR format was employed for the detection of bois noir (BNp), GLRaV-1 and *-3*, and GVA using the same sap extract of grapevines samples as the template. A TaqMan fluorogenic assay was used for the detection of FDp and BNp phytoplasmas and optimized for GLRaV-1 and *-3* and GVA detection by designing a real-time nested PCR following a one-tube RT-PCR format. As grapevine viruses have an RNA genome and are strictly localized to the phloem, as for the phytoplasmas, sampling could be made from the same tissue; RT-PCR allowed use of the same extract without the need for separate nucleic acid extractions. The TaqMan procedure developed in this investigation combined the advantages of RT-PCR with the real-time technique, avoiding the use of hazardous chemicals and reducing total testing time. Further, TaqMan technology provided additional specificity and sensitivity compared to conventional RT-PCR and the FD assay was five orders of magnitude more sensitive than RT-PCR developed earlier. The real-time nested PCR format was 2–5 times more sensitive than the ELISA test. The results indicated coinfection of grapevines by phytoplasmas and viruses in 30% of the samples and mixed virus infection in 69% of virus-infected samples. Real-time nested PCR format, useful for the detection of the viruses, and BN phytoplasma could be performed in the same 96-well microplate allowing up to 20 samples to be checked simultaneously for 4 pathogens. The methods developed in this investigation were effectively employed to monitor infections by phytoplasmas and viruses in field and nursery samples during the grapevine-growing season (Margaria et al. 2009).

A real-time PCR assay conjugated with the fluorescent SYBR® Green I dye was developed for rapid, sensitive, and qualitative detection of “*Candidatus Phytoplasma pyri*,” “*Ca. P. prunorum*,” and “*Ca. P. mali*,” members of apple proliferation (16SrX) group of quarantine importance. The double-stranded DNA-binding dye SYBR Green I is an

economic alternative based on the monitoring of the fluorescence intensity, after each PCR cycle. The dye binds in the minor groove of ds-DNA in a sequence-independent way. Different PCR amplification products could be accurately distinguished, using the dye, by the generation of DNA melting curves and first derivative melting peaks. The primers selected in this investigation specifically amplified a target 217bp fragment from the 16Sr gene region of the 16Sr-X group and not from any other tested phytoplasma groups. An artificial template, consisting of a plasmid clone of a 1785bp DNA fragment of the 16S rRNA gene, 16S/23S rDNA spacer region, tRNA-Ile, and partial 23S rRNA gene of a "*Ca. P. prunorum*" isolate, was employed to establish a calibration curve to evaluate the number of amplified targets per sample. The sensitivity of the real-time PCR assay was similar to that obtained with nested PCR tested with field samples and calibration standards, allowing the detection of 10 copies of the target gene present in the reaction mixture. The populations of phytoplasmas in apricot, pear, and Japanese plum ranged from 9.7×10^3 to 3.0×10^5 cells g⁻¹ of tissue. Apple proliferation (AP) phytoplasma concentrations ranged from 6.5×10^2 to 1.0×10^8 cells g⁻¹ in apple tree tissues. The real-time PCR assay was found to be a high-throughput detection tool capable of detecting all phytoplasmas from apple proliferation group that are subjected to quarantine regulations. In addition, the protocol provides high security with the positive results due to the specificity of the primers used and avoids false-positive results due to post-PCR manipulations, allowing its application in a wide sanitary program in nursery selection (Torres et al. 2005).

Apple proliferation (AP) phytoplasma disease appeared to pose an endemic threat in some apple-growing areas of central and southern Europe and also in North America, necessitating the application of strict quarantine regulations (EPPO 2004; NAPPO 2004). The comparative sensitivity and applicability of the TaqMan real-time PCR and four conventional PCR-based assays, namely PCR fU5/rU4, PCR fOI/rOI, PCR AP5/AP4, and PCR-ELISA, was assessed to detect AP phytoplasma in apple trees. The performance of the methods was evaluated based on sensitivity, specificity, susceptibility to PCR inhibition, handling effort, testing time, assay expenses, and potential risk for operator and environment. Of the 162 isolates of AP DNA, 119 were found to be positive with real-time PCR, 117 with PCR-ELISA, 115 with fU5/rU4 and fOI/rOI, and 114 with AP5/AP4 formats. The TaqMan real-time PCR format combined the highest test sensitivity with highest specificity, and offered the distinct advantage of being insensitive to PCR inhibition by inhibitory compounds of host origin. The specificity of PCR fU5/rU4 and PCR-ELISA was found to be 95.6% and 95.5%, respectively, and were therefore at a lower level in comparison to TaqMan real-time PCR format. In addition, TaqMan real-time format had the simplest and fastest testing process involving the minimum handling steps. However, the high cost of consumables, reagents, and equipment exceeded that of standard PCR format by a factor of four. Nevertheless, the higher material costs could be compensated by considerably lower personnel costs and by saving expenses for hazardous waste disposal. The real-time TaqMan PCR assay for the detection of AP phytoplasma was found to be the most accurate and rapid testing method compared to other methods tested. The possibility for automation provided an advantage over other diagnostic methods for large-scale routine application in certification programs or pathogen surveys (Baric et al. 2006).

A major prerequisite for the management of apple proliferation (AP) disease caused by "*Candidatus Phytoplasma mali*" is the accurate and rapid detection of the pathogen for fulfilling the certification requirements. The relatively low titers and uneven

distribution of the phytoplasmal pathogen make it even more problematic for developing suitable and reliable diagnostic methods. A TaqMan minor groove binding (MGB) probe and optimal PCR conditions were designed for rapid, specific, and sensitive detection of "*Ca. Phytoplasma mali*." The probe could distinguish a single mismatch between "*Ca. P. mali*" and "*Ca. P. prunorum*." The relative position of the mismatch in the MGB probe greatly influenced the specificity of detection. The real-time PCR protocol developed in this investigation was able to detect one plasmid copy in water and 100 copies in healthy plant DNA extracts. A single mismatch within the annealing region of the qAP-16S probe generated a fluorescence signal. Phytoplasma infection was monitored after graft inoculation and evaluated by conventional PCR and real-time PCR formats. The conventional PCR procedure detected infection by "*Ca. P. mali*" at 4 months after inoculation. On the other hand, the real-time PCR assay detected the pathogen at 3 months after inoculation in apple and periwinkle plants. The TaqMan MGB probe successfully detected all three subtypes of "*Ca. P. mali*" (-AP, -AT1, and -AT2) of different geographical origin. No signal was obtained with strains belonging to closely related phytoplasmas, indicating higher level of specificity of the assay. The protocol developed in this investigation was 10–100 times more sensitive than standard PCR using the universal primer pair fU5/rU3, and 10 times more sensitive than two other real-time PCR formats tested. The TaqMan MGB real-time PCR detected the phytoplasma in 100% of infected apple plant materials tested, even before the appearance of symptoms. Because of the higher sensitivity and rapidity of providing the results, the TaqMan MGB real-time PCR format might be useful in the framework of certification programs where early detection of infection status of a suspicious sample could be a critical factor (Aldaghi et al. 2007).

A quantitative, real-time TaqMan PCR assay was developed for the detection and quantification of a group 16Sr-II phytoplasma in extracts of tomato and potato infected by Columbian Basin potato purple top (CBPPT) phytoplasma. Primers and probes were designed from the 16S rRNA gene of CBPPT phytoplasma, which was closely related to the beet leafhopper-transmitted virescence agent (BLTVA). The real-time PCR assay consistently detected the 16Sr-VI phytoplasma in the extracts from infected tomatoes, potatoes, and the aster yellows (AY group 16Sr-I), and potato witches' broom group (16Sr-IX group) phytoplasma in periwinkle plants. A dilution series of 5 pg to 50 ng of DNA from six potato plants showing symptoms of potato purple top disease indicated that there were considerable variations in the pathogen concentration, as reflected by phytoplasma DNA contents in these plants. The threshold cycle (Ct) values confirmed the results of serial dilutions when 25 ng of DNA from eight purple top symptomatic plants were tested. The concentrations of phytoplasma DNA varied by a factor of several thousand in the extracts obtained from infected potato plants (Fig. 5.25). The nested PCR and real-time PCR formats showed that 30% of the leafhopper vectors (*Circulifer tenellus*) contained detectable phytoplasma. As the real-time PCR assay is less labor-intensive than nested PCR assay, it may be more suitable for rapid and quantitative detection of phytoplasmas both in plants and the leafhopper vectors (Crosslin et al. 2006).

Based on the 16S rRNA gene sequence similarity, apple proliferation (AP) phytoplasma ("*Candidatus Phytoplasma mali*") is considered to be closely related to "*Ca. P. pyri*" (16SrX-C) and "*Ca. P. prunorum*" (16SrX-B). Analyses of a nonribosomal DNA fragment, composed of three putative ORFs (in particular ORF2 coding for a protein significantly homologous with a bacterial nitroreductase), proved the existence of at

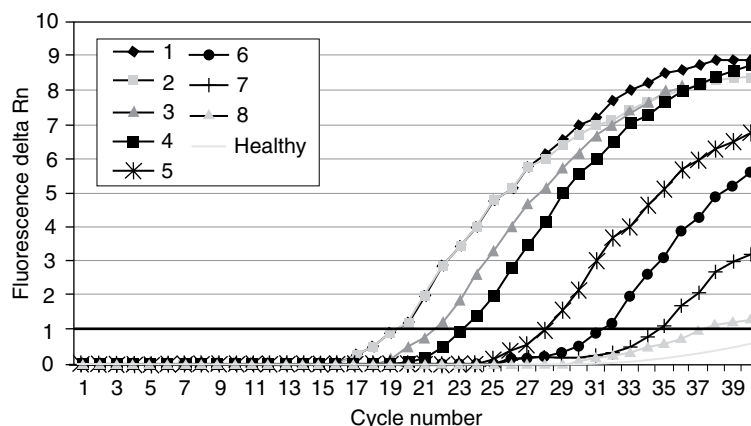


Figure 5.25 Detection of potato purple top phytoplasma by real-time PCR assay. Eight potato plants showing positive reaction in nested PCR assay were analyzed along with a sample from healthy potato plants cultivar Shepody. Source: Crosslin et al. (2006). Reproduced with permission of the American Phytopathological Society, MN, USA.

least three different AP phytoplasma subtypes designated AT-1, AT-2, and AP-15 (Jarausch et al. 2000). Molecular characterization of the genes coding for the ribosomal proteins L22 and S3 revealed the presence of higher genetic heterogeneity within isolates of “*Ca. P. mali*” (Martini et al. 2008). In a later investigation, direct PCR assays carried out using universal primer pairs P1/P7 detected AP phytoplasma in 22 of 33 samples and primer pair AP13/AP10 detected the phytoplasma in 25 of 33 samples. Nested PCR reactions performed using primer pair AP14/AP15 detected the phytoplasma in all samples (100%). The presence of three AP subtypes, namely AT-1, AT-2, and AP-15, was identified in 31 symptomatic apple trees. AT-1 was identified in 31 symptomatic apple trees. AT-1 was dominant in northwestern Italy. The molecular characterization carried out on AT-1 and AP-15 isolates through sequencing showed six nucleotide polymorphisms in the 16S rDNA and three in the spacer regions. The SNPs at nucleotide position 204 (in T-3 sequence, AT-1 subtype) and 1596 (on T-3 and 147 sequences, AT-1) were validated using *FauI* and *HpaII* restriction enzymes, respectively. The same *HpaII* pattern was present in all plant samples infected by AT-1 subtype, but not in AT-2 and AP-15 infected samples. Restriction analysis performed using *FauI* enzyme discriminated AT-1 subtypes detected in 11 samples from those detected in the remaining 19 samples and from AT-2 and AP-15 subtypes. The SNP at nucleotide 204 might therefore be a molecular marker for the identification of two different phytoplasmal genetic linkages within AT-1 subtype. The results indicated a high degree of genetic diversity among AP phytoplasma population in northwestern Italy and the 16Sr rDNA analysis might be useful for the identification of the geographical origin of the isolates of AP phytoplasma (Fig. 5.26) (Casati et al. 2010).

The possibility of mixed infection of citrus by huanglongbing (HLB) and phytoplasma in grapefruit (*Citrus paradisi*) was examined in China. Leaf samples (87) with symptoms of blotchy mottle from symptomatic grapefruit trees and 320 leaf samples from symptomless trees adjacent to the symptomatic trees were collected. Nested PCR assay using universal phytoplasma primer set P1/P7 followed by primer set of U5/rU3 identified 7 (8.0%) positive samples from symptomatic samples, but none from asymptomatic

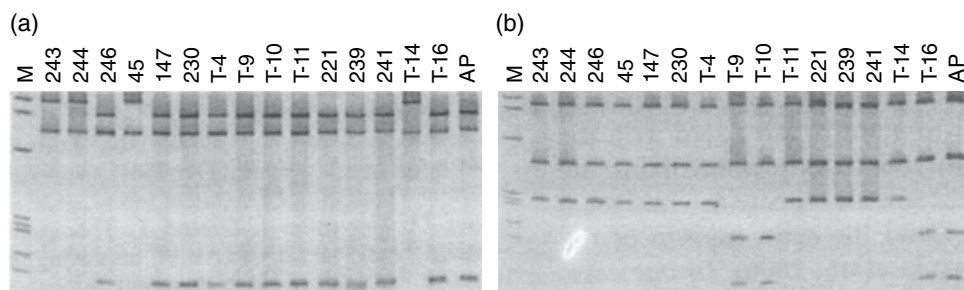


Figure 5.26 Patterns of RFLP obtained following digestion of amplicons from “*Ca. Phytoplasma mali*” isolates with (a) *FauI* and (b) *HpaII* restriction enzymes. (a) Isolates 246, 147, 230, T-4, T-11, 221, 239, 241, AP (AT-1 subtype), T-9, T-16 (AP-15 subtype), and T-10 (AT-2 subtype). (b) Pattern 1 isolates: 243, 244, 246, 45, 147, 230, T-4, T-11, 221, 241, and AP (AT-1 subtype); pattern 2 isolates: T-9, T-16 (AP-15 subtype), T-10 (AT-2 subtype), and AP (AT-1 subtype). M: DNA size markers. Source: Casati et al. (2010). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

samples. Of the 87 symptomatic samples, 77 (88.5%) were positive for “*Candidatus Liberibacter asiaticus*” and five for both phytoplasma and HLB pathogen. Sequence analysis indicated that seven 881 bp amplicons, amplified by nested phytoplasma primer sets P1/P7 and fU5/rU3, shared 100% sequence identity with each other. Phylogenetic analysis and virtual RFLP analysis confirmed the phytoplasma subgroup 16SrII-A. The 16SrII-A* phytoplasma induced HLB-like symptoms which might be mistaken as the disease was due to the HLB bacterium (Lou et al. 2014).

Citrus stubborn disease caused by *Spruioplasma citri* infects most *Citrus* species and cultivars, as well as a wide range of noncitrus plants. Detection of *S. citri* in plants was achieved by culturing in cell-free liquid medium and observing for the presence of helical structure and its mobility using dark-field microscopy (Lee and Davis 1983). PCR assay was developed for the detection of *S. citri* using primers based on gene sequences for spiralin, the most abundant membrane protein. However, this format lacked the required level of sensitivity because only a single copy of spiralin gene was present in the pathogen chromosome. Primers were therefore designed based on the sequences of P89 or P58 genes, which were multicopy genes. P89 is a putative adhesion protein gene present on a plasmid as well as in the *S. citri* genome and P58 is a putative adhesion multigene. When PCR assays were employed, amplicons of expected sizes of 707 bp and 450 bp were readily generated using P89-f/r and P58-6f/4r primer pairs, respectively, and S616 DNA as templates. By serial dilutions, amplicons were detected to a level of 0.2 pg DNA with these primers. Spiralin primers, in contrast, produced visible amplicons (675 bp) only to approximately 0.2 ng (Fig. 5.27a–c). The amplicons produced by *S. citri* DNA templates with P89-f/r and P58-6f/4r were invariably very strong, unlike spiralin primers. The detection of *S. citri* using the new primer was validated by comparing culturing of the pathogen with PCR assays. Fruit columella collected from 384 and 377 individual citrus in two fields were tested by using one part for culturing and the other for PCR assay. PCR results matched those of culturing 85–100% of the time, depending on the primers used. Real-time PCR format proved to be better than the standard PCR format. Quantitative detection of *S. citri* was accomplished using both P58-base/primer pairs. Melt curves showed a single peak from DNA analyzed from infected samples, whereas extracts from healthy tissue and no-template controls yielded negative (flat line) results. Amplicons from real-time PCR were electrophoresed to

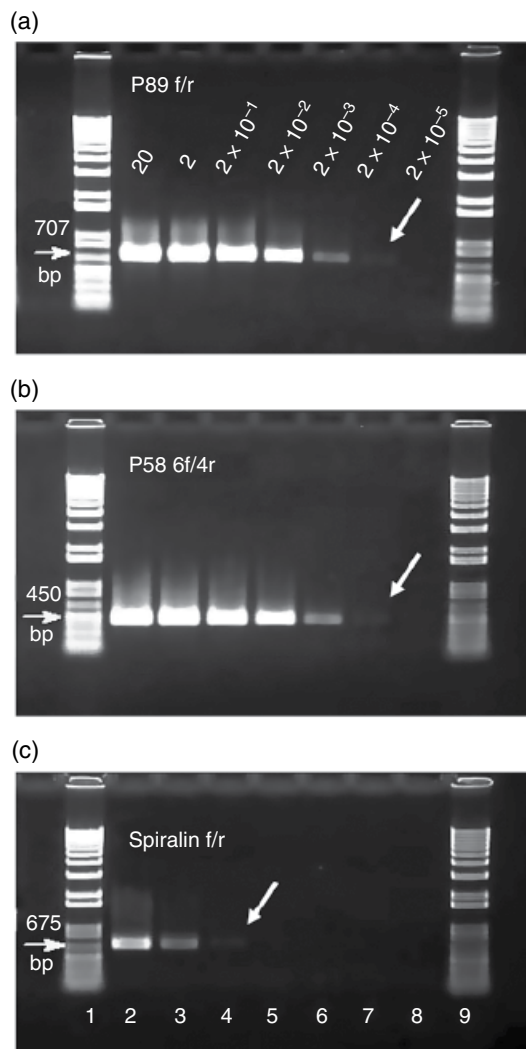


Figure 5.27 Comparative sensitivities of PCR assays using different primers amplifying different amplicons from the DNA of *Spiroplasma citri*. (a) Primer P89f/r producing 707 bp product; (b) primer P58-6f/4r producing a 450 bp product; and (c) Sprialin-f/r (Spln) producing 675 bp product. Lanes 1 and 9: DNA size standards; lanes 3–7: serial dilutions of *S. citri* DNA (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}); lane 8: water control. Source: Yokomi et al. (2008). Reproduced with permission of the American Phytopathological Society, MN, USA.

visualize the presence of amplicons of expected sizes (86 and 119 bp). The real-time PCR assay detected less than 10^{-6} ng of *S. citri* DNA. Two distinct groups of *S. citri* isolates were readily detected in samples by conventional or real-time PCR formats. The group 1 isolates represented a different population that lacked homology in the P58 region characterized in the *S. citri* groups in the field samples. The group 1 isolates were more predominant than group 2 isolates. The results indicated the usefulness of the primers generated from the sequences of P58 or P89, which are multicopy genes, for the detection of *S. citri* in citrus field samples (Yokomi et al. 2008).

5.3.2.3 Loop-Mediated Isothermal Amplification Assay

Loop-mediated isothermal amplification (LAMP) assay has the potential to circumvent the adverse effects of inhibitors present in plant extracts on the sensitivity of real-time PCR and to be amenable for deployment in fields due to its isothermal nature (Tomlinson et al. 2010; Francois et al. 2011). The robustness, simplicity, and speed of LAMP assay mean that it has become the method of choice for detection of microbial plant pathogens in various crop plants. LAMP assay was evaluated for its usefulness for the sensitive detection of flavescentia dorée (FD) phytoplasma infecting grapevine. A set of primers was designed based on the sequences of 23S rRNA of FD phytoplasma where regions of sequence specific for 16Sr-V group phytoplasma were present. The diagnostic sensitivity of 23S rRNA LAMP assay was found to be 100%, as the FDp could be detected in all tested samples of grapevine. The FDp types FD70, FD-C, and FD-D currently differentiated were detected with 23S rRNA LAMP assay. Furthermore, the LAMP assay did not react with grapevine tissues infected by phytoplasma causing bois noir (BN) disease of grapevine, the symptoms of which were indistinguishable from those induced by FDp, revealing the specificity of the LAMP assay. No cross-reactivity was observed with any fungal and bacterial pathogens, including epiphytic bacteria occurring on grapevine plants. The time required for LAMP assay was 1 h whereas a qPCR procedure required 10 h, indicating the rapidity of the LAMP assay. A procedure for homogenization of grapevine samples (leaf vein, flower, or berry) was optimized to allow direct testing of crude homogenated plant tissues with LAMP assay without the need for DNA extraction. The 23S rRNA LAMP assay could be employed for use both in the laboratory and for onsite detection since the assay allowed use of simple, portable, battery-powered equipment (Genie II, OptGene). The interpretation of final results is straightforward, as the assay does not require any complicated data analysis. The assay may be performed in small laboratories, at production sites, customs, or ports with minimally trained staff (Kogovšek et al. 2015).

5.4 Appendix

5.4.1 General/Semiselective Media for Isolation of Bacterial Pathogens from Seed

- Nutrient broth yeast (NBY) extract medium (Vidaver 1967): nutrient broth 8.0g; yeast extract 2.0g; K₂HPO₄ 2.0g; KH₂PO₄ 0.5g; glucose 2.5g; agar 15.0g; distilled water 1000 mL.
- XTS agar medium (Schaad and Forster 1985): nutrient agar (Difco) 23.0g; glucose 5.0g; cycloheximide 200 mg (20 mL of a 100 mg stock solution in 75% ethanol); gentamycin 8 mg (0.8 mL of a 10 mg mL⁻¹ stock solution in 75% ethanol); cephalixin 10 mg (1.0 mL of a 10 mg mL⁻¹ stock solution in 75% ethanol); distilled water 1000 mL. Add the antibiotics after autoclaving the medium.
- CCNT medium for *Burkholderia glumae* (Kawaradani et al. 2000): yeast extract 2.0g; polypeptone 1.0g; inositol 4.0g; cetrimide 10.0mg; chloramphenicol 10.0mg; novobiocin 1.0mg; chlorothalonil 100.0mg; agar 18.0g; distilled water 1000 mL. Adjust the pH to 4.8.
- Yeast peptone glucose agar (YPGA) for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Lelliot and Stead 1987): yeast extract 5.0g; bacto-peptone 5.0g; glucose 10.0g; agar 15.0g; distilled water 1000 mL.

- MSCFF medium for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Maringoni et al. 2006): peptone 5.0 g; meat extract 3.0 g; sucrose 5.0 g; agar 15.0 g; distilled water 1000 mL. Add the following after autoclaving the basal medium: skim milk powder 5.0 g; congo red 0.05 g; chlorothalonil 0.01 g; thiophanate methyl 0.01 g; nalidixic acid 0.01 g; nitrofurantoin 0.01 g; oxacillin 0.001 g; and sodium azide 0.001 g.
- Bacterial canker of tomato (BCT) medium for *Clavibacter michiganensis* ssp. *michiganensis* (Ftayeh et al. 2011): mannitol 2.5 g (Merck); yeast extract 2.0 g; K_2HPO_4 1.0 g; KH_2PO_4 0.1 g; NaCl 0.05 g; $MgSO_4 \cdot 7H_2O$ 0.15 g; $FeSO_4 \cdot 7H_2O$ 0.015 g; $MnSO_4 \cdot 7H_2O$ 0.015 g; H_3BO_3 0.6 g; agar 15.0 g; water 1000 mL, pH 7.0–7.1. After autoclaving at 121°C for 15 min and cooling to 50–55°C under stirring, add nalidixic acid (20 mg), trimethoprim (100 mg), polymyxin B sulfate (20 mg), 8120 international units mg^{-1} , the fungicide OpusTop (BASF) 50 μ L (containing epoxiconazole at 84 $g L^{-1}$), and fenpropimorph (250 $g L^{-1}$).
- Semiselective broth yeast extract (NBY agar) (Tegli et al. 1998): rhamnose 5.0 g; yeast extract 2.0 g; KH_2PO_4 0.5 g; K_2HPO_4 2.0 g; NH_4Cl 1.0 g; LiCl 10.0 g; $MgSO_4 \cdot 7H_2O$ 0.25 g; Tris-HCl 1.2 g; sodium azide 2.0 g; cycloheximide 0.1 g; polymyxin B 0.04 g; bromocresol purple 1.0 mL (15% solution in ethanol); agar 15.0 g; distilled water 1000 mL.

5.4.2 Stem-Printing Technique for Evaluation of Seed Transmission of *Pantoea stewartii* ssp. *stewartii* in Corn Seedlings (Block et al. 1998)

- Raise the plants from infected seeds in greenhouse; at two–three-leaf stage cut the stem off 2–3 cm above soil line; cut cross-section of each stem; press gently onto agar media to leave a stem imprint containing plant sap on agar and incubate at 28°C for 24–48 h.
- Examine the plates for development of bacterial colonies with yellow color and subculture onto fresh media.
- Confirm the identity of the bacteria developing in the media by enzyme-linked immunosorbent assay (ELISA).
- Inject two plants at the two-leaf growth stage with a PBS suspension (approximately 10^8 CFU mL^{-1}) of each yellow isolate.
- Observe the inoculated plants for development of characteristic streaking and wilting symptoms associated with *P. stewartii* ssp. *stewartii* infection.
- Subculture the nonyellow bacterial colonies; test them by ELISA to ensure that they are not strains of Stewart's wilt pathogen.

5.4.3 Detection of *Acidovorax avenae* ssp. *citrulli* (Aac) by MAb-Captured-Sandwich ELISA (MC-sELISA) in Seed Extracts (Himananto et al. 2011)

5.4.3.1 Detection of the Pathogen in Culture

- Coat 96-well microplates with 100 μ L of MAb 11E at 2 μ g mL^{-1} diluted in carbonate buffer; incubate overnight at 4°C or for 2 h at 25°C; wash the plates with 400 μ L PBST containing 1.5 mM KH_2PO_4 , 137 mM NaCl, 20 mM Na_2HPO_4 , and 2.7 mM KCl (pH 7.2) (PBS) + 0.05% Tween 20 (pH 7.2).
- Prepare serial dilution of the pathogen (0 – 10^8 CFU mL^{-1}) in an extraction buffer (PVPBST+) consisting of 0.06 M Na_2SO_3 , 2% polyvinylpyrrolidone 40 (w/v), egg albumin at 2 $g L^{-1}$ and Tween 20 in PBS; add 100 μ L of each dilution of the pathogen to the wells individually and incubate for 1 h at 25°C.

- iii) Wash the plates separately with PBST as done earlier (step i above); add 100 μ L of 1/4000 anti-AcMPC rPAb diluted in 0.5% bovine serum albumin (BSA) in PBST and incubate for 1 h at 25°C followed by washing the plates as earlier.
- iv) Add goat antirabbit immunoglobulins conjugated with alkaline phosphatase (ALP) diluted 1/10,000 in 0.5% BSA in PBST (100 μ L/well) and incubate for 1 h at 25°C.
- v) Observe the development of chromogenic (color) signal and record absorbance values at 405 nm.

5.4.3.2 Detection of the Pathogen in Artificially and Naturally Infested Seeds

- i) Soak healthy (noninfested) seed (100) in 20 mL *Aac* suspension (10^8 CFU mL⁻¹); infiltrate in vacuo for 20 min in a vacuum jar and air dry the seed overnight at room temperature.
- ii) Mix the infested with noninfested seed to have seed with different levels of infestation (0, 0.1, 0.3, 1, and 5%) and prepare three replicates for each level of contamination with the pathogen.
- iii) Prepare seed wash by incubating noninfested seed (100) in 10 mL PVPBST+ for 1 h at 25°C with agitation (200 rpm).
- iv) Prepare the seed macerate by crushing the seed (100) in 10 mL PVPBST+ using a homogenizer.
- v) Pass the seed wash and macerate through two layers of cheese cloth to remove seed debris and use the filtrate for testing by ELISA format.

5.4.4 Detection of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in Tomato Seeds by Immunomagnetic Separation (IMS) Plating Technique (de León et al. 2008)

5.4.4.1 Coating of Magnetic Beads

- i) Coat the immunomagnetic beads (IMBs) precoated with sheep antirabbit IgG (Dynabeads M-280, Dynal Norway) with antisera specific to the pathogen by washing the beads three times with phosphate-buffered saline 0.1 M, pH 7.2 (PBS); collect the beads using a magnetic particle concentrator (Dynal) and resuspend the beads in PBS to obtain the desired concentration of beads.
- ii) Incubate the IMBs with anti-*Cmm* antiserum for 24 h at 4°C, with gentle shaking as per the manufacturer's instructions, and wash the beads three times with PBS containing 0.1% bovine serum albumin (PBS-BSA) and 0.05% Tween-20 to remove unattached antibodies.
- iii) Resuspend the beads in PBS-BSA to have a stock suspension (10^8 IMB mL⁻¹) and store at 4°C until required.

5.4.4.2 Standardization of Immunoseparation Technique

- i) Prepare different dilutions of the selected antiserum (anti-*Cmm*) (1/200, 1/400, 1/800, 1/1600, 1/3200, and 1/6400) and coat the IMBs (10^5 mL⁻¹) with the antiserum dilutions.
- ii) Add aliquots of anti-*Cmm*-coated IMBs to 1 mL *Cmm* suspension (5.7×10^3 CFU mL⁻¹) to obtain 10^5 IMB mL⁻¹ and incubate for 1 h at room temperature with gentle shaking.

- iii) Wash the IMBs three times with 1 mL of PBS-BSA containing 0.05% Tween-20 for 10 min and resuspend the IMBs in 1 mL of PBS.
- iv) Spread 0.1 mL of the suspension on yeast-peptone-glucose agar (YPGA) medium containing yeast extract (5 g), bactopectone (5 g), glucose (10 g), agar (15 g), and distilled water (100 mL) and incubate for 5 days at 25°C.
- v) Enumerate *Cmm*-like colonies developing on the YPGA medium.

5.4.5 Detection of *Xanthomonas campestris* pv. *carotae* by PCR Assay (Meng et al. 2004)

5.4.5.1 Extraction of Pathogen DNA from Seeds

- i) Incubate the seed samples (10 g, 10,000 seeds) in 0.85% NaCl (100 mL) at 4°C for 16–18 h in 250 mL flasks separately; add two drops of Tween-20; and place the flasks on a rotary shaker (250 rpm) for 2 min.
- ii) Filter the seed wash through four layers of sterilized cheese cloth; centrifuge at 10,000 g for 10 min and resuspend the pellet in 0.85% NaCl (10 mL).
- iii) Add 0.5 or 1.0 mL suspension to 1.5 mL Eppendorf tube; centrifuge the contents at 10,000 g for 10 min and resuspend the pellet as detailed below for DNA extraction by different methods.
- iv) The NaOH + PVP method: resuspend the pellet in 100 µL of 0.5 NaOH + 0.5% polyvinylpyrrolidone (PVP) (w/v); add 5 µL of this suspension to 495 µL of 20 mM Tris-HCl (pH 8.0) and use 2 µL of this extract in a 50 µL PCR reaction.
- v) Modified Dellaporta method: resuspend the pellet in 600 µL of Dellaporta buffer and 1% sodium dodecylsulfate (SDS) and use 2 µL of this DNA extract in a 50 µL PCR reaction.
- vi) CTAB method: resuspend the pellet in 800 µL of 2% CTAB buffer and use 2 µL of the extract in 50 µL PCR reaction.
- vii) Qiagen DNeasy Tissue Kit method (Qiagen Inc.): follow the manufacturer's instructions; use 4 µL of the extract in 25 µL PCR reaction.

5.4.5.2 Polymerase Chain Reaction (PCR) Assay

- i) Perform the PCR in 50 µL reaction mixture containing 1 × PCR buffer (20 mM Tris-HCl (pH 9.0), 37.5 mM KCl) and 7.5 mM Triton X-100), 250 µL each of dATP, dCTP, dGTP, and dTTP, 1 µM each of forward and reverse primers; 2 Units of *Taq* DNA polymerase (Perkin Elmer Cetus, CA), 4 ng of bacterial total genomic DNA in a DNA Thermal Cycler 480 (Perkin Elmer), an Eppendorf Mastercycler gradient or a Primus 96 plus PCR System (MWG Biotech Inc. NC)
- ii) Carry out 30 cycles of PCR amplification with each cycle of 94°C for 1 min, 65°C for 45 s and 72°C for 2 min.

5.4.6 Detection of *Burkholderia glumae* in Rice Seeds by Real-Time PCR Assay (Sayler et al. 2006)

5.4.6.1 Preparation of Seed Wash

- i) Wash rice seed sample (2 g, approximately 20 seeds) in 2 mL sterile distilled water containing 0.1% Tween-20.
- ii) Mix the aqueous suspension on a rotary shaker at 150 rpm for 30 min.
- iii) Maintain duplicates for each sample.

5.4.6.2 Real-Time PCR Assay

- i) Prepare serial dilutions (10^0 , 10^1 , 10^3 , and 10^5) of bacterial cell suspensions.
- ii) Perform real-time PCR using a Thermo-Fast 96-well reaction plate and Thermo-Fast Caps (AB gene Epsom, Surrey, UK) in the M × 3000 P real-time PCR machine (Stratagene Corporation, La Jolla CA).
- iii) Dispense to each well a 25 μ L reaction mixture containing 12.5 μ L of 2 × SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and 12.5 μ L primer (3 pmol each of forward and reverse primer and 1 μ L of bacterial cells or seed wash/plant extract).
- iv) Provide the conditions for amplification: 95°C for 15 min, 40 cycles each of 95°C for 15 s, 60°C for annealing for 30 s and 72°C extension for 15 s, 95°C for 1 min and 55°C for 30 s.
- v) Construct a standard curve to calculate the correlation between the bacterial cell numbers and the PCR Ct values by assaying the series of dilutions prepared already; determine the bacterial numbers using a spectrophotometer (turbidimetric) assays followed by plate counting.

5.4.7 Detection and Quantification of *Burkholderia* spp. in Rice Seeds by Real-Time PCR Assay (Nandakumar et al. 2009)

5.4.7.1 Isolation of DNA for Real-Time PCR Assay

- i) Transfer the bacterial strains (reference and cultured ones) to King's B broth (KBB); incubate on an orbiting shaker for 18 h at 200 rpm at 35°C; collect the bacterial cells ($OD_{600} = 0.5$) from 1 mL of culture by centrifuging at 8600 g for 3 min and separate the pellets for DNA isolation.
- ii) Extract the genomic DNA from pelleted preparation for each test strain, using the Bio-Rad AquaPure Genomic DNA Kit (Bio-Rad) as per manufacturer's recommendations.
- iii) Simultaneously enumerate the number of CFU in 1 mL of bacterial culture by serial dilution and plating on KBA and maintain three replicates for both DNA extraction and population counts.

5.4.7.2 Real-Time PCR Assay

- i) Design the real-time PCR primers and TaqMan probes (Allied Biosystems) using Primer3 software based on published information for the *B. glumae* strains employing appropriate primers and probes for real-time reactions.
- ii) Label the TaqMan probe with 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethyl rhodamine (TAMARA) at the 3' end.
- iii) Test different probe concentrations (50–300 nM), primer concentrations (2.5, 5, 10, 15, and 20 pmole) and annealing extension times (20, 30, 40, and 60 s) using a uniform template concentration of DNA of reference strain.
- iv) Check the quality of DNA and real-time pairs using conventional PCR format.
- v) Use the reaction mixture (30 μ L) consisting of 15 μ L of 2 × master mix, 1 μ L each of forward and reverse primers (5 pmol μ L⁻¹), 1 μ L of probe (100 nM) and varying amounts of DNA template and sterile water.
- vi) Provide the following PCR amplification conditions: 10 min preincubation period with a 15 s denaturation at 95°C, leading to 40 cycles of annealing and extension at 60°C for 20 s.
- vii) Maintain nonDNA template as a negative control and three replicates for each sample.

5.4.7.3 Preparation of Standard Curve

- i) Prepare different concentrations of DNA, namely 0.25, 2.5, 25, and 250 pg and 2.5, 25, 250, and 2500 ng, from the reference strain of the pathogen (*B. glumae* ATC33671) to correspond to 10^1 to 10^8 CFU mL⁻¹ and maintain three replicates for each concentration of DNA.
- ii) Prepare the standard curve by plotting Ct (threshold cycle) values for each DNA concentration which correspond to the pathogen population (cell number).
- iii) Use the standard curve to predict the quantity of target bacterial DNA from pure cultures and seed samples with unknown concentrations of pathogen cells.

5.4.8 Detection of *Pseudomonas syringae* pv. *phaseolicola* in Bean Seed Washings by Membrane BIO-PCR Assay (Schaad et al. 2007)

- i) Use the specially designed 96-well microtitre plate fitted with surfactant-free 0.45 µm pore size cellulose nitrate filter (Sartorius Corp) that allows complete contact with the agar medium; dispense aliquots of 400 µL of each sample into triplicate wells each of two plates; place the plates on a 96-well vacuum suction device (Whatman Polyfiltronics) to remove the liquid and repeat this process twice with additional 400 µL aliquots so that 1.2 mL of the sample may be filtered through each well.
- ii) Spread 100 µL of each dilution of the pathogen onto five separate plates of KB and MSP agar plates and incubate under the same conditions to determine mean CFU mL⁻¹ of each sample.
- iii) After filtering the samples, place one of the 96-well plates onto KB or MSP soft agar in the original sterile plastic container; tap lightly to allow contact with agar; place the lid to prevent contamination and incubate for 32–34 h for KB and 48–52 h for MSP medium.
- iv) Wash each well with 200 µL of sterile water using a multichannel micropipette; transfer the sample to microfuge tube for performing PCR or store at –20°C.
- v) Use the second 96-well plate for membrane PCR assay without incubating on agar media; wash each well and use 12.8 µL of the sample for classical real-time PCR.

5.4.9 Detection of *Acidovorax avenae* subsp. *citrulli* in the Seeds of Watermelon by IMS-Real-Time PCR Assay (Bahar et al. 2008)

5.4.9.1 Immunomagnetic Separation (IMS)

- i) Suspend seed samples (5000 seeds) in 500 mL of 0.1 M PBS buffer in two tubulation flasks (2 L); mix the contents for 1 h at room temperature using a magnetic stir bar and a stir plate (Nuvo II, Thermolyne).
- ii) Apply vacuum to the seeds and disrupt at 15 min interval during incubation to extract the deep-seated bacteria in the seed.
- iii) Pass the suspension through four layers of cheese cloth; collect the filtrate; pass through one layer of Whatman No. 1 filter paper overnight into a 1 L flask; centrifuge the filtrate (seed wash) at 8000 g for 15 min and decant the supernatant.
- iv) Resuspend the pellet in 6 mL of PBS-BSA (0.1 M PBS containing 0.1% bovine serum albumin (BSA) and add immunomagnetic beads (IMB, Dynal) coated with anti-*Aac* antibodies (40 µg/10⁶ IMB) to the suspension and incubate for 1 h at 40°C on an end-over-end sample mixer (Dynal) with gentle agitation (30 rpm).

- v) Concentrate IMB-*Aac* complex using a magnetic concentrator (MPC, Dynal); wash three times using 8 mL PBS-BSA; wash with 8 mL sterile deionized water; resuspend the IMB in 500 μ L sterile deionized water and transfer to a new tube.
- vi) Concentrate the IMB using the magnetic particle concentrator (MPC); remove the excess water; add 20 mL sterile deionized water and boil the suspension at 100°C for 10 min to release bacterial DNA.
- vii) Centrifuge the suspension at 16,000 g for 15 min to pellet the IMB and separate the supernatant containing bacterial DNA to be used for PCR reactions.

5.4.9.2 Real-Time PCR Assay

- i) Perform real-time PCR in aliquots of 25 μ L reaction solution using a Bio-Rad SuperIQ master mix, with 25 pM BX-S or *Aac* primers and 5 μ L template DNA solution.
- ii) Provide the following conditions for reaction cycles: denaturation at 95°C for 3 min followed by 35 cycles of denaturation for 15 s and annealing and extension at 60°C for 40 s.
- iii) Conduct real-time PCR reactions using a Cepheid Smart Cycler and determine cycle threshold (Ct) for each primer set using a threshold of 30 fluorescent units.
- iv) Use a SYBR Green assay for BX-S primer set and TaqMan assay for the *Aac* primer set using the *Aac* P2 TaqMan probe and maintain three replicates for each primer set.

5.4.10 Detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*) by Direct PCR and Immunocapture (IC-) PCR Assays in Watermelon Seeds (Xiao et al. 2007)

5.4.10.1 Selection of Antibodies and Primers Specific to *Aac*

- i) Prepare anti-*Aac* polyclonal antisera containing antibodies against antigen Ab and Ab1 using New Zealand white rabbits.
- ii) Design primers WFB1 and WFB2 based on the sequences of the 16S ribosomal RNA (rRNA) from the standard strain of *Aac*.

5.4.10.2 Detection of *Aac* by Standard PCR Format

- i) Use aliquots of 25 μ L reaction mixture combining 200 μ mol L⁻¹ of dNTP (dATP, dCTP, dGTP, dTTP), 0.25 μ mol L⁻¹ of each primer WFB1 and WFB2, 1 μ mol L⁻¹ of *Taq* DNA polymerase, 2.5 μ L 10 $\times$ PCR buffer with MgCl₂ and 2–4 μ L of bacteria cell lysates as template.
- ii) Carry out DNA amplification in PTL-200 Peltier Thermal Cycler (MJ Research) under following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 65°C for 30 s, primer annealing at 65°C for 30 s and elongation at 72°C for 30 s.
- iii) Complete the PCR by incubating the tubes at 72°C for 5 min.
- iv) Determine the PCR products by electrophoresis on 1% agarose gels.

5.4.10.3 Detection of *Aac* by IC-PCR Assay

- i) Prepare a dilution of 1: 2000 of the antiserum specific to target pathogen with sterilized distilled water; dispense 200 μ L aliquots into 0.2 mL Eppendorf tube and incubate for 3 h at 37°C.

- ii) Rinse three times with washing buffer PBST containing 0.01 mol L^{-1} of phosphate buffered saline (PBS) and 0.2% Tween-20 and add $200 \mu\text{L}^{-1}$ of 0.5% dried skimmed milk and incubate for 30 min.
- iii) Add $200 \mu\text{L}$ of lysates of target bacterial cells after removing the milk powder, and incubate overnight at 4°C .
- iv) Rinse three times with PBST followed by one rinse with sterile distilled water.
- v) Use all reagents except the template and adopt all steps as in standard PCR procedure (Section 5.4.10.2 above).

5.4.11 Detection of "*Candidatus Liberibacter solanacearum*," Causative Agent of Potato Zebra Chip Disease by PCR Assay (Munyaneza et al. 2012)

5.4.11.1 Extraction of DNA from Plant Tissues

- i) Macerate potato tissues (400 mg: stem, petioles, and leaf tissues) with 1 mL extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, and 10 mM mercaptoethanol) using BioReba sample bags and a Homex6 homogenizer (BioReba); collect 300 mL of macerate; mix with $80 \mu\text{L}$ of lysozyme (50 mg mL^{-1} in 10 mM Tris-HCl, pH 8.0), and incubate for 30 min at 37°C .
- ii) Add $500 \mu\text{L}$ of CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 0.2% mercaptoethanol) to the homogenate; incubate for 30 min at 65°C and place the bag at room temperature for 3 min.
- iii) Add ice-cold chloroform ($500 \mu\text{L}$); vortex the samples to mix thoroughly; centrifuge at 14,000 rpm for 10 min; transfer the aqueous layer to a new microfuge tube containing 0.6 volume of isopropanol; place the tube on ice for 20 min to precipitate the DNA and centrifuge as before.
- iv) Wash the pellet with ice-cold 70% ethanol; centrifuge at 14,000 rpm for 2 min; air dry the pellet; resuspend the pellet in $100 \mu\text{L}$ sterile water and store at -40°C until the PCR analysis.

5.4.11.2 PCR Amplification

- i) Prepare $50 \mu\text{L}$ reaction solution containing $1 \times$ Green GoTaq reaction buffer (Promega), $100 \mu\text{M}$ dNTPs, 20 pmol of each primer, $2 \mu\text{L}$ of DNA extracts, and 1 U of GoTaq Polymerase (Promega).
- ii) Provide the following PCR cycling conditions: 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, followed by final incubation at 72°C for 5 min (MJ Research).
- iii) Analyze the amplicon ($10 \mu\text{L}$) by electrophoresis in 1.5% agarose gels stained with ethidium bromide, and observe under ultraviolet light to detect the presence of 1168 bp 16S rDNA band (positive for "*Ca. L. solanacearum*") in the sample of potato tissue.

5.4.12 Detection of *Erwinia carotovora* subsp. *atroseptica* (Eca) by Loop-Mediated Isothermal Amplification (LAMP) Assay in Potato (Li et al. 2009c, 2011)

5.4.12.1 Preparation of Genomic DNA of the Pathogen

- i) Crush bacterial culture or tissue from symptomatic plant material; suspend in 100 mL sterile distilled water or TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0); add 50 mL of 3% KOH to 50 mL of suspension and mix for 3 min to lyse the bacterial cells.

- ii) Add 21.9 μL of HCl to neutralize the suspension; dilute the sample to 1:10 and use 2 μL of diluted sample as the template in 25 μL LAMP assay.
- iii) Store the sample extracts in duplicate at 4°C and -20°C for up to 113 days for testing at an interval of 1 week by LAMP assay.

5.4.12.2 Preparation of Primers

- i) Design a LAMP primer set for efficient stem-loop formation to target the polyketide synthase (PKS) gene *Cfab* using PrimerExplorer V4 software program and synthesize the primers (by Integrated DNA Technologies, Coralville, IA, USA).
- ii) BIP primers for the PKS gene of *Eca* consists of B1 (22-nt), a TTTTT linker and B2 (19-nt); FIP primers consist of F1c (20-nt), a TTTT linker and a complementary sequence to F2c (18-nt); primers B3 (19-nt) and F3 (19-nt), loop primers, Loop F1 (20-nt), and Loop B1 are also included to increase amplification efficiency.
- iii) Analyze all six primers in silico against GenBank sequences for potential homologous sequences to determine specificity.

5.4.12.3 LAMP Assay

- i) Optimize the procedure by evaluating the effect of changing Bst enzyme (New England Biolabs) concentration, Mg concentration, Betaine (Sigma) concentration, and duration of the DNA amplification. The optimal conditions are as follows: final volume of 25 μL consisting of 0.8 μM each of FIP and BIP primers, 0.2 μM each of F3 and B3 primers, 0.4 μM each of Loop F1 and Loop B1 primers, 1 M Betaine, 400 μM each of dNTPs, 4 mM MgSO_4 , 1 $\times$ Bst buffer (New England Biolabs), and 8 U of Bst DNA polymerase large fragment (New England Biolabs) and 2 μL of sample template.
- ii) Incubate the contents at 65°C for 50 min and deactivate at 80°C for 10 min to terminate the reaction.
- iii) Electrophorese aliquots of 2–5 μL of LAMP product in 1% agarose gels (0.5 $\times$ TBE) containing ethidium bromide; mix the products with SYBR Green for visualization under UV light.

5.4.13 Detection of Flavescence Dorée Phytoplasma in Grapevine by Reverse-Transcription PCR Assay (Margaria et al. 2007)

5.4.13.1 Extraction of Leaf Sap

- i) Place the samples of leaf or midvein tissue (0.2 g) in extraction bags (Bioreba, Reinach, Switzerland); homogenize in grape grinding buffer (15 mM Na_2CO_3 , 34.9 mM NaHCO_3 , 2% polyvinyl-pyrrolidone (PVP-) 40, 0.2% bovine serum albumin (BSA), 0.05% Tween-20, and 1% $\text{Na}_2\text{S}_2\text{O}_5$, pH 9.6) at a final dilution of 1:20 using a Rixon drill press; add the crude extract (2 μL) to 25 μL sterile buffer (0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100); denature for 10 min at 95°C and place the extract on ice for 5 min.
- ii) Design the primers by aligning with Clustal Software the transcribed region of the 16S rRNA from at least one phytoplasma for each of the 15 groups available in sequence databases; primers selected: FD-221 F and FD-483R.
- iii) Add the denatured extract (2 μL) to a final reaction volume of 25 μL consisting of 1 $\times$ PCR buffer (Applied Biosystems, CA), 3 mM MgCl_2 , 5 mM DTT, 50 mM forward primer, 200 μM each of dNTP, 4 units of RNase OUT (Invitrogen Life

- Technologies, CA), 1 unit of Thermoscript (Invitrogen), and 1.25 units of AmpliTaq polymerase (Applied Biosystems).
- iv) Provide the following conditions for amplification: 30 min reverse-transcription at 52°C; followed by 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 20 s, and final extension at 72°C for 10 min.
 - v) Analyze the amplicons in 2% agarose gel in Tris-borate-EDTA buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0).

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6

Biology and Infection Process of Bacterial and Phytoplasmal Pathogens

Bacterial and phytoplasmal pathogens are primitive microorganisms classified as prokaryotes, whereas fungal pathogens are placed under eukaryotes. They are capable of causing diseases in plants, humans, and animals. Fossil prokaryotes have been recorded in rocks of c. 3.5 billion years of age, and it appears that prokaryotes were the only form of life on Earth for over 2 billion years. Fossils of eukaryotic organisms have not been observed in rocks older than c. 1.6 billion years (Schumann 1991). The bacterial species *Erwinia amylovora* was the first to be demonstrated as a causative agent of fire blight diseases of apple and pear by Burril in 1878. *Agrobacterium tumefaciens* was found to be the incitant of crown gall diseases affecting several plant species (Smith and Townsend 1907). Several other bacterial pathogens were established later as the causes of many economically important crop diseases.

6.1 Biology of Bacterial Pathogens

6.1.1 General Characteristics of Bacterial Pathogens

Bacterial cells are much smaller in size compared to fungal cells and may measure about 1 μm in diameter, whereas the average fungal spore has a diameter of 50–200 μm . Bacterial cells may be rod-shaped, spherical, ellipsoidal, spiral, comma-shaped, or filamentous. Most bacteria are strictly saprophytic in nature and are beneficial to humans, as they readily decompose large quantities of organic wastes produced by industries and the death of animals, plants, and other organisms. Furthermore, they also play an important role in building up the nutritional levels of soils by fixing atmospheric nitrogen and breaking down complex organic matter. All plant pathogenic bacteria except *Streptomyces* spp. are rod-shaped and measure 0.3–1.0 μm by 0.5–3.5 μm . About 200 species of 1600 bacterial species are known to infect different kinds of plants. The cell walls of bacteria of most species are enveloped by a viscous, gummy material. When the gummy material is thin and diffuse, it forms a slime layer around the bacterial cell. On the other hand, if it is thick, forming a mass around the bacterial cell, it is known as a capsule. Most of the plant pathogenic bacteria possess delicate, thread-like flagella, organs of locomotion which are appreciably longer than the bacterial cells themselves. The flagella may vary in number and position in the bacterial cells. Some bacterial species may have a single flagellum and others may have a tuft (group) of flagella at one

end of the cells (polar flagella). Some species have flagella distributed over the entire surface of the cell (peritrichous flagella).

The bacterial protoplasm is enclosed by a distinct cell wall that can be differentiated from the outer cytoplasmic membrane. Bacteria can be separated into two biologically different groups based on their ability to retain the stain developed by Hans Christian Gram. The Gram-positive bacteria (retaining the stain) have a relatively thick uniform cell wall, whereas Gram-negative bacteria (losing the stain) have a thinner cell wall with an additional outer layer of polysaccharides and lipids. The lipids present in the layer prevent absorption of certain substances (such as penicillin), making them insensitive/resistant to those compounds. *Bacillus* spp. are known to form endospores that are resistant to adverse environmental conditions. Other plant pathogenic bacterial species do not form endospores and are therefore sensitive to desiccation and other stresses. All bacterial species produce individual cells, whereas *Streptomyces* spp. are usually spiral-shaped and produce conidia in chains on aerial hyphae.

Bacterial cells multiply on appropriate media, forming colonies that exhibit variations in size, shape, color, elevation, and form of edges. The properties of bacterial colonies may be useful in the identification of certain bacterial genera. The size of colonies may vary from 1 mm to several centimeters in diameter and they may be circular, oval, or irregular with smooth, wavy, or angular edges. The elevation of the colonies may be flat, raised-dome-like, or wrinkled, and appear white, yellow, red, or gray. The bacterial cell contains nuclear material which is not separated from the cytoplasm by a nuclear membrane as in eukaryotes. The internal cell contents constitute the protoplast which consists of a cytoplasmic or protoplast membrane. The extent of selective permeability to various substances into and out of the cell wall is controlled by the protoplast membrane. Cytoplasm contains a complex mixture of proteins, lipids, carbohydrates, and different kinds of other organic compounds, minerals, and water. The nuclear material consists of a large circular chromosome composed of DNA which forms the primary body of genetic material of the bacterial species concerned. It appears as a spherical, ellipsoidal, or dumbbell-shaped structure within the cytoplasm. Additional smaller circular genetic materials known as plasmids are also present in single or multiple copies in the bacterial cells. The plasmids are capable of independently replicating the chromosome and can govern some characteristics such as pathogenicity, resistance to chemicals/antibiotics, and tumor production. Several nonessential genes are present in the plasmids, which can move or be moved between bacteria or even between bacteria and plants.

With a simple morphological structure, bacteria do not such have a complex life cycle as fungi. During asexual and sexual reproduction, no additional structures are produced in the bacteria. During asexual reproduction, bacteria produce daughter cells by a process known as binary fission. The cytoplasmic membrane grows toward the center of the cell, forming a transverse membranous partition dividing the cytoplasm into two approximately equal parts. Two layers of cell-wall material, continuous with the outer cell wall, are laid down between the two cytoplasmic layers. As the partition of the cytoplasm is in progress the DNA, which is in the form of circular chromosome, condenses into an amorphous mass and then elongates into a dumbbell-shaped structure. The DNA then divides into two equal pieces and becomes distributed into the new daughter cells that are formed from the dividing single cell. The process of binary fission can be repeated indefinitely. When optimum conditions are available bacteria can divide once every 20–50 min, reaching astonishing population levels rapidly.

Variability in the genetic characteristics of organisms may be mainly the result of sexual recombination or mutation. In the case of some bacterial species, the process known as conjugation, considered similar to sexual reproduction, has been observed. Two physiologically opposite cells (+ and –) come into contact side by side and a small fragment of DNA from the male cell (donor) is transferred to the female cell (receptor). The female cell then multiplies by binary fission, leading to the production of a population of cells possessing the characteristics of the both donor and receptor cells. Variations in the genetic characteristics may occur in three other modes in addition to conjugation. (i) Mutation may occur in a very small percentage of bacterial cells, resulting in permanent changes in some of the characteristics of the bacteria. (ii) The genetic material in some bacteria is liberated from one cell either by secretion or rupture of the cell wall. A fragment of the DNA so released gains entry into a genetically compatible bacterial cell of the same or closely related species and the recipient cell becomes genetically different. This phenomenon is designated transformation. (iii) The phenomenon of transduction also leads to changes in the genetic constitution of bacteria. A bacteriophage is involved as a vector in this phenomenon for the transfer of genetic material (a fragment of the bacterial DNA) from one bacterial cell to another bacterial cell. The vector acquires a fragment of DNA of the infected bacterial cell which is lyzed later, liberating the bacteriophage. When the bacteriophage infects another bacterial cell, the DNA fragment of the lyzed cell becomes integrated with the DNA of the freshly infected bacterial cell. Various characters of bacteria may be altered by this process involved in the transfer of bacterial DNA, or by spontaneous changes due to mutation caused by environmental conditions and exposure to certain chemicals applied for the management of diseases caused by bacterial pathogens. However, the frequency of such genetic changes in bacterial pathogens is very low (Narayanasamy 2001).

6.2 Disease Cycles of Seedborne Bacterial Pathogens

Disease cycles of phytopathogenic bacteria have been studied to gather information on the importance of seed as a source of inoculum, the existence of other sources of inoculum of epidemiological importance, host range, and levels of susceptibility of cultivars of the crop plant species. This information may be useful to develop short- and long-term strategies for effective management of diseases caused by seedborne bacterial pathogens belonging to different genera.

6.2.1 *Xanthomonas* spp.

Seedborne bacterial pathogens may infect seeds as in cereals directly or reach the seeds after infecting the fruits or vegetables containing seeds. Visible symptoms may be produced by the bacterial pathogens on infected seeds or fruits or they may remain dormant. Bacterial pathogens cause rotting of infected fruits or vegetables, which may also be invaded by other bacterial species. Bacterial leaf blight (BLB) disease is one of the devastating diseases of rice in SE Asian countries, Australia, Africa, America, and India. *Xanthomonas oryzae* pv. *oryzae* (Xoo) induces leaf blight and vascular wilt, resulting in drying up of foliage and the ultimate death of infected rice plants. Destructive manifestation of the disease is termed “kresek.” Opaque and turbid drops of bacterial ooze drying into yellowish beads may be observed on the infected leaves during dry

weather. Pathogen–host interactions have been investigated by inoculating rice leaves. Populations of *Xoo* were monitored over time to determine their growth kinetics in rice leaves. Both compatible and incompatible populations increased equally in rice leaves for about 2–4 days from 10^3 to 10^4 colony-forming units (CFUs) to 10^7 – 10^8 CFU/leaf. In some rice cultivars, the development of incompatible strain was reduced compared to that of compatible strain. A threshold level of *Xoo* was required before the host responded and bacterial multiplication began to reduce. Spread of *Xoo* in rice leaves was more rapid and extensive with compatible than incompatible interactions (Leach et al. 1989). *X. oryzae* pv. *oryzae* (*Xoo*) is considered as a model organism for analysis of host–plant–pathogen interaction, since more than 30 races differing in virulence and 25 resistance genes in rice have been identified. The complete genome sequence of *Xoo* strain MAFF311018 has been identified (Zhu et al. 2000; Ochiai et al. 2005).

Xanthomonas citri subsp. *citri* (*Xcc*) (= *X. campestris* pv. *citri* and *X. axonopodis* pv. *citri*), considered to be a hemibiotrophic pathogen, grows initially epiphytically on leaf surfaces and gains entry into the host through stomata or wounds followed by colonization of the mesophyll parenchyma. The pathogen multiplies in the apoplast (intercellular spaces) in the susceptible plants. Bacterial pathogens possess several virulence factors to enhance infection levels and become successfully established in susceptible host plants (Laia et al. 2009; Yan and Wang 2012). The formation of biofilms by bacterial pathogens is an important virulence factor with a crucial role in the early stages of infection by increasing persistence of the epiphytic populations of *Xcc*. Multiple mutants of *Xcc* impaired in biofilm formation consistently showed reduction in population growth in planta, and could only induce mild symptoms in susceptible citrus leaves indicating that inability to produce biofilm might reduce the pathogenic potential of *Xcc* (Rigano et al. 2007; Li and Wang 2011). The possibility of using biofilm formation-inhibiting compounds for restricting the infection and development of citrus canker disease was therefore explored.

D-leucine and 3-indolylacetonitrile (IAN) were found to effectively prevent biofilm formation by *Xcc* on different abiotic surfaces as well as on citrus leaves at concentrations lower than the minimum inhibitory concentration (MIC). The effects of D-leucine and IAN on expression of genes governing biofilm formation in *Xcc* strain 306 were investigated by employing qRT-PCR assay. IAN significantly repressed expression of chemotaxis and motility genes (*cheA*, *pilB* *motB*), while D-leucine did not exert any influence on the expression of the genes tested. Further, treatment of planktonic and biofilm cells of *Xcc*, either alone or in combination, significantly increased the susceptibility of the pathogen to copper (CuSO_4) by a factor of c. 10 compared with untreated bacterial cells. However, expression of any of the copper resistance genes *copA*, *copB*, and *copL* was not affected by treatment of *Xcc* cells with D-leucine or IAN or in combination in the presence of CuSO_4 . Spray application of D-leucine and IAN at 6 h as preinoculation or coinoculation with the pathogen reduced the number of canker lesions produced by *X. citri* subsp. *citri* in grapefruit leaves. Pathogen populations were also reduced by D-leucine and IAN, as revealed by qRT-PCR assay. The results indicated the effectiveness of this novel approach of employing biofilm-inhibiting compounds as a disease management strategy (Li and Wang 2014).

Xanthomonas spp. possess *hrp* (hypersensitive response (HR) and pathogenicity) genes. The functions of *hrp* genes are the characteristic features of the type III secretory system. The pathogenicity of and elicitation of resistance by bacterial pathogens may be controlled by the *hrp* genes. The number of *hrp* genes may vary depending on the

bacterial species/pathovar. Three biochemical functions, namely gene regulation, protein secretion, and induction of HR, are ascribed to the *hrp* genes (Gopalan and He 1996). In addition to *hrp*-controlled pathogenic factors (secreted via type III secretory system), many other virulence factors are required for induction of different symptoms in susceptible plants. The water-soaking symptom associated with several bacterial diseases in the early stages of infection is due to the action of extracellular polysaccharides (EPS) produced by the bacterial pathogens. The amounts of EPS produced by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) appeared to be related to the level of virulence of *Xoo* strains. The most virulent strain BX08 of *Xoo* produced higher amount of EPS ($43.62 \mu\text{g mL}^{-1}$) than the strain BX01, which produced low amounts of EPS ($32.6 \mu\text{g mL}^{-1}$). Furthermore, the compositional variation of EPS may have some influence on the virulence of *X. oryzae* pv. *oryzae* (Singh et al. 2006).

The pathogenicity of *Xanthomonas* spp. and *Pseudomonas* spp. depends on a specialized type III secretions system (TTSS) which secretes some helper and accessory proteins into an extracellular environment that supports the injection of effector proteins, the major substrate class of the TTSS system into the host cell. The specialized protein system is encoded by a 23 kb chromosomal *hrp* gene cluster which contains six operons *hrpA* to *hrpF*. The *hrp* mutants are unable to infect susceptible plants and induce symptoms and also fail to elicit hypersensitive response (HR) in resistant plants. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) produces AvrXa7 protein, the product of the pathogen gene *avrXa7*, which is a virulence factor. The *avrXa7* gene is a member of the *avrBs* avirulence gene family that encodes proteins targeted to plant cells by the type III secretion (T3S) system. The specificities of virulence and avirulence were associated with the central repeat domain, which in *avrXa7* consists of 25.5 direct repeat units. Mutations in three C-terminal nuclear localization motifs abolished both avirulence and virulence activities in rice (Yang et al. 2000). AvrXa7 is the only type III secretion system effector from *Xoo* with a major contribution to pathogen growth and lesion formation in bacterial leaf blight (BLB) disease. The growth of *Xoo* in plants was drastically arrested, due to inactivation of even a single effector gene representative of strains from Japan, Korea, and Philippines (Yang and White 2004).

Virulence analysis and race classification of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) were accomplished by inoculating 91 representative isolates collected from 22 rice-growing provinces of China onto 24 near-isogenic rice lines containing a single resistance gene or two to four genes. Most isolates of *Xoo* were unable to infect pyramided lines, except IRBB51. Thirteen rice lines with a single gene for resistance were therefore used for further experimentation of *Xoo* in China. IR 24 and IRBB10 were susceptible to the isolates with several exceptions, whereas IRBB5, IRBB7, and IRBB21 were resistant. Based on the nature of interactions between isolates of *Xoo* and near-isogenic rice lines, six single-gene rice cultivars were chosen as differentials and the isolates (285) were classified into nine races. The race frequencies varied from 4.91% to 24.21%. In addition, the virulence of representative strains of eight Philippines races on 13 rice lines with a single-gene resistance was also determined (Liu et al. 2007).

The homologous suicide plasmid integration technique was applied to generate a phosphoenolpyruvate synthase (*ppsA*-) disrupted mutant OSPAM. This mutant could not grow in the medium with pyruvate or C4-dicarboxylates as the sole carbon source, indicating a disruption in *ppsA* function. Further, the mutant had reduced level of virulence on rice, although it induced a hypersensitive response in tobacco. The level of virulence, as in the wild-type strain, was restored in the mutant by complementation

of the disrupted gene. The results suggested that *Xanthomonas oryzae* pv. *oryzae* followed only the PPSA route in gluconeogenesis, which is required for virulence on rice plants (Yang et al. 2008).

The role of endogenous superoxide anion production in the virulence of *Xoo* was studied. The production of endogenous superoxide anion in wild-type strains and *avrXa7* mutants of *Xoo* was assessed by electron spin resonance technique. Localization of superoxide anion in the intra- and extracellular fractions of *Xoo* was carried out. The presence of superoxide anion in multilocations of *Xoo* cells was detected. The extracellular fraction was found to be the major location of superoxide anion production. The levels of endogenous superoxide anion were positively correlated with the virulence of *Xoo* strains, indicating the role of superoxide anion in the virulence of the strains of *X. oryzae* pv. *oryzae* (Li et al. 2009b).

Progressive loss of sensitivity to antibiotics or fungicides in bacterial plant pathogens is likely to occur due to excessive application of chemicals for restricting the incidence and spread of the diseases caused by them, and this has been a great concern for growers and administrators. Molecular basis of resistance of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), causing bacterial leaf blight (BLB), and *X. oryzae* pv. *oryzicola*, causing bacterial leaf streak (BLS), was studied. Single-colony isolates of *X. oryzae* pv. *oryzae* (534) were tested for their sensitivity to streptomycin on plates. Four strains (0.75%) of *Xoo* were resistant to streptomycin and the resistance factor (the ratio of the mean median effective concentration inhibiting growth of resistant isolates to the sensitive isolates) was approximately 226. Field isolates of *Xoo* with resistance to streptomycin were detected and isolates with less sensitivity were also present in the pathogen population in Yunnan Province, China. On the other hand, none of the isolates of *X. oryzae* pv. *oryzicola* exhibited less sensitivity to streptomycin. The genes *str* or mutations in *rpsL* and *rrs* genes (encoding 16S rRNA) were considered to be responsible for streptomycin resistance in other plant pathogens. However, neither the presence of the *strA* gene nor mutations in the *rpsL* or *rrs* genes was observed in *X. oryzae* pv. *oryzicola*. The results suggested that resistance mechanisms other than the presence of *strA* or mutations in *rpsL* or *rrs* genes might be involved in the development of resistance to streptomycin in *X. oryzae* pv. *oryzae* (Xu et al. 2010b).

Isolates of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (323), obtained from symptomatic rice plants in four provinces of China, were tested for their baseline sensitivity to zinc thiazole, a novel bactericide with strong antibacterial activity against *Xoo*. All isolates had similar level of sensitivity to zinc thiazole ($0.1\text{--}16.8\text{ mg L}^{-1}$) over the 2 year period (2009–2010) tested. The baseline sensitivity was distributed as a unimodal curve with a mean EC_{50} value of $6.79 \pm 1.61\text{ mg L}^{-1}$. The risk of mutation to resistance of zinc thiazole in *Xoo* was further evaluated under the laboratory and field conditions. Zinc thiazole mutants were generated through UV-irradiation, culturing on zinc-thiazole-amended nutrient agar (NA) plates and culturing on zinc-thiazole-treated rice plants. The mutants had resistance factors ($\text{RF} = \text{EC}_{50}$ value of mutants/ EC_{50} value of the wild-type parent of the mutant concerned) of $12.4\text{--}186.1$ with a mean RF value of 44.1. Mutants obtained via UV-irradiation, culturing on NA plates, and culturing on rice plants had mean RF values of 51.8, 24.5, and 14.4, respectively. The resistance to zinc thiazole of all mutants decreased after 20 successive transfers on unamended medium (free of bactericide) or 10 successive inoculation-reisolation on untreated rice plants. No significant difference was found in bacterial growth and sensitivity to bismethiazol between zinc-thiazole-resistant mutants and their parents. However, a significant reduction in the

pathogenicity of zinc-thiazole-resistant mutants relative to their parents, particularly for mutants generated through UV-irradiation, was observed (Zhang et al. 2013).

Isolates of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), exhibiting resistance to the systemic fungicide amicarbazol (2-amino-4-methylthiazole-5-carboxanilide) which is a potent inhibitor of succinate dehydrogenase (SDH, EC1.3.99.1), were detected in in vitro tests. The mechanism underlying the induction of resistance to amicarbazol was investigated by employing molecular techniques. Mutants of *Xoo* were generated by exposure to UV radiation. The SDH activity of the wild-type strain of *Xoo* was strongly inhibited by amicarbazol. In contrast, UV-induced mutants were insensitive to the fungicide. A mutation of Histidine₂₂₉ (CAC) to Tyrosine₂₂₉ (TAC) was identified in *sdhB* which encoded the iron-sulfur protein subunit of SDH. The wild-type strain transformed with *sdhB* showed resistance to amicarbazol, confirming that mutation of His₂₂₉ (CAC) to Tyr₂₂₉ (TAC) conferred resistance to amicarbazol in the wild-type strain of *X. oryzae* pv. *oryzae* (Zhang et al. 2010).

In a later investigation, the nature and molecular basis of resistance of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) to the fungicide bismethiazol was investigated. Three *Xoo* mutants (1-1, 2-1, 4-1-1) showing resistance to the fungicide were compared with the wild-type strain (ZJ173) for their pathogenicity on rice plants treated with 300 µg mL⁻¹ bismethiazol. The mutants were pathogenic to treated rice plant leaves, indicating that the resistance to bismethiazol was stable in vivo. The mutants grew faster than ZJ173 during the logarithmic phase, but at the same rate during the decline phase. The degree of bismethiazol resistance of the field populations of *Xoo* in China was assessed by examining 781 isolates of the pathogen from rice leaf samples collected from different provinces of China from 2007 to 2009. The isolates were inoculated on rice plants treated with 300 µg mL⁻¹ bismethiazol. The tested strains were considered as resistant if they showed >70% inhibition compared to the wild-type strain ZJ173. Of the field isolates (505) tested, 62 were found to be resistant to bismethiazol with resistance frequencies, varying over 0–21.1% in different provinces and years. No relationship between fungicide resistance assessments carried out in vitro and in vivo could be observed. The levels of resistance of *Xoo* assessed in field isolates might serve as baseline information for the formulation of management system for rice bacterial leaf blight disease (Zhu et al. 2013).

The endogenous avr/R (avirulence/virulence) interactions are unclear for rice leaf streak disease caused by *Xanthomonas oryzae* pv. *oryzicola*. The genes *avrXa7* and *avrXa10* from *X. oryzae* pv. *oryzae* (*Xoo*) failed to elicit disease-resistance-associated HR and development of leaf streak was not inhibited in cultivars with the corresponding *R* genes after introduction into *X. oryzae* pv. *oryzicola*, despite the ability of this pathovar to deliver an AvrXa10:Cya fusion protein into rice cells. Furthermore, coinoculation of *X. oryzae* pv. *oryzicola* and *Xoo* carrying *avrXa10* inhibited the HR of rice cultivar IRBBIO. Inhibition was quantitative and dependent on the T3S system of *X. oryzae* pv. *oryzicola*. It is possible that one or more *X. oryzae* pv. *oryzicola* type III effectors interfere with avr/R gene-mediated recognition or signaling and subsequent defense response in the host (Makino et al. 2006). The core *hrp* cluster of xanthomonads contains six operons from *hrpA* to *hrpF*. *Xanthomonas oryzae* pv. *oryzicola* also possesses clusters of *hrp* genes that control elicitation of HR in nonhost tobacco and pathogenicity in rice plants. *X. oryzae* pv. *oryzicola* was shown to contain three separate DNA regions for HR induction in tobacco and pathogenicity in rice. Analysis of the genome of this pathogen provided a fundamental base for understanding pathogenicity determinants in comparison with *X. oryzae* pv. *oryzae* (Zou et al. 2006).

Xanthomonas oryzae pv. *oryzicola*, causing bacterial leaf streak (BLS) disease of rice, is seedborne. This pathogen induces characteristic water-soaked to translucent interveinal streaks on the leaves and the streaks coalesce to form large patches, ultimately leading to blighting of leaves. *X. oryzae* pv. *oryzicola* may survive in the seed from season to season, but not in the crop debris left in the soil. Disease development is favored by moderate temperatures of 26–31°C. *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* suspensions, when sprayed on leaves of maize or Italian ryegrass (*Lolium multiflorum*), concentrated on pickle hairs, microhairs, and bases of cushion hairs in maize leaves. These pathogens reached maximum population on Italian ryegrass at 3 days after application, as revealed by scanning electron microscopy (Huang and DeCleene 1989). *X. oryzae* pv. *oryzicola* uses a type III secretion (T3S) system, which has genes for pathogenicity and hypersensitive reaction (HR). Many of the *hrp* (hypersensitive response and pathogenicity, *hrc*-conserved and *hra*-associated) genes have roles in pathogenesis. The *hrpE3* showed homology to *hpaE* of *X. campestris* pv. *vesicatoria* (Xcv). The secretion of the HrpE3 was dependent on T3S and subcellular localization of HrpE3 was cytoplasmic and nuclear in plant cells. A mutation in the *hrpE3* reduced the virulence of *X. oryzae* pv. *oryzicola* by decreasing the lesion length and pathogen growth in planta (Cui et al. 2013).

Xanthomonas axonopodis pv. *malvacearum* (*Xam*) causes cotton bacterial blight, angular leaf spot, or black arm at different stages of crop growth. The pathogen becomes seedborne when the bolls are infected. It may also pass along the fibers and infect the seeds externally and internally. *Xam* may survive in dry leaves for several years. Following germination, the infected seed may serve as the primary source of inoculum for the spread of the disease to young cotton plants through wind-splashed rain and dew. The transcriptome investigation of the cotton-*Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) interaction was performed. Clones from a cDNA library were used to identify host genes expressed in upland cotton leaves, following inoculation with *Xam*. The cDNA was prepared from inoculated and noninoculated leaves of cotton line Im 216 with high broad and stable resistance to bacterial blight pathogen. Suppression subtractive hybridization was used to make a normalized cDNA library that was differential between transcripts of inoculated and noninoculated leaves. Polymerase chain reaction (PCR-) amplified cDNAs of the 121 genes, as well as of 13 previously identified genes, were arrayed onto glass slides. These microarrays were used to analyze transcripts in Im 216 leaves over the extended period of 8–60h after inoculation. Microarray analysis indicated that 98% of the genes were significantly upregulated at one or more of the sampling times. Of these, 63% had sequence similarity to the plant genes associated with defense responses, that is, to genes that function in disease development/defense, protein synthesis/turnover, secondary metabolism, signaling, stress/programmed cell death, or code for pathogenesis-related (PR-) proteins or retrotransposon-like proteins. Some genes (17%) exhibited the highest differential abundance during the 24h after inoculation, prior to HR recognition microscopically. The majority of the genes showed differential expression at 60h postinoculation, when healthy cells or neighboring dying or dead cells were major participants in the response (Patil et al. 2005).

Superoxide dismutase (SOD) genes of *Gossypium hirsutum* were characterized by assessing the SOD activity in cotton cotyledons inoculated with virulent and avirulent strains of *Xanthomonas axonopodis* pv. *malvacearum* race 18 (*Xam* 18). The contribution of SOD to the oxidative burst in the hypersensitive reaction (HR) was investigated. The cDNA fragments encoding cotton SOD genes were isolated by RT-PCR assay,

sequenced, and their similarities to known Cu/ZnSOD (cytoplasmic and chloroplastic), MnSOD, and FeSOD were established based on amino acid sequences. Transcriptional expression of cotton SODs during the development of HR in cotton was determined using a semiquantitative RT-PCR analysis. Cu/ZnSOD_{cyt} and Cu/ZnSOD_{dchl} transcripts were similar in both compatible and incompatible interactions. However, in the incompatible interaction MnSOD was induced at 6 h postinoculation (hpi) and peaked at 10–12 hpi. The results suggested that MnSOD might contribute to the SOD activity observed at 4 hpi, playing a role in the early apoptic events of the HR in cotton-*Xam* interaction. In addition, catalase1 was transcriptionally stable in cotton-*Xam* interaction with a slightly increased level in incompatible interaction, resulting in hypersensitive response (Voloudakis et al. 2006).

Highly virulent strains (HVS) of *Xam* could infect all commercial cultivars of cotton tested, including 101-102B considered to be “immune” to the disease. High-quality draft sequences of highly virulent strain GSPB2388 from Sudan and a strain of race 18 (GSPB2388 and GSPB1386) contained 4665 and 4620 protein-coding genes, respectively. A total of 181 and 178 potential virulence-related genes, including genes encoding a major group of type III effectors, were identified in these 2 strains. The differential effectors and sequence diversity between HVS and race 18 might be useful to identify the key factors that contribute to the high virulence of HVS. The average nucleotide identity (ANI) between *Xam* and *X. axonopodis* pv. *citri* was shown to be 98.4%, suggesting that these two strains might belong to the same species (Zhai et al. 2013.).

Xanthomonas campestris pv. *campestris* (*Xcc*) causes black rot disease of cabbage, cauliflower, lettuce, and other *Brassica* (cruciferous) crops. The formation of V-shaped lesions on leaves, followed by blight and sudden collapse of interveinal tissues, are commonly observed in infected plants. The pathogen is seedborne and the floral transmission of bacteria to seeds is a permissive pathway, compared to transmission through vascular system (Darsonval et al. 2008, 2009). Transmission of *Xcc* to cauliflower blossom via flies (*Calliphora vomitoria*) resulting in seed infestation was investigated. Cauliflower blossom was inoculated with *Xcc* by brush or pollination with bluebottle flies, a vector of *Xcc* in polytunnels. In approximately 30% of seed batches of breeding lines, high densities of *Xcc* ($>10,000$ CFU g⁻¹) were present both before and after seed treatment with warm water. The infestation with *Xcc* in seed derived from fly-inoculated plants was confirmed in seedling grow-out test. Brush inoculation resulted in an average of 45% of seed batches being contaminated with high densities of *Xcc*. The flies were contaminated with *Xcc* by allowing them to feed on agar-grown pathogen colonies. *Xcc* populations decreased rapidly and survived for a maximum period of 5 days in the flies. It is possible that the flies contaminated with *Xcc* could function as a vector for the transfer of *Xcc* to cauliflower blossoms and ultimately to the seed (van der Wolf and van der Zouwen 2010).

Systemic colonization of cauliflower plants (*Brassica oleracea*) by *Xanthomonas campestris* pv. *campestris* (*Xcc*) upon leaf infection or alternatively after flower infection was studied under polytunnel conditions in 2007 and 2008. Plants were spray-inoculated at the 8-leaf stage or during flowering. A high percentage of systemic infection was observed in plants inoculated at all stages of plant growth. The average estimated infection incidences for stem base and peduncle infections were 16% and 19%, respectively. The presence of *Xcc* was detected in 61% of 23 seed samples harvested from plants with inoculated flowers. However, seed transmission of *Xcc* to plants could not be confirmed by grow-out tests under conditions favorable for disease development (RH > 95%

and 28°C). *Xcc* was detected in 59/60 seed samples harvested from leaf-inoculated plants with one exception. Investigation of the population dynamics of *Xcc* on inoculated flowers showed that 52% of flowers were infected with *Xcc*. During development of siliques, infection percentage decreased slowly and at 56 days postinoculation (dpi), 20% of the superficially disinfected siliques were infected with *Xcc*. Infection estimates indicated that 0.18% of the seeds were infected and 1–10% of infected siliques contained infected seeds (van der Wolf et al. 2013).

Xanthomonas campestris pv. *campestris* (*Xcc*) may survive in plant debris only for a short period. When the infected seed germinates, the bacteria in the infested seed coat that emerges out along with the cotyledons may serve as the primary source of inoculum. The secondary inoculum is disseminated by raindrop splashes from the infected leaves. Semiselective media FS and NSCAA or mCS20ABN were employed for the isolation of *Xcc* from 107 seed lots of *Brassica*. The population of *Xcc* was greater on FS medium than on mCS20ABN medium (Roberts et al. 2004).

X. campestris pv. *campestris* (*Xcc*) strain NRRLB-1459 was chromosomally tagged with green fluorescent protein gene (*gfp*) and its presence in the chromosome of *Xcc* was confirmed by Southern hybridization. The virulence related gene of the transformant was not affected by the insertion of *gfp*. The infection process of *Xcc* was followed in planta after inoculation with the transformed strain. Using a fluorescence microscope, the migration and distribution of *gfp*-labeled *Xcc* strain was visualized in real time. The *gfp*-labeled bacterial cells could be easily visualized. A time delay of 2 days between the detection of the presence of *Xcc* in plant cells and the appearance of visible necrosis was observed, indicating the potential of the technique for early detection of *Xcc* in infected cabbage plants (So et al. 2002).

The infection of plants by bacterial pathogens depends on their ability to use type III secretion systems (TTSS) and to deliver effector proteins into the eukaryotic (plant) cells for the initiation of infection and subsequent disease development (pathogenesis). In bacteria–plant interactions, one effector may function as an avirulence factor, making the pathogen vulnerable to detection of its presence by the plant surveillance system and hypersensitive response (HR) in the resistant host, carrying a corresponding resistance (*R*) gene. However, the same effector can also sustain the growth of the pathogen by acting as a virulence factor to modulate host physiology in the susceptible host lacking the corresponding *R* gene. A bifunctional TTSS effector AvrXccC belonging to the AvrB effector family in *Xanthomonas campestris* pv. *campestris* 8004 was identified. This effector is required for full virulence in the susceptible cabbage (*Brassica oleracea*) and avirulence in the resistant host mustard (*Brassica napiformis*). Expression of *avrXccC* in mustard-virulent strain *Xcc* HRI3849A resulted in the conversion of its virulence into avirulence. The effector AvrXccC was anchored to the plant plasma membrane and the N-terminal myristoylation site (amino acids 2-7: GLcaSK) was found to be essential for its localization. In addition, the avirulence function of AvrXccC for host recognition depended on its plasma membrane and the expression of *avrXccC* was *hrpG/hrpX*-dependent. Further, secretion of AvrXccC displayed *hrp*-dependency and the core sequence for AvrXccC translocation was defined to the N-terminal 40 amino acids (Wang et al. 2007).

Transmission of *Xanthomonas campestris* pv. *campestris* (*Xcc*) from flowers to seeds and from seeds to seedlings was determined in compatible and incompatible interactions. Transmission from seeds to seedlings were compared for *Xcc*, for *X. citri* pv. *phaseoli* var. *fuscans* (*Xcp*) in compatible interactions with bean, and for *Escherichia coli*

(*Ec*) (pathogenic to humans) in null interactions with bean. Colonization of germinating seeds and seedlings is considered as a critical step in the establishment of bacterial diseases. Populations of *X. campestris* pv. *campestris* ATC33913 increased up to 1.8×10^4 CFU/contaminated seed and 2.4×10^6 CFU/contaminated seedling. The two harvested seed lots showed contamination rates of 0.6% and 1.4% and 4 day old seedlings showed corresponding contamination rates of 0.4% and 3.0%, respectively. The dynamics of bacterial population sizes during seedling development were analyzed by inoculating the seeds by vacuum infiltration with *Xcc* (incompatible interaction), *Xcp* (compatible interaction), and *E. coli* (null interaction). Population sizes did not vary at 2 h after inoculation in compatible and incompatible interactions, but a reduction in pathogen population occurred in null interaction. It was not until 14 days after germination that *Xcp* began to multiply at a faster rate than *Xcc* or *Ec* on bean seedlings, indicating that the bacteria behaved in a similar manner at emergence. No visible symptoms appeared on seeds and seedlings following seed contamination with *Xcc*. The results implied that seedling colonization might not require bacterial pathogenicity determinants. No significant defense responses were induced in the plant for the incompatible interaction until the bacteria were introduced within the plant tissues. The results indicated that colonization of seedlings relied mostly on the surface colonization and not on parasitic relationships. Further, the transmission of incompatible bacteria to seedlings from contaminated seed suggested the possible role of seeds of nonhost plants as an inoculum carrier for seedborne bacteria. It is possible that since nutrients are available in sufficient quantities on germinating seeds and seedlings, bacterial pathogens do not need to initiate a molecular dialogue with the plant to multiply efficiently in this environment. Genes of type III secretion system genes are not induced in nutrient-rich medium. In contrast, these genes are induced in the phyllosphere where nutrient availability is limited, necessitating the induction of these genes (Darrasse et al. 2010).

The development of resistance to fungicides and bactericides in bacterial pathogens causing crop diseases is of great concern to growers and consumers because of increased environmental pollution due to the frequent and excessive application of these chemicals to reduce incidence and spread of the bacterial diseases. The levels of resistance of 56 native isolates in Trinidad and seven reference strains of *Xanthomonas campestris* pv. *campestris* (*Xcc*) to copper were assessed using buffered (pH 7.0) and unbuffered (pH 5.6) nutrient agar media. All isolates of *Xcc* were pathogenic to *Brassica oleracea* cultivar Copenhagen Market and induced typical cabbage black rot disease symptoms. The resistant isolates grew on buffered media amended with ≥ 2000 ppm copper, indicating the existence of highly resistant *Xcc* isolates in Trinidad. A strong relationship was observed between the length of time of continuous applications of copper formulations applied for the control of black rot disease and proportion of isolates exhibiting copper resistance. Sensitivity or resistance to copper did not influence the virulence of *Xcc* isolates at 10 days after inoculation (Lugo et al. 2013).

Xanthomonas campestris pv. *vesicatoria* infects pepper and tomato causing bacterial spots on leaves, stem, and fruit and seedling blight. Infected seeds form the primary sources of inoculum for the spread of the disease over short and long distances. Four phenotypically and genotypically distinct *Xanthomonas* spp. causing bacterial spot disease of pepper and tomato have been distinguished: *Xanthomonas euvesicatoria* (group A encloses most of the strains previously known as *X. campestris* pv. *vesicatoria* or *X. axonopodis* pv. *vesicatoria*); *X. vesicatoria* (group B); *X. perforans* (group C); and *X. gardneri* (group D). Strains of A, B, and D are pathogenic to tomato and pepper,

whereas group C strains infect tomato only. Further, groups A and B are distributed worldwide and strains of C and D groups have limited distribution (Jones et al. 2004; Beran and Mráz 2013). In addition, *X. campestris* pv. *vesicatoria* exists in the form of several races that show variations in virulence and sensitivity to chemicals. A total of 16 races existing in Barbados were identified and 11 of them were detected in bell pepper and tomato for the first time. Four races could infect only pepper and one race was confined to tomato. Races P1T1 and P6T2, each amounting to 16% of strains from pepper, replaced race P1 (the predominant race) which contributed to 4% incidence. On tomato, races P1T1 and P1T2 accounted for 36.1% and 21.0%, respectively, and race T1 was replaced by these two races. Races sensitive to zinc, but resistant to copper, became resistant to zinc and reverted by 40% to copper sensitivity. The results indicated that changes in virulence of races of *X. campestris* pv. *vesicatoria* and their sensitivity to zinc and/or copper have to be monitored continuously (Gore and O'Garro 1999).

Xanthomonas campestris pv. *vesicatoria* (*Xcv*) possesses a number of genes (designated *hrp* genes), most of which are clustered in a 23 kb chromosomal region required for pathogenicity on susceptible plants and induction of hypersensitive response (HR) on nonhost plant species. Nine *hrp* genes encode the components of type III protein secretion apparatus that is conserved in Gram-negative plant and animal bacterial pathogens. *Xcv* secreted proteins into the culture medium in a *hrp*-dependent manner. Substrates for the Hrp secretion machinery were found to be pathogenic factors and avirulence proteins such as AvrBs3. The AvrBs3 protein governed recognition (HR induction) when *Xcv* infected pepper plants carrying the corresponding resistance gene *Bs3*. The AvrBs3 protein contained eukaryotic signatures, such as nuclear localization signature (NLS), demonstrated to act inside the host plant cell. It was suggested that AvrBs3 might be transferred into the plant cell via the Hrp type III pathway and recognition of AvrBs3 might take place in the plant cell nucleus (Bonas et al. 2000).

Xanthomonas spp. possess a 23 kb chromosomal *hrp* gene cluster which contains six operons (*hrpA* to *hrpF*) and mutations, and any one of these operons may result in the loss of pathogenicity of the mutants. A few of the nonchromosomal proteins essential for pathogenicity of *X. campestris* pv. *vesicatoria* (*Xcv*) have been identified. HrpB2 and HrpE were secreted and essential for secretion of other proteins by the TTSS. HrpE was found to be a major subunit of the Hrp pilus, an extracellular appendix considered to be associated with the TTSS, whereas HrpF is essential for pathogenicity but not for secretion in *Xcv* (Rossier et al. 2000; Koebnik 2001; Weber et al. 2005).

The formation of filamentous structures, the Hrp pili, at the surface of cells of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) was visualized by immunoelectron microscopy, involving the use of antisera specific to HrpE, HrpF, and AvrBs3. HrpE was shown to be the major pilus subunit, as revealed by heavy labeling of Hrp pili by HrpE-specific antibodies. The other TTSS-secreted proteins HrpF and AvrBs3 were also in close contact with Hrp pilus during and/or after their secretion. The gene *hrpE* was unique to the genus *Xanthomonas* and showed no similarity to the other pili genes. Mutations in *hrpE* abolished pathogenicity or the ability to elicit HR in plants. Further, HrpE was shown to be essential for all TTSS secretion of HrpF, AvrBs1, and AvrBs3, indicating its indispensability for maintaining a functional TTSS (Weber et al. 2005). The *hpa* (*hrp*-associated) genes that contribute to, but are not essential for, pathogenic interaction of *Xcv* with host plants were identified by the analysis of nonpolar mutants in the *hrp* gene cluster (Nöel et al. 2002; Büttner et al. 2004). The gene *hpaA* encoded a protein required for the HR and full pathogenicity. HpaA secreted by the TTSS was

localized to the plant cell nucleus when expressed in planta (Huguet et al. 1998). Direct evidence indicating the occurrence of translocation of AvrBs3 from *Xcv* into host plant cells was provided by the technique of immunocytochemistry. Immunocytochemistry procedures for pepper cells expressing avrBs3 via *Agrobacterium*-mediated gene transfer under the control of the constitutive CaMV 35S promoter were developed. The subcellular localization of AvrBs3 was determined in serial transverse sections of susceptible pepper (ECW) leaf tissue expressing avrBs3, using an AvrBs3-specific PAb and alkaline phosphatase-coupled secondary antibody. The DNA-specific staining with 4',6-diamidino-2-phenylindole (DAPI) showed AvrBs3 protein could be detected in the nucleus of some cells. The translocation of AvrBs3 could be visualized in situ in native tissues of both susceptible and resistant plants. AvrBs3 could be detected in the nucleus as early as 4 h postinoculation and was dependent on a functionality of TTSS and the presence of the putative translocator HrpF (Szurek et al. 2002).

Changes in the DNase and RNase activities were assessed in leaves of pepper cultivar Early Calwonder inoculated with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) race 1 (virulent) and race 2 (avirulent). Two major bands of RNase activity with apparent molecular mass of 18 and 27 kDa were detected in pepper leaf extracts. A 25 kDa band with both RNase and DNase activity, a nuclease, was intra- and extracellularly produced by race 1 (compatible interaction). The activity was at an optimal level at pH 5.0–7.5 and was strongly inhibited by EDTA and Zn^{2+} in both compatible and incompatible interactions with race 1 and race 2, respectively. Marked increases in DNase activity (25 kDa band) were detected in pepper leaves infected by both race 1 and race 2 of *Xcv*. High-intensity bands appeared at 4 and 8–12 h after inoculation with incompatible and compatible races of *Xcv*, respectively. The enhancement of DNase activity did not appear to be related to the growth of *Xcv* in planta, since the pathogen population remained essentially constant during 0–12 h after inoculation. It is possible that bacterial nucleases, along with other extracellular degradation enzymes of pathogen origin, might exploit the host plant nutrients after host cell lysis (Buonauro et al. 2001).

Localization of the avirulence gene *avrRxv* in *X. campestris* pv. *vesicatoria* (*Xcv*) to the cytoplasm and possibly in association with plasma and nuclear membranes was observed in both resistant and susceptible tomato plants. The AvrRxv cysteine protease catalytic core was essential for host recognition by the bacterial pathogen. The introduction of mutations in this domain affected the ability of AvrRxv to elicit HR and the inhibition of pathogen development in resistant tomato plants. Based on the expression profile analysis, 420 of approximately 8600 tomato genes were identified as differentially modulated by the expression of a functional AvrRxv, including over 15 functional classes of protein and a large number of transcription factors and signaling components (Bonshtien et al. 2005). In another study, *Bs4* (disease resistance gene) promoter was characterized and its ability to regulate the level of expression of bacterial type III effector proteins in planta was assessed. *Bs4* was constitutively expressed at low levels, as revealed by RT-PCR assay. Transcript abundance did not have a significant influence following infection with *avrBs4*-containing *Xcv* strains (Schornack et al. 2005).

In order to identify *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) genes that are specifically induced during its interaction with tomato, an adaptation of a recombinase- or resolvase-based in vivo expression technology was applied. Analysis of the genes showed that a *citH* (citrate transporter) homologous gene contributed to *Xcv* virulence on tomato. The *citH* product facilitated citrate uptake required for the pathogen growth. The *citH* promoter, specifically induced by citrate and citrate content from tomato leaf

apoplast, was considerably reduced following growth of wild-type strain and *citH*-complemented strain of *Xcc*, but not the *citH* mutant. The promoter activity of the *citH* gene was induced as early as 2.5 h after syringe infiltration of *Xcc* into tomato leaves, and the enhanced activity continued up to 96 h after inoculation. An operon containing a two-component regulator system homologous to *tctD/tctE* influencing *citH* expression in *Xcv* was detected. The expression of *hrp* genes did not seem to be affected in the *citH* mutant and this mutant could not be complemented for growth in planta when coinoculated with the wild-type strain, indicating that citrate uptake in the apoplast of tomato plants was essential for *Xcv* virulence (Tamir-Ariel et al. 2011).

Strains of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) have been grouped based on host plant species infected and into races based on the disease response on differential cultivars of tomato and pepper. Based on the competitive ability of strains of *Xcv*, tomato race 1 (T1) strains and tomato race T3 strains occurring in Florida, USA were differentiated. Tomato race T3 strains of *Xcv* were found to be antagonistic to tomato race 1 (T1) strains in vitro. All T1 strains and 11 strains of other *Xanthomonas campestris* pathovars were inhibited by tomato race T3 strains. Production of an antagonistic compound against selected strains of *Xcv* by T3 strains was detected. The compound causing antagonism was secreted extracellularly and was diffusible through agar media. Strain 91-118 (T3 strain) was not sensitive to its own antagonistic agent, which was inactivated at a temperature of 80°C and by pronase and proteinase K. These properties have been shown to be shared by most bacteriocins (BCNs). The sensitivity of *Xcv* strains to inhibitory BCN-like compounds produced by T3 strain varied considerably. Strains belonging to *Xcv* groups A, B, C, and D (differing phenotypically and genotypically) exhibited variations in sensitivity to BCN-like compounds secreted by T3 strains. The antagonistic activity of T3 strains was associated with at least three different regions of DNA of *X. campestris* pv. *vesicatoria*. Southern hybridization analyses showed that the strains of *Xcv* could be classified into three bacteriocin (BCN-) producing groups designated BCN-A, BCN-B, and BCN-C. These groups could be differentiated by variations in the inhibitory spectra and levels of activity in plate assays. Mutations that inactivated expression of each BCN group individually in a wild-type T3 strain had inhibitory activity, confirming that multiple BCNs were produced by T3 strains. T3 strains inhibited T1 indicator strains in leaves of susceptible genotype of tomato, providing direct evidence for a direct selective advantage for T3 strains in the infection court. BCN-A expression was essential and the dominant component in the suppression of development of sensitive T strain in tomato leaf tissue. The results indicated that production of BCN-A could play a major role in mediating the population dynamics between strains of *X. campestris* pv. *vesicatoria* in tomato leaves (Tudor-Nelson et al. 2003).

Bacterial spot (BS) disease of pepper is caused by *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*. *X. euvesicatoria* is seedborne and infested pepper seeds form the primary source of inoculum for disease incidence and subsequent spread. If the pathogen is introduced into the greenhouse, the prevailing high temperature, humidity, and plant density favor rapid development of the disease, indicating the importance of the use of disease-free pepper seed to avoid the introduction of the pathogen in the confined environments of greenhouse (Leite et al. 1995).

Pathways of seed infection by *X. euvesicatoria* were investigated to facilitate development of effective disease management strategies to limit seed infection by the pathogen. The colonization of pepper blossoms by *X. euvesicatoria* and the relationship between inoculum concentrations and seed infestation were studied. Pepper fruits

developed from inoculated blossoms were asymptomatic. However, real-time PCR assay detected *X. euvesicatoria* in 39% of seed lots assayed and viable colonies were identified as *X. euvesicatoria* by PCR assay. Further, 16% (15/95) of the seed lots yielded seedlings exhibiting disease symptoms under conditions of 29°C and 90% RH. The pathogen was isolated from the seedlings showing symptoms on YDC medium, and PCR assays confirmed the identity of the pathogen isolated as *X. euvesicatoria*. At 96 h after inoculation, populations of *X. euvesicatoria* were up to 1×10^5 CFU/blossom on stigmas, whereas stilar and ovary tissues had populations ranging from 1×10^5 to 1×10^6 CFU/blossom. Inoculum concentration showed a positive relationship with percentage of infested seed lots.

The temporal population dynamics of *X. euvesicatoria* and *Acidovorax citrulli* (a known blossom-colonizing pathogen of watermelon) were compared. *X. euvesicatoria* and *A. citrulli* could be recovered immediately after inoculation from the stigmas, while these pathogens were only detectable from style and ovary tissues 6 and 12 h, respectively, after inoculation. *A. citrulli* colonized pepper blossoms more efficiently than *X. euvesicatoria* at 96 h postinoculation. It appeared that *A. citrulli* could colonize stigmas of nonhost blossoms and the host–pathogen interaction might not be an important factor for blossom colonization. Inoculation of pepper blossoms even with low concentration of *X. euvesicatoria* (10 CFU/blossom) resulted in infestation of $\leq 14\%$ of seed lots, indicating the substantial risk posed when pepper blossoms were colonized by *X. euvesicatoria*, leading to appreciable levels of seed infestation. The results revealed the role of pepper blossoms as a site of *X. euvesicatoria* ingress and subsequent seed infestation with asymptomatic pepper fruits (Dutta et al. 2014).

Bacterial blight disease is caused by *Xanthomonas campestris* pv. *vignicola* which infects the cowpea crop at all stages of growth and also seeds in storage, becoming an important constraint in cowpea production. The role of infected seeds, plant debris, and infested soil in survival of the pathogen was investigated under laboratory and field conditions at the International Institute of Tropical Agriculture (IITA), Nigeria. Populations of *X. campestris* pv. *vignicola* in plant debris varied from 1.4×10^6 to 7.6×10^8 CFU mL⁻¹ in 1996 and from 1.8×10^8 to 8.0×10^8 CFU mL⁻¹ in 1997. Soil population of the pathogen in soil was much lower in both years. During the off-season, bacterial population ranged from 0.4×10^8 to 3.0×10^8 CFU, whereas the soil population was below the detectable levels at 8 months after harvest. Pathogen population in infected seeds ranged from 5.8×10^8 to 19.8×10^8 CFU mL⁻¹ in 1996 and from 9.8×10^8 to 25.0×10^8 CFU mL⁻¹ in 1997, indicating that seeds harbored very high levels of pathogen populations compared with infested plant debris and infested soil. As no detectable levels of pathogen population were recorded, the possibility of survival of *X. campestris* pv. *vignicola* in soil is limited. Infected seeds and plant debris left on the soil after harvest were considered to be the major sources of primary inoculum for the newly planted cowpea crops (Okechukwu and Ekpo 2008).

Xanthomonas campestris pv. *vitians*, incitant of bacterial leaf spot disease, can be seedborne in lettuce. The ability of *X. campestris* pv. *vitians* to enter and translocate in the vascular system of lettuce plants and to contaminate seeds externally and/or internally was assessed. Lettuce plants (8–11 weeks old) were stab-inoculated with the bacterial suspension at lower stem 0.5 cm above soil line (Section 6.6.1). The location of *X. campestris* pv. *vitians* within the stems of stab-inoculated 8–9 and 11 week old lettuce plants was determined by examining the stem sections taken every 2 cm from point of inoculation at each time point of 4, 8, 12, and 16 h postinoculation and pressing

onto XCS medium. Colonies of xanthomonads-like bacterial colonies could be observed, and the location from which they were recovered roughly corresponded to the location of the vascular bundles of the lettuce stem (Fig. 6.1). The identity of the bacteria isolated on XCS medium was confirmed by polymerase chain reaction (PCR) assays, using B162 primer pair as *X. campestris* pv. *vitians*. The pathogen was readily recovered from the surfaces of stem sections of spray-inoculated plants and surface disinfection resulted in elimination of the bacteria from stem sections. However, the pathogen could be recovered from crushed-disinfected stem pieces and populations from inside stems ranged from 0 to 10^6 CFU g⁻¹ of stem tissues. Direct plating of individual seeds revealed the presence of *X. campestris* pv. *vitians* in three 1000 seed samples, as confirmed by PCR assays. However, no xanthomonads-like bacteria was recovered from any of the

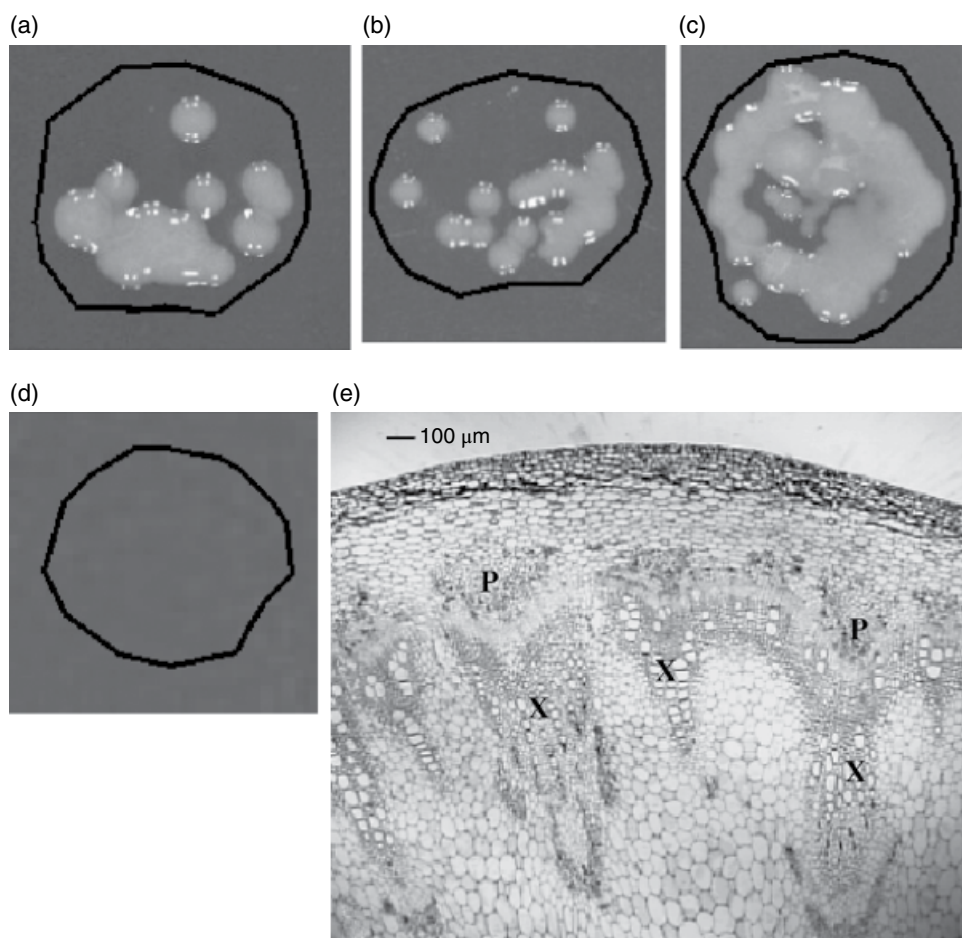


Figure 6.1 Detection of *Xanthomonas campestris* pv. *vitians* (Xcv) by isolation from stab-inoculated lettuce and visualization of phloem and xylem from which the pathogen could be isolated. Isolation of Xcv from stem sections taken at different locations: (a) 2–4 cm at 4 h postinoculation (hpi); (b) 0–2 cm from point of inoculation at 12 hpi; (c) 4–6 cm from point of inoculation at 16 hpi; (d) 0–2 cm from uninoculated plant (control); (e) cross-section of lettuce stem showing phloem and xylem under light microscope. Source: Barak et al. (2002). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

crushed lettuce seed samples. Seed-wash assays showed that seed contamination level in spray-inoculated plants was about 10^4 CFU g⁻¹ of seed. The results indicated that *X. campestris* pv. *vitians* could move within the vascular system of lettuce plants without inducing any visible symptoms, as stab- and spray-inoculated plants did not show any symptoms of bacterial leaf spot disease. Seed produced from diseased lettuce plants were contaminated at a level of about 2% systemic infection of plants. Although seeds were asymptomatic, they could favor survival of *X. campestris* pv. *vitians* in association with lettuce debris as stem tissues are likely to persist longer than the leaves left in soil. Symptomless systemic infection of lettuce plants might also lead to external contamination of seed in the absence of disease symptom (Barak et al. 2002).

The role of alternative or collateral host plants in serving as sources of inoculum for the bacterial pathogens of crops has been studied in different pathosystems. The ability of several weed species belonging to Asteraceae, Chenopodiaceae, Malvaceae, Polygonaceae, and Portulacaceae infected by *Xanthomonas campestris* pv. *vitians*, causative agent of lettuce bacterial leaf spot disease, to serve as sources of inoculum was assessed. Different weed species were spray-inoculated with the suspension of the pathogen cells and incubated in the greenhouse at 24°C and 95% relative humidity for 4 weeks. Pathogen population recovered by leaf washings from inoculated plants was expressed in CFU. Bacterial populations from weed species differed significantly ($P < 0.0001$). *Lactuca* sp. supported pathogen development to the maximum extent ($>10^5$ CFU g⁻¹ of plant). The intensity of disease symptoms induced by *X. campestris* pv. *vitians* was severe in *Lactuca* spp., while other species consistently developed mild symptoms or no visible symptoms appeared on them. The results indicated that *Lactuca* sp. could be a potential source of inoculum for disease incidence on lettuce crops (Toussaint et al. 2012).

6.2.2 *Pseudomonas* spp.

Bacterial plant pathogens belonging to the genus *Pseudomonas* may exist as epiphytes and/or endophytes in their host plant species. As epiphytes, they may develop nonparasitically on plant surfaces, primarily for physical support, although some damage due to epiphytic development of pseudomonads may occur. As endophytes, they may exist in plant tissues without inducing substantial adverse effects or deriving benefit other than residency (Kobayashi and Palumbo 2000). *Pseudomonas syringae* pv. *phaseolicola* (*Psp*), causing bean halo blight disease, is seedborne and the contaminated seeds could be the primary inoculum for spread of the disease over short and long distances (Grogan and Kimble 1967). The pathogen infects leaves and pods, inducing characteristic water-soaked lesions on leaves, pods, stems, or petioles which later turn into brown spots surrounded by yellow areas (halos). *Psp* could penetrate from external pod lesions into the underlying bean seeds (Taylor et al. 1979).

Pseudomonas syringae pv. *phaseolicola* (*Psp*) shows a differential reaction on a set of bean cultivars due to variation in the virulence of different isolates of *Psp*. Nine races of *Psp* have been distinguished, based on the virulence to differential varieties of bean (Taylor et al. 1996). The ability of *Psp* to induce disease in bean has been demonstrated to be due to the presence of a pathogenicity island, localized to a 150 kb plasmid that includes genes which are either essential for pathogenicity on bean and soybean or capable of contributing to aggressiveness in an additive manner (Jackson et al. 1999; Tsiamis et al. 2000). Further, strains of *P. syringae* pv. *phaseolicola* possess the *argK-tox* gene cluster governing the biosynthesis of the toxin phaseolotoxin which appears to

increase the virulence of the strains. As the isolates capable of synthesizing phaseolotoxin (Tox⁺) are considered to be of epidemiological significance, this DNA region has been commonly used as a target for PCR detection and identification of *Psp* strains (Mitchell 1978; Schaad et al. 1995).

The *lux* genes of *Photobacterium luminescens* have been shown to be effective for monitoring metabolic activity of bacteria. Bioluminescence is a measure of metabolic activity rather than cell viability and multiplication. The genes of *P. luminescens* were employed to monitor the population dynamics of *P. syringae* pv. *phaseolicola* (*Psp*) in bean plants. The plasmid pGLITE containing the *luxCDABE* genes was introduced into *Psp* race 7 strain 1449B. The growth of *Psp* in the leaf intercellular spaces was assessed by determining the viable counts and the bioluminescence of leaf disc homogenates. The direct measurement of bioluminescence was consistently positively correlated with the bacterial population as determined by bacterial cell counting. The bioluminescence assessment was found to be a potential alternative to the labor intensive and time-consuming enumeration procedures (Paynter et al. 2006).

Infected seed may not show any obvious symptoms of disease or may have wrinkled or buttery yellow patches on the seed coat. The pathogen *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) is Gram-negative, aerobic, rod-shaped, 1.5 µm × 0.7–1.2 µm in size, motile with at least one polar flagellum. Optimal temperatures for growth are 25–30°C. In addition to dissemination through infected seeds, the pathogen may be disseminated from bacterial exudation by water-splash and wind during rainfall. The pathogen may persist on dry bean seeds and actively multiply after germination. It could survive both as parasite within the plant tissues and as an epiphyte (Arnold et al. 2011).

Studies on molecular genetics of *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) have provided the basic information on microbial pathogenicity and plant defenses. The first characterization of *hrp* genes that encode the type III secretion system (T3SS), which delivers proteins directly into plant cells, was accomplished using *Psp*. In addition, comparative analysis of the whole-genome sequences of *Psp* and other *P. syringae* pathovars greatly helped to understand the evolution of host specificity. Bacterial pathogens manipulate their hosts by delivering effector proteins into the plant cell cytoplasm through the conserved type III secretion system (T3SS) (Casper-Lindley et al. 2002). Mutants of *hrp* of *Psp* were generated using Tn5 insertion. The mutants lacked pathogenicity to bean as well as the ability to elicit HR in tobacco (a nonhost). The *hrp* genes have also been shown to be conserved in other *P. syringae* pathovars and are clustered on a pathogenicity island that consisted of at least six operons (Alfano et al. 2000). Phaseolotoxin is produced by *Pseudomonas syringae* pv. *phaseolicola* (*Psp*), which induces characteristic haloes on infected leaves and pods. The chlorotic halo is due to the action of phaseolotoxin that inhibits ornithine carbamoyl transferase, a critical enzyme in the urea cycle (Moore et al. 1984). Influence of temperature on phaseolotoxin production appears to be a major factor. The temperatures of 18–20°C are optimal, while toxin production is inhibited at 30°C (Nüske and Fritsche 1989). The presence of specific plant components is required for the biosynthesis of phaseolotoxin by *Psp*, in addition to low temperature (Hernández-Morales et al. 2009). The investigation, carried out using a collection of 138 isolates of *Psp* from commercial fields in Spain, cast doubt on the importance of phaseolotoxin in pathogenesis. The majority of the isolates (95) did not produce phaseolotoxin in vitro, as revealed by the *Escherichia coli* inhibition bioassay. Most of the isolates (94) failed to generate the expected PCR amplification product from primers designed to ORF6 of the

phaseolotoxin gene cluster, indicating widespread existence of *Tox⁻* strains of *Psp* in the field, contradicting the earlier view that *Tox⁻* strains have little or no epidemiological significance (San José et al. 2010).

Pseudomonas syringae pv. *tomato* (*Pst*), causal agent of tomato bacterial speck disease, induces necrotic lesions on the leaves, stem, and fruits. Infection of fruits may lead to infestation of tomato seed in which *Pst* may survive, especially inside cavities present in the seed surface (Devash et al. 1980). Seedlings growing from infested seeds either develop visible symptoms or remain symptomless. Asymptomatic plants, grown under high relative humidity, contain very high populations of *Pst* which are likely to become potential reservoirs of inoculum for disease outbreaks (McCarter et al. 1983). *P. syringae* pv. *tomato* is Gram-negative, aerobic, rod-shaped, motile with polar flagella, pathogenic to tomato and *Brassica*, and induces hypersensitive response (HR) on nonhost tobacco. It can survive as a saprophyte in plant debris, soil, and on leaf surfaces as epiphyte. Pathogen dispersal may occur through infested seed, wind, and rain splash. Disease development is favored by leaf wetness and cool temperatures (13–25°C). *Pst* exists in the form of different strains. The strain *P. syringae* pv. *tomato* DC3000 is a model strain extensively used for investigating plant–microbe interactions due to its genetic tractability and pathogenicity on tomato, *Arabidopsis thaliana* (model plant) and *Brassica* spp. (Preston 2000).

Lesions on leaves induced by *Pseudomonas syringae* pv. *tomato* (*Pst*) are surrounded by yellow chlorotic haloes which are associated with the toxic activity of the phytotoxin coronatine (COR) and its derivatives. A phaseolotoxin-like substance has also been detected in some strains of *Pst* (Bagdaache et al. 1990; Bender et al. 1999). The COR molecule has two components, namely coronafacic acid (CMA) and a polyketide and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from alloseleucine (Parry et al. 1994; Couch et al. 2004). During pathogenesis, COR has been shown to play important roles. COR was involved in suppression of stomatal closure, growth, and persistence of *Pst* in the foliage to tomato (Uppalapatti et al. 2008). The coronatine biosynthetic gene clusters of many strains of *Pst* were plasmid-borne. On the other hand, the coronatine gene clusters of *Pst* DC3000 strain were chromosomal in origin (Moore et al. 1989; Cuppels and Ainsworth 1995).

The primary pathogenicity and virulence factors of *Pst* have been demonstrated to be dependent on type III secretion system and toxin biosynthesis. Epiphytic growth is a crucial phase in the life cycle of *Pst* and efficient strains possess a range of traits, including chemotaxis, attachment, microcolony formation, nutrient acquisition, antibiosis, and resistance to UV inactivation (Preston et al. 2000). In response to infection of tomato by *Pseudomonas syringae* pv. *tomato* DC3000, a neutral leucine aminopeptidase (LAP-N) and an acidic LAP (LAP-A) are expressed constitutively during floral development. To investigate the mechanism underlying the strong induction of LAP, tomato plants were inoculated with COR-producing and deficient *Pst* strains and LAP-A expression was monitored. LAP-A RNA and activity were detected only with COR-producing *Pst* strains. COR treatment of excised shoots induced increases in RNAs for jasmonic acid (JA-) regulated PR-proteins gene PR-1. The results indicated that COR mimicked the wound response (Pautot et al. 2001).

Many of the type III secretion system (TTSS) effectors appear to have been acquired by horizontal gene transfer among bacterial strains. In various bacterial strains, over 150 effector genes have been collectively identified. In the *Pseudomonas syringae* pv. *tomato* DC3000 strain, the presence of more than 40 TTSS substrates has been

demonstrated. The effectors injected into host cells have a crucial role in promoting virulence of bacterial pathogens. The effectors delivered by *Pst* (>30) showed diverse enzymatic activities, such as cysteine protease (López-Solanilla et al. 2004). An in situ immunogold labeling technique was applied to localize AvrPto and HrpW proteins in *Pst* cells. The majority of these proteins were found to be localized specifically along the Hrp pili, but not along the flagella or randomly distributed in the intercellular spaces (Jin et al. 2001). Another class of *hrp* genes encoded secreted proteins, some of which were shown to be extracellular components of TTSS as in the case of Hrp. In *P. syringae*, *hrp/hrc* genes encode effector proteins. A tripartite mosaic Hrp pathogenicity island (PAI) is formed by the *hrp/hrc* genes. *P. syringae* pv. *syringae* 61 and *P. syringae* pv. *tomato* DC3000 are flanked by exchangeable effector locus and conserved factor locus. The conserved locus was found to be essential for pathogenicity, while the exchangeable effector locus had only a minor role in the growth of bacteria in tomato (Collmer et al. 2000). Electron microscopy and immunocytochemistry techniques were employed to visualize the formation of pilus in vitro from *Pst* DC3000 strain. Following labeling with specific antibodies, HrpA pili developed rapidly in a nonpolar manner soon after the detection of the *hrpA* transcript and extended up to 5 µm into the surrounding media. The basal bodies of flagella could be clearly differentiated from the structures at the base of pilus. Another protein HrpX, also secreted via TTSS, was revealed by the immunogold labels associated with the pilus in vitro (Brown et al. 2001).

Mutants of *Pseudomonas syringae* pv. *tomato* DC3000, defective in *hrcC* which encoded an outer membrane protein required for type III-mediated secretion, produced the phytotoxin coronatine (COR) in vitro. The *hrcC* mutants could produce COR in planta, which induced chlorotic lesions that did not turn necrotic as the lesions induced by the wild-type strain. The results suggested lack of *hrp/hrc* secretion system might not affect COR production, but mutations in this system might have regulatory effects on the formation of virulence factors such as COR (Bender 2000). Cornofacic acid (CFA) and coronamic acid (CMA) that are distinct components of COR are intermediate molecules in the COR biosynthetic pathway. The role of COR (encoding a response regulator) in *Pst* DC3000 is not clear. In another study, a COR mutant of *Pst* DC3000, defective in the biosynthesis of COR, was investigated to assess the effects of the components of COR. The CFA and CMA induced less severe symptoms on tomato compared with wild-type strain. In addition, a mutation in *hrpL*, which encoded an alternate RNA polymerase sigma factor needed for the expression of genes encoding components of TTSS, inhibited production of COR in *Pst* DC3000. Furthermore, the COR mutant of *Pst* DC3000 had reduced and delayed expression of *hrpL* and was impaired in its ability to elicit HR reaction in *Nicotiana benthamiana*. The results indicated that *corR* impacted directly the expression of the *hrp* regulon in *P. syringae* (Sreedharan et al. 2006).

Pseudomonas fuscovaginae is seedborne in rice and its endophytic development was investigated by using a rifampicin-marked strain of the seedborne strain of *P. fuscovaginae* (*Pf^R*). Rice seeds were grouped into five categories based on the percentages of seed discoloration (0, ≤25, ≤50, ≤75, and ≤100). The relative seedborne bacterial concentration increased significantly ($P < 0.001$) with increasing levels of seed lot infection/discoloration (Fig. 6.2). Seeds with high levels of surface discoloration were either empty or, if filled, failed to germinate. The rifampicin-marked strain *Pf^R* could be recovered from all growth stages of rice plant, with the highest number of recovered cells being at the seedling stage. The *Pf^R* strain was present in most tested seeds indicating that, even

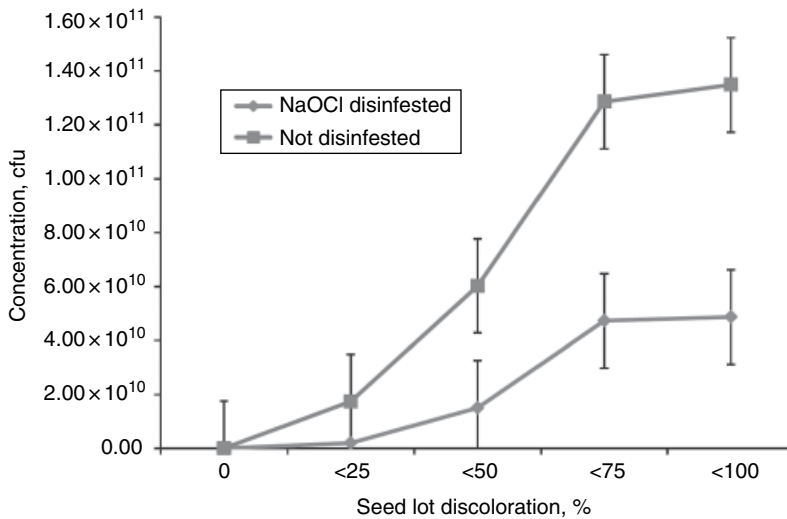


Figure 6.2 Relative populations of *Pseudomonas fuscovaginae* isolated from rice seeds with or without treatment with sodium hypochlorite (NaOCl). Populations of *P. fuscovaginae* expressed as CFU/100 seeds with different levels of seed discoloration in cultivar Amaroo. Source: Adorada et al. (2015). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

without recognizable discoloration, seed transmission of the pathogen might occur. Crushed discolored seeds carried a greater number of pathogen cells than the noncrushed discolored seeds. The recovery of the rifampicin-resistant strain, inoculated onto rice seeds from different rice seeds at different growth stages, indicated the endophytic colonization of *P. fuscovaginae*. Low levels of pathogen cells were detected in visibly clean seed, indicating that even clean or healthy-looking seeds might not represent a disease-free seed lot. It is therefore desirable to disinfect the rice seeds with sodium hypochlorite or an appropriate chemical to improve the sanitary status of the rice seed lots (Adorada et al. 2015).

6.2.3 *Burkholderia* spp.

Burkholderia (*Pseudomonas*) *glumae* causes rice seedling and panicle blight and grain rot disease. Seedling rot in the nursery boxes is one of the most important diseases in Japan. The pathogen is seedborne and grows epiphytically on the plant through the booting stage. Multiplication of the pathogen is rapid on the surface of emerging panicles and infection of flowers occurs just after emergence (Goto and Ohata 1956). Both *B. glumae* and *B. gladioli* produced similar symptoms on rice. However, strains of *B. glumae* were generally more aggressive and induced more severe symptoms compared with that of *B. gladioli*. Some of the strains of *B. glumae* were found to be avirulent on both seedlings and panicles. Avirulent strains did not produce the yellow-pigmented toxin, toxoflavin, in the medium, whereas virulent strains produced toxoflavin in culture. Growth responses of these pathogens to temperatures were similar, the optima ranging over 38–40°C for *B. glumae* and 35–37°C for *B. gladioli* (Nandakumar et al. 2009). *B. gladioli*, causing bacterial grain rot and leaf sheath browning, was consistently isolated from infected leaf sheaths and grains and was pathogenic to rice plants inducing the disease symptoms. It could infect orchidaceous (oncidium, cymbidium, dendrobium) gladiolus

plants and onion bulbs. The isolates from infected plants were identified as *B. gladioli* which could cause grain rot of rice, in addition to *B. glumae* (Ura et al. 2006).

Bacterial isolates (428) obtained from crushed seed of 11 farmers' rice seed samples were distinguished by BOX-PCR fingerprinting of genomic DNA. *Burkholderia glumae* and *B. gladioli* isolated from those seed extracts were pathogenic to rice plants, the former being more virulent. Infection was rapid and characterized by the induction of necrotic lesions on leaf sheath; the gradual wilting of leaves was seen later. Infection by *B. glumae* caused the ultimate death of entire plant and it is recognized as the causative agent of seed rot and seedling rot (Cottyn et al. 2001). The association of *B. glumae* and *B. gladioli* with rice bacterial panicle blight (BPB) disease occurring in Louisiana and southern USA was established by isolation, pathogenicity tests, biochemical and physiological methods, and real-time PCR assay. *Burkholderia plantarii* was detected in paddy water. Graminaceous weeds infected by *B. plantarii*, when immersed in water, could release the pathogen which survived for at least 4 days at 30°C. *B. plantarii* might be dispersed by rain splash at least 30 cm upward under field conditions. Harvested rice seed sprayed with *B. plantarii* released from graminaceous weeds at the flowering stage retained the pathogen. Seedlings growing from these infested seeds, sown in the nursery boxes, developed bacterial seedling blight symptoms, indicating the potential of infected weed species as sources of inoculum for the bacterial seedling blight disease and possibility of rice seeds receiving inoculum through paddy water (Miyagawa and Inoue 2002).

Burkholderia glumae produces the toxin toxoflavin composed of two proteins (TRP-1 and TRP-2). The transposon mutants (*Tox*⁻) were found to be nontoxigenic and non-pathogenic. They were not able to produce yellowish pigments in the same way as the wild-type strain, indicating that toxoflavin production is required for pathogenicity of *B. glumae* (Suzuki et al. 1998; Yoneyama et al. 1998). Of the two toxoflavin biosynthesis-related proteins, TRP-1 possessed characteristics of a methyltransferase and was encoded by *toxA* gene of *B. glumae*. A chromosomal DNA region (8.2 kb) of the pathogen contained the *tox* operons (*toxABCDE*), and an upstream regulatory gene (*toxR*) involved in toxoflavin biosynthesis was cloned and sequenced. The sequence downstream of *toxA* contained four open reading frames (ORFs) – *toxB*, *toxC*, *toxD*, and *toxE* – which encode polypeptides with molecular masses of 23.3, 61.6, 34.9, and 38.3 kDa, respectively. All the polypeptides had the same transcriptional orientations of *toxA*. The disruption of *tox* operons resulted in the generation of mutants which had the ability to produce toxoflavin, but were unable to induce typical chlorosis of infected rice panicles. The information encoded by *tox* operons might be polycistronic for all five genes, as indicated by RT-PCR assays. The *toxR* gene product (37.5 kDa) might be a member of LysR family of regulatory molecules and an activator that allowed transcription of the *tox* operons. ToxB and ToxE proteins exhibited similarity to the GTP cyclohydrolase II and the deaminase, respectively, involved in riboflavin synthesis in other organisms. The results suggested that biosynthesis of toxoflavin might follow a similar biosynthetic pathway as riboflavin, at least in part (Suzuki et al. 2004).

Determination of the gene sequences controlling toxin production and resistance to toxin would be useful to develop probes that could be employed for disease diagnosis and differentiation of pathovars of bacterial pathogens. Toxoflavin functions as a major virulence factor. The expression of genes involved in the biosynthesis and transport of toxoflavin was shown to be regulated by N-octanoyl homoserine lactone (C8-HSL) (Kim et al. 2004). In a further investigation, the mechanism of flagellum biosynthesis

under the control of quorum sensing (QS) in *Burkholderia glumae* was investigated. A major deviation from the current concept for the regulation of bacterial polar flagellum was revealed in *B. glumae*. The C8-HSL-deficient mutant of *B. glumae* was aflagellate (lacking flagellum), and was therefore unable to swim and swarm at 37°C as for the wild-type strain. A functional role of FlhDC as a regulator of the polarly flagellated *B. glumae* was examined. The QSmR, an IcIR-type transcriptional regulator, was identified in the hierarchical gene regulation of *B. glumae*. Since QSmR regulates *flhDC* expression, it represents a novel flagellar regulon controlled by QS. The nonmotile *qsmR*, *flhA*, and *flhDC* mutants produced toxoflavin, but these mutants were nonpathogenic. Variations in the typical regulatory mechanisms were detected in *B. glumae* which contained FlhDC, generally present in peritrichous flagellum systems. This investigation revealed that the functional flagella of *B. glumae* have a critical role in pathogenicity, suggesting that either QS or flagellum formation could constitute a good target for the control of rice grain rot (Kim et al. 2007).

The roles of shikimate pathway genes *aroA* and *aroB* in the virulence, growth, and UV-tolerance of *Burkholderia glumae* were studied. Some strains of *B. glumae* exhibited phenotypes producing at least two (yellow-green and purple) pigment compounds in casein-peptone-glucose agar medium. The strains LSUPB114 and LSUPB116 were pigment-deficient mutant derivatives of the virulent and pigment-proficient strain 411gr-6 with min-*Tn5gus* insertions in *aroA* encoding 3-phosphoshikimate-1-carboxyvinyltransferase and *aroB* encoding 3-dehydroquinate synthase respectively. These enzymes are involved in the shikimate pathway which results in the synthesis of aromatic amino acids. The results showed that *aroA* and *aroB* were required for normal virulence in rice and onion, growth in M9 minimal medium, and tolerance to UV-light. They were not found to be essential for the production of the phytotoxin toxoflavin by *B. glumae*. The shikimate pathway might be involved in the pathogenesis by *B. glumae* without a significant role in the production of toxoflavin, a major virulence factor of *B. glumae* (Karki and Ham 2014).

6.2.4 *Acidovorax* spp.

Acidovorax avenae subsp. *citrulli* (= *Pseudomonas pseudocaligenes* subsp. *citrulli*, *P. avenae* subsp. *citrulli*) infects watermelon, melon, and other cucurbits causing bacterial fruit blotch (BFB) disease. Marketable fruit yield losses of up to 90% have been reported. The BFB-associated lesions on fruits have a greasy appearance, while mature watermelon leaves show distinct dark to reddish-brown lesions along the veins. Fruits at advanced stages of disease collapse into watery rot due to invasions by secondary colonizing organisms. *Acidovorax avenae* subsp. *citrulli* (*Aac*) is a Gram-negative, rod-shaped bacterium, measuring 0.5 × 1.7 µm and possesses a simple polar flagellum. The pathogen is strictly aerobic, positive for oxidase activity, and develops well at 41°C. The bacterial colonies are smooth, round, cream-colored, and nonfluorescent. The primary inoculum of *A. avenae* subsp. *citrulli* is the infected seed. The female watermelon blossom colonization could result in seed infestation by *Aac*, even though BFB symptoms were not induced in the fruits.

The role of watermelon infection (inoculation) in infestation of seeds by *Acidovorax avenae* subsp. *citrulli* (*Aac*) was studied. Inoculation of watermelon blossoms with 1 × 10⁷ or 1 × 10⁹ CFU concentration of *Aac*/blossom resulted in fruits infestation (98%), but the fruits remained asymptomatic. Immunomagnetic separation (IMS-) PCR assay

detected *Aac* in 44% of the seed lots assayed. *Aac* colonies were recovered from 70% of IMS-PCR-positive seed lots, and 62% of them gave rise to seedlings with bacterial fruit blotch symptoms. The green fluorescent protein (GFP-) expressing mutant AAC8-IST induced lesions on watermelon seedlings and, when inoculated onto watermelon seed by vacuum infiltration, led to BFB seedling transmission. When watermelon blossoms were inoculated with GFP mutant, 44% of the seed lots were IMS-PCR-positive. Green fluorescent colonies were recovered with AAC8-IST mutant strain. The internal tissues (pulp) of fruit from blossoms inoculated with *Aac* contained the pathogen, as revealed by IMS-PCR assays. Blossom inoculation with AAC8-IST mutant resulted in higher level of fruit and seed infestation than the wild-type strain. The results indicated that female watermelon blossom might have a role as sites of ingress for seed infestation by *Aac*, even though the fruits might be asymptomatic (Walcott et al. 2003). Seeds from pericarp-inoculated fruits showed fruit rind penetration by *Aac*. These fruits had superficial contamination of the testae and perisperm-endosperm layers, while seed from stigma-inoculated fruits with pistil penetration led to infection of seed embryos (Dutta et al. 2012a).

The duration of survival of *Acidovorax avenae* subsp. *citrulli* (*Aac*) in citron melon (*Citrullus lanatus* var *citroides*) seeds was determined. Citron melon seed lots infected with *Aac* were produced by inoculating either pistils (stigma) or pericarps (ovary walls) of female blossoms in the field. A total of 17 *Aac* isolates from 14 different haplotypes belonging to two different groups (I and II) were used for inoculation. Infection of all seeds (100%) was confirmed and the seeds were stored at 4°C and 50% RH for 7 years. The viability of *Aac* from individual seed lots was determined by isolation from macerated seeds on semiselective medium, as well as growing seeds for 14 days and scoring for bacterial fruit blotch (BFB) symptoms. The survival period of bacterial isolates of group I and II was similar. However, *Aac* survival was significantly greater in seed lots generated via pistil inoculation (52.9 and 29.4%) than via pericarp inoculation (23.5 and 17.6%). Repetitive extragenic palindrome (REP-) PCR assay on *Aac* isolated from citron melon seed lots after storage displayed similar fingerprinting patterns to those of the reference strains originally used for blossom inoculation, indicating the absence of cross-contamination. The results indicated that *A. avenae* subsp. *citrulli* might survive or overwinter in citron melon seeds for at least 7 years and the infection site (pistil/pericarp) could significantly influence the BFB pathogen survival in citron melon seed (Dutta et al. 2014b).

Infested seeds form the primary sources of inoculum of cucurbit bacterial fruit blotch (BFB) disease. The influence of seed inoculum load on BFB seedling transmission was investigated under greenhouse conditions. Watermelon seed lots (128 seeds/lot) containing one seed inoculated with *Acidovorax avenae* subsp. *citrulli* at levels ranging from 1×10^1 to 1×10^7 CFU were used to study the effect of seed inoculum load on seedling transmission and spatiotemporal spread of the BFB disease. The proportion of seed lots that yielded BFB-infected seedlings was lower for lots with one seed infested with 1×10^3 (46.6%) and 1×10^1 (16.7%) CFU of *A. avenae* subsp. *citrulli*. The relationship between *A. citrulli* seed inoculum level and frequency of pathogen detection in seed lots using immunomagnetic separation combined with a real-time PCR assay also followed a sigmoidal pattern ($R^2 = 0.997$, $P = 0.0034$). All samples (100%) from seed lots (10,000 seeds/lot) with one seed containing $>1 \times 10^5$ CFU tested positive for *A. citrulli*. On the other hand, 75% of samples from lots with one seed containing 1×10^3 CFU tested positive for the pathogen and only 16.7% of samples with one seed containing

10 CFU tested positive. Based on the results, it was recommended that zero tolerance for seedborne *A. avenae* subsp. *citrulli* should be adopted for effective BFB disease management in watermelon and other cucurbit crops (Dutta et al. 2012b).

The ability of *Acidovorax avenae* subsp. *citrulli* (*Aac*) to colonize female watermelon blossoms and the relationship between blossom inoculum dosage and seed infestation were investigated. *Aac* colonized stigmas and styles of female blossom, reaching populations of c. 10^7 – 10^8 CFU/blossom at 96 h postinoculation. The population increase of *Aac* was reduced by pollination and the growth of the pathogen in the style was significantly less than in stigma of blossoms with c. 1×10^3 *Aac* CFU/blossom resulting in 36–55% infestation of seed lots within symptomless fruits. About 14% of seedlings growing from the infested seed lots developed bacterial fruit blotch (BFB) symptoms. A strong positive relationship between *Aac* inoculum levels applied to blossoms and the percentage of infested seed lots was discernible, as revealed by the seedling grow-out assay ($R^2 = 0.34$). Furthermore, a strong positive linear correlation between *Aac* blossom inoculum dose and mean percentage of BFB-infected seedlings ($R^2 = 0.99$) was evident, as noted in seedling grow-out assays. The results suggested that blossom colonization by *Aac* might be a potential risk, resulting in enhanced seed infestation also under field conditions (Lessl et al. 2007).

In a later investigation, the pathways of ingress of *Acidovorax citrulli* into watermelon seeds were investigated. When *A. citrulli* inoculum (1×10^6 CFU) was applied to stigmas versus ovary pericarps of female flowers, a significantly higher percentage of watermelon seeds was infected up to 7 days postinoculation (dpi). Observations under immunofluorescence microscope revealed that with stigma inoculation, *A. citrulli* colonized style and ovary tissues by 1 dpi and the pathogen colocalized with pollen germ tubes in these tissues. *A. citrulli* cells penetrated the epicarp and mesocarp tissues by 1 dpi, but did not reach endocarp until 4 dpi when inoculated on pericarp. Manual pollination followed by stigma inoculation resulted in >53% infection of seeds by *A. citrulli*. On the other hand, the presence of *A. citrulli* could not be detected in seeds/ovules generated by stigma inoculation without pollination (chemically induced parthenocarp). Colonization of watermelon ovules by *A. citrulli* occurred at a faster pace following stigma inoculation than pericarp inoculation. However, no difference in the levels of infection in mature seeds was evident, due to types of floral tissue inoculated with *A. citrulli*. The results indicated that pollen germ tubes might have a role in the ingress of *A. citrulli* into watermelon seeds via stigmas (Dutta et al. 2015).

The epiphytic populations of *Aac* may initiate BFB outbreaks under field conditions. Under favorable environmental conditions, the risk of BFB disease incidence may be high even if the seed lot contamination with *Aac* is at a low level. *A. avenae* ssp. *citrulli* could overwinter in decaying infested fruit rind tissues or infested seeds that may develop into volunteer plants in subsequent crops (Walcott 2005). Bacterial fruit blotch (BFB) disease, caused by *Acidovorax avenae* subsp. *citrulli* (*Aac*), is considered as one of the most serious disease in the USA because the cucurbit seed producers may incur up to 100% yield loss. There is a zero-tolerance limit applied by importing countries for cucurbit seed lots; all seeds have to be tested and certified to be free of *Aac* (Ha et al. 2009).

Transplant house conditions, including high temperatures, high relative humidity, and high plant populations, favor rapid development of BFB disease caused by *Acidovorax citrulli*. Bacterial populations present in the lesions on the leaves are dispersed by overhead irrigation to seedlings under transplant house conditions. *A. citrulli*

was found to be dispersed by overhead irrigation from infected seedlings to 95% of the neighboring healthy seedlings, with 80% of them displaying enhanced levels of disease severity. In contrast, when subirrigation by floating was adopted, the neighbors of the infected plants did not exhibit disease symptoms and were not colonized by the pathogen. Assessment of *A. citrulli* populations in different parts of seedlings showed that cotyledons were the most colonized part of the plant. Images of fluorescent binocular and confocal laser-scanning microscopy of seedlings infected with *A. citrulli* strain labeled with green fluorescent protein (GFP) showed that the pathogen formed abundant aggregates on the surfaces of cotyledons followed by extensive colonization of intercellular spaces of parenchymatous tissues. The pathogen then moved through vascular system of hypocotyls, resulting in infection of emerging leaves. The results indicated that prevention of secondary spread of *A. citrulli* in melon nurseries might be achieved by adopting subirrigation and avoiding overhead irrigation system (Chalupowicz et al. 2015).

Different cultivars of watermelon, muskmelon, honeydew melon, acorn squash, butternut squash, yellow squash, zucchini squash, cucumber, and pumpkin were evaluated in 1997 and 1999 for foliar and fruit susceptibility to bacterial fruit blotch (BFB) disease and for seed transmission of *Acidovorax avenae* subsp. *citrulli* (*Aac*) under field conditions. The pathogen was introduced into the field on infected watermelon transplants or by misting a suspension of *Aac* onto fruits of cucurbits. Watermelon, muskmelon, and honeydew melon plants showed more extensive foliar and fruit symptoms than other cucurbits tested, indicating their high level of susceptibility to the disease. However, seed transmission of *Aac* could be detected in all cucurbits in one or other year of testing. All cucurbits have to be considered as a potential source of introduction of *Aac* into transplant house or field (Hopkins and Thompson 2002). An efficient method of inoculating melon (*Cucumis* spp.) genotypes – seed vacuum-infusion assay – was developed to assess their levels of susceptibility/resistance to BFB disease. Plant introductions (PIs) of watermelon were evaluated for their resistance to BFB disease. Significant variability in the reactions of PIs was observed, facilitating the selection of entries with desirable level of resistance to the BFB disease (Wechter et al. 2011).

The behaviors of many bacterial species are regulated in a cell-density-dependent manner of quorum sensing (QS). *N*-acylhomoserine lactones (AHLs) function as a mediation of cell-density-dependent communication among Gram-negative bacteria. The bacteria-to-bacteria communication is associated with quorum-sensing process, based on the production of AHLs. The role of AHL-type quorum sensing (QS) system in growth, swimming motility, and virulence in *Acidovorax avenae* subsp. *citrulli* strain XJL12 was investigated. The AHL synthase gene of QS system from strain XJL12, designated *aac1*, was cloned and characterized and an *aac1* disruption mutant was generated. The *aac1* mutant XJL13 lacked the ability to produce AHL molecules, whereas the complemented corresponding strain CPXJL13 produced wild-type levels of AHL. In addition, *aac1* mutant had decreased growth rate relative to the wild-type in minimal medium and was partially impaired in swimming motility. Pathogenicity assessment showed that the *aac1* mutant possessed a lower level of virulence on watermelon fruits and melon seedlings when compared with wild-type strain. However, the *aac1* mutation in strain XJL12 did not affect the biofilm formation, exopolysaccharide production, or induction of hypersensitive response in tobacco (nonhost). The results suggested that the AHL-type QS might play a role in the virulence of the pathogen causing bacterial fruit blotch (BFB) disease in cucurbits (Fan et al. 2011).

The effect of mutation in specific gene(s) on the growth and virulence of *Acidovorax avenae* subsp. *citrulli* (Aac) (= *Acidovorax citrulli*) was investigated. A mini-Tn5 mutant MJ22-3 showed reduced virulence on melon seedlings. The gene disrupted by Tn5 was identified as *leuB* which encoded a putative 3-isopropylmalate dehydrogenase, an enzyme that appeared to be essential for leucine biosynthesis. The *leuB* gene in-frame deletion mutant $\Delta leuB$ as well as the Tn5-inserted MJ22-3 were unable to grow in minimal medium without leucine and exhibited reduced swimming motility. However, the mutations did not affect the biofilm formation or induction of HR on tobacco by the mutants. When inoculated at a low population (10^4 CFU mL⁻¹), the *leuB* gene mutants showed a significantly slower growth rate and attenuated virulence on melon cotyledons as compared to the wild-type strain MH21. However, inoculations with mutants at high population (10^8 CFU mL⁻¹) or low concentration (10^4 CFU mL⁻¹) inoculation supplemented with 0.01% leucine led to growth rates and disease severity at levels similar to that of the wild-type strain. The results suggested the requirement of leucine for both in vitro growth and full virulence of *A. avenae* subsp. *citrulli* on melon seedlings (Ren et al. 2014).

Secreted effector proteins have a vital role in the interaction between bacterial pathogens and their host plants. Clear understanding of the melon-*Acidovorax citrulli* pathosystem is essential to limit seed-to-seedling transmission of the pathogen for effective management of bacterial fruit blight (BFB) disease of cucurbits. The type IV pili and flagella have an important role in virulence, twitching motility, and biofilm formation (Bahar et al. 2009). The type III secretion system (T3SS) and especially the *hrcC* gene encoding the type III pilus protein were found to be necessary for pathogenicity, but not required for the colonization of germinating seeds during early stages of seed-to-seedling transmission of *A. citrulli* (Johnson et al. 2009).

The type VI protein secretion system (T6SS) has been shown to be essential for the virulence of many Gram-negative bacterial pathogens. A T6SS gene cluster approximately 25 kb in length and comprising 17 genes was identified in *A. citrulli* AAC00-1 genome. Seventeen core T6SS genes were deleted individually to generate mutants from wild-type strains. Of these, four mutants – $\Delta vasD$, $\Delta impK$, $\Delta impj$, and $\Delta impF$ – were impaired in seed-to-seedling transmission of BFB in melon and in biofilm formation. Another *A. citrulli* T6SS protein ImpJ was identified as a trimeric cytoplasmic protein that interacts with components of phage-like and membrane-anchoring complexes of the T6SS. The VCA109-like ImpF was required for biofilm formation by *A. citrulli* on glass surfaces and for seed-to-seedling transmission. *A. citrulli* grew as an epiphytic nonpathogen on melon seeds during the initial stages of seed germination. Based on this finding, the role of T6SS in the putative switch to pathogenic growth on emerging melon seedlings was investigated. BFB seed-to-seedling transmission and biofilm formation were reduced for the mutants $\Delta vasD$, $\Delta impK$, $\Delta impj$, and $\Delta impF$, indicating the indispensable nature of biofilm formation for the colonization of melon seed tissues during the early stage of seed germination. The results provided evidence in support of the existence of a functional T6SS needed for biofilm formation and seed-to-seedling transmission of *A. citrulli* in lemon (Tian et al. 2015).

6.2.5 *Clavibacter* spp.

Clavibacter michiganensis subsp. *michiganensis* (Cmm) may survive in tomato seeds, plant debris, and soil. Survival of Cmm in tomato stems in soil under widely different

field conditions was determined quantitatively employing a laboratory assay, using wild-type strains of the pathogen and semiselective SCM agar medium. *Cmm* survived for significantly longer periods in tomato stems left on the soil surface than in stems buried in the soil conditions existing in California, Ohio, and Morocco. *Cmm* was recovered in high concentrations from tomato stems on soil surface for 314 days in Ohio and California and for 132–194 days in two locations in Morocco. The half-life of *Cmm* in stems on soil surface was 23–25 days in the USA and 8–12 days in Morocco. The half-life of *Cmm* was reduced significantly in stems buried in the soil. The predicted survival times were 542 and 122 days in the USA and Morocco, respectively. The use of SCM agar and wild-type strains of *Cmm* allowed the pathogen to be tracked in infected stems over time both qualitatively and quantitatively. The results indicated that the survival and half-life of *Cmm* in plant debris might vary depending on tissue exposure and geographic locations with different environmental conditions (Fatmi and Schaad 2002).

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), a quarantine pathogen causing tomato bacterial canker disease, infects tomato fruits and subsequently infests (externally) and/or infects tomato seeds (internally), where it is able to survive for long periods. Seed infection may allow long-distance dissemination and its introduction into disease-free areas through infected seeds (Kaneshiro and Alvarez 2003). The pathogen might become quiescent or enter the viable or nonculturable state (VBNC) induced by dry conditions, lack of required nutrients, or chemical treatments as in the case of many other bacterial species (Ordax et al. 2006). Estimates of populations of *C. michiganensis* subsp. *michiganensis* on naturally infected seed differed considerably, possibly due to different conditions to which the tomato plants were exposed. A population level between 10^2 and 10^4 CFU/seed were reported on naturally infected tomato seeds. When seed lots containing 58 to 1000 CFU g^{-1} seed were sown, the incidence of bacterial canker in tomato seedlings was 0.05–4%, indicating a high positive correlation between CFU g^{-1} seed and disease incidence (Fig. 6.3) (Hadas et al. 2005).

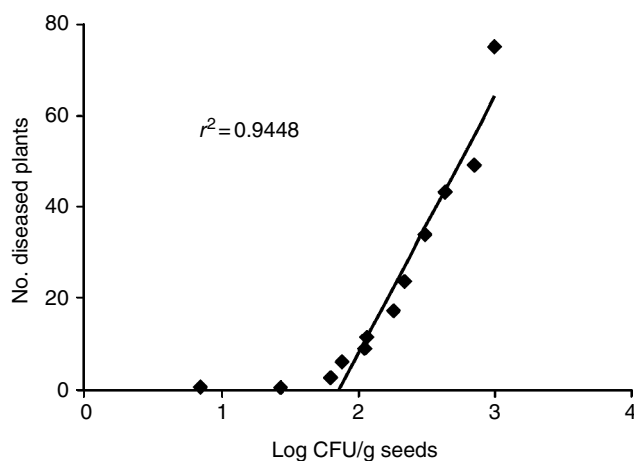


Figure 6.3 Correlation between seed contamination levels and severity ratings of tomato bacterial canker disease. Contamination levels of tomato seeds by *Clavibacter michiganensis* subsp. *michiganensis* measured as CFU g^{-1} of seed and number of diseased seedlings infected per 200 seeds show high positive relationship ($r^2 = 0.9448$). Source: Hadas et al. (2005). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

Infected or contaminated seed and transplants have been shown to be of prime importance for disease transmission, since as few as 0.01–0.05% contaminated seed or transplants could cause an epidemic under favorable conditions (Chang et al. 1991). A sensitive method of indexing of tomato seed lots for *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is therefore a crucial factor in effective management of bacterial canker disease. The detection assay most often employed is based on dilution plating method. A new selective medium recommended by the European and Mediterranean Plant Protection Organization (EPPO) and International Seed Health Initiative (ISHI) was not effective in detecting *Cmm* in asymptomatic plants or seeds that contained low populations of the pathogen. The new bacterial canker of tomato (BCT) medium was found to be more efficient in recovering low populations of *Cmm*; its mean plating efficiency amounted to $\leq 89\%$ within 7 days for all 30 strains of *Cmm* from different sources tested, and other bacteria associated with tomato seed were effectively inhibited to an extent of 98–100%. The new medium could be used for detection of *Cmm* in tomato seed and in symptomless tomato plantlets (Ftayeh et al. 2011).

The spread of tomato bacterial canker disease in greenhouses was studied. Regardless of the year of isolation, location, or tomato cultivar, the strains of *C. michiganensis* subsp. *michiganensis* (*Cmm*) in each greenhouse and location belonged to the same haplotype, suggesting that strains of *Cmm* originated from the previous greenhouse population. Disbudding and defoliation using either scissors or by hand were carried out in the same direction to promote the spread of *Cmm* in greenhouse. The distribution of diseased plants indicated a random distribution in a quadrant along a furrow of plants (two adjoining rows of plants). The results indicated that disbudding and defoliation contributed highly to the secondary spread of bacterial canker in commercial greenhouses. The primary inoculum in the greenhouse-grown tomato plants in Okayama, Japan appeared to originate each year from residual plant debris in the soil, rather than new introduction from infected seeds (Kawaguchi et al. 2010).

In addition to seed transmission, *C. michiganensis* subsp. *michiganensis* (*Cmm*) may be spread by clipping tomato seedlings in transplant beds, sprinkler or fog irrigation practices, and also from latently infected tomato transplants. Furthermore, grafting in tomato nursery production, requiring wounding of both rootstock and scion, provides a rapid way of spreading the pathogen from plant to plant. Systemically infected tomato plants, although fewer in number, may have high leaf epiphytic populations of *Cmm* which could serve as inocula for secondary infections. Several solanaceous and nonsolanaceous plant species have been reported to be susceptible under artificial inoculated conditions. These plant species, if infected under natural conditions, may provide additional inoculum to tomato crops. *Cmm* could persist for up to 1 year in tomato plant debris, which could also serve as a source of inoculum (Gleason et al. 1991; de León et al. 2011). The spread of *Cmm* through contaminated tools occurred only to a limited distance (<4 plants) under greenhouse conditions. In contrast, touching symptomless tomato plants bearing guttation droplets prior to touching nearby plants resulted in the spread of *Cmm* over long distances within rows (>22 plants). A large population of *Cmm* was exuded in the guttation fluid and was not influenced by the inoculation procedure applied, leaf age, or the volume of guttation droplets. Population size of *Cmm* and the incidence of leaflets with epiphytic bacteria were significantly higher in plants placed in a guttation-induction chamber with high humidity. The results suggested that exudation of *Cmm* through guttation droplets might favor the build up of large epiphytic population on leaflets, providing conditions for epidemic breakouts (Sharabani et al. 2013a).

The genome of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) represents a single circular chromosome of 3.3 Mbp with high G + C content (72%), carrying a pathogenicity island (PAI) of 129 kb with a lower G + C content (65.5%), designated *chp/tomA* region. Two subregions of PAI, namely the *chp* region, with several genes encoding proteins involved in sugar uptake and metabolism, and *tomA*, encoding a tomatinase, have been identified. A *chp/tomA* PAI deletion mutant Cmm27 did not induce disease symptoms and was able to colonize tomato xylem and reach a lower markedly concentration as compared with the wild-type strain (Kaupo et al. 2005; Gartemann et al. 2008; Chalupowicz et al. 2010). The wild-type strain Cmm382 harbors two circular conjugative plasmids, pCM1 (27.4 kb) and pCM2 (70 kb), which individually carry a major virulence gene. The type strain Cmm382 secretes a wide range of cell-wall-degrading enzymes, namely polygalacturonase, pectate lyase, xylanases, and endoglucanases, and a large group of serine proteases of different families. The serine proteases were induced in planta and might be involved in infection by *C. michiganensis* subsp. *michiganensis* (Chalupowicz et al. 2012).

The infection process and development of disease as population dynamics of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) were studied using axenically grown tomato plants. A spontaneous rifampicin-resistant strain of *Cmm* was tagged with a marker that expressed a green fluorescent protein (GFP) in a stable way with a similar level of virulence as the wild-type strain of *Cmm*. In vitro plants were drop-inoculated at the stem base. Population dynamics were assessed by dilution-pour-plating in a selective medium. At 3 h after inoculation, *Cmm* could be detected in low titers in roots, stems, and leaves. At 16 dpi, *Cmm* had spread to plant parts and high densities of pathogen population (c. 10^{10} CFU g⁻¹) were observed in the infected plant tissues. Further development of characteristic symptoms such as canker, wilting, and general growth reduction was observed. Observations under epifluorescence stereo- and confocal laser scanning microscopy revealed the presence of *Cmm* in vascular and parenchymatic tissues of in vitro tomato plants which could be used effectively for investigations on tomato–*Cmm* interactions. GFP-tagged strain was useful for in situ visualization of the pathogen and its movement from one tissue to another within tomato plants (Lelis et al. 2014).

Genes for seven putative serine proteases (ChpA–ChapG) belonging to the trypsin subfamily and homologous to the virulence factor *pat-1* were identified on chromosome of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) NCPPB382. All proteases have signal peptides, indicating export of these proteins. The genes encoding serine proteases are clustered in a chromosomal region of about 50 kb with a significantly lower G + C content than common for *Cmm*. The genes *chpA*, *chpB*, and *chpD* were found to be pseudogenes, as frame shifts and/or in-frame stop codons were present in them. The genes *chpC* and *chpG* were inactivated by the insertion of an antibiotic resistance cassette. The *chpG* mutant retained virulence to the level as in the wild-type strain. The titer of the *chpC* mutant was substantially reduced in inoculated plants and only faint disease symptoms were produced by this mutant. However, complementation of the *chpC* mutant by the wild-type allele restored full virulence. The chromosomal gene *chpC* of *Cmm* was the gene identified for the first time in this investigation (Stork et al. 2008).

Bioluminescent mutants of *Cmm* were employed to visualize the pathogen colonization dynamics in seed transmission and translocation of *C. michiganensis* subsp. *michiganensis* in grafted plants (Xu et al. 2010a). The effect of tomato plant age at the time of inoculation with *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) on severity of bacterial

wilt and canker disease was assessed in six greenhouse experiments. The period for development of wilt and death of tomato plants, designated window of vulnerability, ranged from transplanting to 17–18-leaf stage. Yield accumulation was drastically reduced in plants inoculated within the period of window of vulnerability, compared with those inoculated after this period. The expression of virulence genes, namely *celA* encoding a secreted cellulose and serine protease-encoding *pat-1*, *chpC*, and *ppaA*, was induced during early stages after inoculation in plants within the window of vulnerability. Differences in *Cmm* populations between plants inoculated within and outwith of this period were insignificant after the first week postinoculation, indicating that differences in disease severity, yield loss, and expression of virulence determinants were not correlated with *Cmm* population levels. As the period of window of vulnerability was found to be a critical factor for disease development, precautionary measures applied during this period might have a season-long effect on plant mortality, resulting in reduction/prevention of yield losses (Sharabani et al. 2013b).

In order to investigate colonization patterns and movement of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), an enhanced green fluorescent protein (GFP) bioreporter was utilized. An EGFP reporter that could be expressed and stably maintained in *Cmm* was constructed to allow direct and precise visualization of fluorescent cells by confocal laser-scanning microscopy (CLSM). The wild-type strain Cmm382 and mutants Cmm100 and Cmm27 labeled with EGFP had similar growth rate in liquid Luria-Bertani (LB) broth at 28°C. The interaction of *Cmm* with tomato was divided into two phases: an endophytic stage and a symptom-inducing stage. CLSM observations on longitudinal section images of tomato stems inoculated with Cmm382 strain at 3 dpi showed extensive multiplication and aggregates of the pathogen cells that filled the lumen of xylem vessels. The transition of the endophytic stage into the second stage required prior extensive multiplication of the pathogen in xylem to reach a high concentration, as revealed by the presence of dense bacterial cells filling the vessel lumen. A cluster of *Cmm* cells forming a biofilm-like structure was observed in a cross-section of the xylem vessels in infected stems. Specific and firm adherence of *Cmm* to the spiral secondary-wall thickening of xylem vessels was a very striking phenomenon observed in infected tomato stems. Further, a clear preference for colonization of protoxylem as opposed to the metaxylem vessels was revealed by SEM observations. It is possible that *Cmm* might migrate through apoplast and invade the protoxylem, but not the metaxylem, during early development of the vessels. *Cmm* could colonize tomato xylem in both acropetal and basipetal directions. Colonization patterns were largely dependent on the expression of genes located on the *chp/tomA* PAI and two pathogenicity plasmids pCM1 and pCM2. The results indicated that the presence of partial serine proteases was sufficient for endophytic multiplication of *Cmm* at infection site, but not for progressive colonization of plant tissues in remote location (Chalupowicz et al. 2012).

The effect of temperature at the early stages of infection of tomato by *C. michiganensis* subsp. *michiganensis* (*Cmm*) in net-houses and greenhouses was assessed. Tomato seedlings were transplanted and inoculated monthly over a period of 2 years. The inoculated plants wilted and died earlier in the spring (21–24°C) and autumn (18–23°C) than in the winter (15–18°C) and summer (28–31°C). The time taken for 50% of inoculated plants to wilt or die (T_{50}) was 2 and 3–4 months after inoculation, respectively. A highly significant correlation was observed between the average temperatures during the first month after inoculation and T_{50} . The shortest T_{50} mortality (70 days) occurred

at an average temperature of 26°C. The expression of virulence genes *pat-1*, *celA*, *chpC*, and *ppaA* of *Cmm* was higher in plants inoculated in the spring than in those inoculated in the summer. In another set of experiments, seedlings were inoculated and maintained in a controlled environment of growth chambers for 2 weeks. Subsequently, the plants were transplanted and maintained in commercial-type greenhouses for 4–5 months. The temperatures prevailing in the first 48 h after inoculation affected the population sizes of *Cmm* and virulence gene expression and had season-long effects on bacterial canker development (Sharabani et al. 2014).

6.2.6 *Curtobacterium* spp.

Curtobacterium flaccumfaciens pv. *flaccumfaciens* (*Cff*), causing bacterial wilt disease of common bean, is seedborne. The pathogen possesses the following characteristics: Gram-positive, aerobic rods producing yellow colonies on media, strictly oxidative, oxidase-negative, and capable of hydrolyzing casein and galactose. Sucrose, erythritol, mannitol, sorbitol, and m-inositol were used by the pathogen as sole carbon sources (González et al. 2005). Yellow variant of *Cff* induces yellow discoloration on white seed coat of the great northern cultivar US1140. Based on the intensity of yellow discoloration in seed coat, four categories of seeds were distinguished: appearance healthy or white seeds (0–25%); slight infection (25–50%); moderate infection (51–75%); and severe infection (>75%). Bacterial population in the seeds, seedling emergence, plant height, and incidence and severity of bacterial wilt were significantly positively/negatively related to the degree of yellow discoloration of the seed coat. Seedlings (14 days old) growing from the discolored seeds revealed the systemic distribution of *Cff* throughout the entire seedling at the time of expression of wilt symptoms. However, some seedlings from discolored seeds remained asymptomatic, but *Cff* could be detected in such seedlings. The pathogen was not detectable in seedlings that developed from seeds without yellow discoloration. The results indicated that seedlings developing from discolored seeds, irrespective of induction of disease symptoms, might be potential sources of inoculum for the incidence and spread of common bean bacterial wilt disease caused by *C. flaccumfaciens* pv. *flaccumfaciens* (Hsieh et al. 2006).

Methods of isolation of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) from symptomatic and asymptomatic bean seeds and protocols for identification of *Cff* isolated from infected seeds by immunoassay and PCR technique have been provided by the European and Mediterranean Plant Protection Organization (EPPO 2011). Variability in seed transmission of *Cff* in dry bean (*Phaseolus vulgaris*) cultivars with differing levels of resistance to bacterial wilt disease was assessed. Plants of six dry bean cultivars were inoculated with a *Cff* isolate by stem puncture, and disease severity was scored using a rating scale. Plant-to-seed transmission of *Cff* differed, depending on the level of resistance of bean cultivars. Transmission of *Cff* from plant-to-seed did not occur in three resistant cultivars with disease severity scores ranging over 0.0–0.5, whereas three susceptible cultivars with disease severity scores ranging over 1.0–8.6 allowed plant-to-seed transmission up to 74.2%. It is possible that in cultivars with high levels of resistance, agglutination of bacterial cells in xylem vessels was prevented by a structure that apparently blocked further colonization and subsequent movement of pathogen, resulting in lower levels of disease severity. Isolates of bacteria obtained from seeds were identified as *C. flaccumfaciens* pv. *flaccumfaciens* by the Microbiolog 2TM method and PCR assay, using specific primers (Camara et al. 2009).

6.2.7 *Pantoea* spp.

Stewart's wilt disease, caused by *Pantoea stewartii* subsp. *stewartii* (*Pss*) (= *Erwinia stewartii*), induces wilting and leaf blight preceded by the production of water-soaked lesions on young leaves of sweet corn and maize. The corn flea beetle, *Chaetocnema pulicaria*, is the principal vector of *Pss*. The beetle vector was reported to be the overwintering host for *Pss* (Elliott and Poos 1940). Seedborne infection by *Pss* was demonstrated by isolating the pathogen from the endosperm and chalazal region of infected maize kernels by Ivanoff (1933). Transmission of *Pss* from seeds to plants was found to be at the rate of 10.6% in the laboratory on agar slants and 2–13% in the greenhouse (Rand and Cash 1933; Frutchey 1936).

The estimates of higher rates of seed transmission of the pathogen to maize plants by earlier investigations were re-examined, as it related to the risk of introducing *Pss* into other countries via seed produced in the USA. Seed transmission of *Pss* was evaluated by the stem-printing technique, which involved cutting and pressing a cross-section of each stem onto agar media. Prints showed *Pss* colonies after 24–48 h. The transmission rates from seed produced on the inoculated plants was 0.066% (28 of 42,206 plants) and the transmission rate from seeds produced from naturally infected plants was 0.0029% (1 of 34,924 plants). The seed transmission in naturally infected seeds was significantly lower than in seeds from artificially inoculated plants. However, the seed transmission of *P. stewartii* pv. *stewartii* in good-quality commercial corn may be remote (Block et al. 1998).

In a later investigation, the rates of transmission of *Pss* from seeds to seedlings were assessed from field grow-outs of seedlings grown from seed infected with *Pss*. Sweet corn hybrid Jubilee seedlings were inoculated repeatedly with pin-prick inoculators and suspension of *Pss* was injected into ear shanks of the primary ears of each adult plant. Single kernels were assayed for *Pss* to estimate the percentage of infected kernels. A total of 51 infected seedlings was observed from an estimated 82,595 infected seeds, corresponding to 0.062% seed-to-seedling transmission of *Pantoea stewartii* subsp. *stewartii* in the hybrid Jubilee which was highly susceptible to Stewart's wilt disease. In earlier reports the involvement of flea beetles in the transmission of *Pss* was not considered, resulting in higher estimates of seed transmission of the pathogen to seedlings (Michener et al. 2002).

Bacterial populations attached to a plant surface are referred to as "biofilms," and the bacteria in biofilms are considered to be resistant to adverse conditions such as desiccation and extreme temperatures. The formation of biofilm may also function as an important virulence factor. *Pantoea stewartii* subsp. *stewartii* (*Pss*) produces stewartan exo/capsular polysaccharide (EPS) in a cell-density-dependent manner governed by *EsaI*/*EsaR* quorum sensing (QS) system. Biofilm development and colonization of host tissues by the wild-type (WT) strain and QS regulatory mutant strains were studied. The stewartan EPS synthesis governed by *EsaI*/*EsaR* QS system was required for effective adhesion of bacterial cells and the development of spatially defined 3D biofilms. A nonvirulent mutant lacking the *esaI* gene was able to adhere strongly to surfaces, in addition to the development of densely packed less structurally defined biofilms in vitro. Adhesion was blocked by treatment with exogenous *N*-acylhomoserine lactone (AHL). Likewise, QS mutants lacking *EsaR* repressor attached inefficiently to surfaces forming amorphous biofilms heavily enmeshed in excess EPS. The two QS mutant strains were localized at the site of infection. In contrast, the WT strain

dispersed efficiently within the xylem primarily in a basipetal direction. Observations using epifluorescence microscopic imaging of infected tissues showed that the WT strain of *P. stewartii* subsp. *stewartii* specifically colonized annular rings of protoxylem. On the other hand, indiscriminate growth of QS mutant strains filling the xylem lumen was observed (Koutsoudis et al. 2006).

Pantoea stewartii subsp. *stewartii* (*Pss*) contains Group I *hrp* gene clusters which are activated by complex regulator pathways encoding HrpS and HrpL (Frederick et al. 2001). The *hrp* type III secretion regulon of *Pss* is regulated by a cascade involving the HrpX/HrpY two-component system, the HrpL enhancer-binding protein, and the HrpL alternate sigma factor. The gene *hrpXY* is both constitutive and autoregulated. HrpY controls *hrpS* and HrpS activates *hrpL*. These regulator genes are sequentially arranged in the order *hrpL*, *hrpXY*, and *hrpS* and constitute three operons (Merighi et al. 2005). Each bacterial pathogen appeared to respond differently to external signals and growth conditions, so that regulation of *hrpS* by specific and global regulators may be fine-tuned for each pathogen's niche. The genetic analysis indicated that the HrpX sensor kinase and HrpY response regulator were at the head of a complex cascade of regulators controlling of *hrp/hrc* secretion and *wt*s effector genes. Only some of the multiple signals controlling the *hrpS* promoter were mediated by HrpY (Merighi et al. 2006).

6.2.8 *Xylella* sp.

Citrus variegated chlorosis (CVC) caused by *Xylella fastidiosa*, a Gram-negative and xylem-limited bacterium, was first reported to infect sweet orange (*Citrus sinensis*) in Brazil and grapevine and other economically important horticultural crops and urban shade trees (Purcell and Hopkins 1996). CVC is transmitted by sharpshooter insects (Cicadellidae), contaminated plant wood, and natural root grafts. The infected fruits are much smaller in size and become unacceptable for either the juice or fresh market. The fresh weight of CVC-affected sweet orange fruits of three cultivars showed a reduction of about 25% compared to healthy fruit. Of the 15 symptomatic fruits analyzed, all samples from the peduncles and central axis septa contained *X. fastidiosa* DNA as revealed by PCR assays. The presence of *X. fastidiosa* was detected in 80% of fruit peels and endocarp and also in 50% of the samples of locular membranes. By employing PCR assays, the presence of *X. fastidiosa* was checked. The pathogen was detected in 20–22% of the seed coat samples and in 15–16% of the embryo samples from seeds of CVC-affected fruits of three sweet orange cultivars.

The pathogen was isolated from 3 month old PCR-positive seedlings of three cultivars, namely Pera, Natal, and Valencia. The identity of the bacterial isolates was confirmed as *X. fastidiosa* by PCR assay (Fig. 6.4). The pathogen concentration in ten PCR-positive seed coats and embryos of each cultivar were determined by quantitative PCR format. Extracts (50 µL) from seed coats or embryos contained approximately 1250 cells of *X. fastidiosa*. Seedlings (250) growing from seeds from CVC-infected fruits were indexed for the presence of the pathogen. *X. fastidiosa* was detected in 23.6% of seedlings, indicating the transmission of the pathogen to seedlings. The aerial parts of the developing seedlings showed the presence of *X. fastidiosa*, which was also detected in the developing primary root. As *X. fastidiosa* is limited to the xylem, it was considered by many researchers that transmission of *X. fastidiosa* through seeds was impossible.

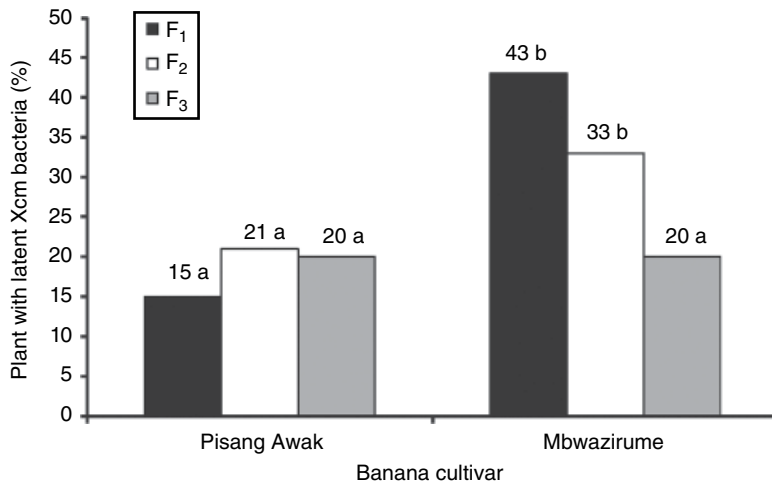


Figure 6.4 Transmission of latent *Xanthomonas campestris* pv. *musacearum* (*Xcm*) in bunch tissues to subsequent generations up to F₃ in banana cultivars Pisang Awak and Mbwasirume. Source: Ocimati et al. (2015). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

This investigation appears to be the first report of the seed transmission of *X. fastidiosa* to sweet orange seedlings (Li et al. 2003).

The incidence of citrus variegated chlorosis (CVC) disease has only been observed on sweet oranges for over two decades. In spite of high inoculum pressure and the availability of sharpshooters known to be the vectors of CVC, lime and mandarin were not infected. The mechanisms underlying this apparent resistance of lime and mandarin to CVC were investigated by inoculating the seedlings under greenhouse conditions. Responses of 11 citrus varieties to inoculation with *X. fastidiosa* were assessed by visual observations (%), isolation of the pathogen (%), and determination of occluded vessels (%), using light microscope. The percentages of seedlings exhibiting symptoms increased over time (6–8 months). Sweet orange and Valencia seedlings were infected to the extent of 63% and 53.8%, respectively. Average percentages of positive isolations and occluded vessels were higher in sweet orange than in mandarin or Rangpur lime. The xylem anatomy of the varieties examined did not show any obvious differences, suggesting that the apparent resistance of citrus varieties might not be related to any physical variation in xylem structure (Garcia et al. 2012).

6.3 Disease Cycles of Propagule-Borne Bacterial Pathogens

Propagules such as tubers, corms, bulbs, setts, or rhizomes carry bacterial pathogens, and such infected propagules form the primary sources of inoculum for the disease incidence and spread over the same or far-away locations. Potato tubers are known to carry different microbial plant pathogens which may occur alone or in combination, forming a complex. Both qualitative and quantitative losses in yield and the possibility of carrying the pathogens to subsequent generations are of great concern to all involved in crop production.

6.3.1 *Erwinia* spp.

The soft rot pathogens infecting potato tubers have been placed in the genus *Pectobacterium* as proposed by Hauben et al. (1998). *Erwinia carotovora* subsp. *carotovora* (*Ecc*), *E. carotovora* subsp. *atroseptica* (*Eca*), and *E. chrysanthemi* were designated *Pectobacterium carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *atrosepticum*, and *P. chrysanthemi* as per the proposal. The nomenclatures have yet to be widely adopted by plant pathologists, however. These bacterial species are Gram-negative, nonsporing, facultative anaerobes, and characterized by the production of large quantities of extracellular pectic enzymes. The development of disease symptoms (soft rot) depends primarily on the production of these enzymes, in addition to a wide range of other plant cell-wall-degrading enzymes (CWDEs) (Collmer and Keen 1986). *Erwinia carotovora* causes soft rot diseases in a wide range of plant species. In potato *E. carotovora* causes wilt, soft rot, and black leg diseases in the field and during storage of seed tubers. Tuber soft rot and aerial stem rot may occur when the plants are wounded, and tuber soft rot is favored by oxygen-limited conditions (Pérombelon 2002). *E. carotovora* subsp. *atroseptica* (*Eca*), *E. carotovora* subsp. *carotovora* (*Ecc*), and *E. chrysanthemi* are associated with potatoes with predominance of one species in different locations and environmental conditions. *Eca* is the principal pathogen of blackleg (stem rot) in Scotland. Planting of seed tubers contaminated with *Eca* led to reduced emergence, reduction in crop cover, and rotting of tubers in the field. *Eca* contamination of tubers above the threshold level of 10^2 – 10^3 CFU mL⁻¹ of seed tuber peel extract increased the level of blackleg disease incidence in the field (Bain et al. 1990). Among the soft rot erwinias *Ecc* has the broadest host range worldwide, while *Eca* could infect many plant species in the tropical and subtropical countries and maize and dahlia in temperate regions (Young et al. 1992).

The possibility of transmission of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) from mother tuber to progeny tubers was assessed by detecting *Eca* in progeny tubers of 12 different potato cultivars by employing by dilution plating, enzyme-linked immunosorbent assay (ELISA), immunofluorescence cell staining (IF), and polymerase chain reaction (PCR-) based assay. The transmission of *Eca* from the inoculated mother tuber to progeny tubers was detected by different methods with varying efficiency. The sensitivity of PCR assay for a direct detection of *Eca* in crude peel extract was less (10^7 – 10^8 cells mL⁻¹) because of inhibition of PCR amplification by tuber-derived inhibitors. IF technique was found to be the most effective for direct detection with detection threshold of 10^4 – 10^5 cells for six potato cultivars. After a 48 h enrichment of peel extract in polypectate medium at 20°C, the sensitivity of IF and PCR improved significantly and *Eca* at 10 cells mL⁻¹ could be detected in the progeny tubers of six and seven out of 12 potato cultivars tested by these assays, respectively. The results indicated that no relationship could be noted between tuber transmission of *Eca* to progeny tubers and blackleg disease incidence under field conditions (Fraaije et al. 1996).

Latent infection of potato tubers by *Erwinia* spp. is common in most commercial seed tuber stocks. The bacterial population might range from undetectable (<10 cells g⁻¹ peel) to high concentrations (up to 10^6 cells g⁻¹ peel). The pathogen may be present intercellularly in lenticels and in wounds, usually beyond the phelloderm layer, and less frequently in the xylem (Billing 1987; Pérombelon 1992). Pathogen population, irrespective of its size, appeared to be dormant or quiescent in tuber tissues. The non-availability of free water might restrict further development of the pathogen.

The relationship between contamination of potato seed tubers with *Erwinia carotovora* subsp. *atroseptica* (*Eca*), blackleg disease development, and the incidence and level of progeny tuber contamination in field-grown potato crops was investigated during 1998–2000. Seed tubers were inoculated by vacuum infiltration with a streptomycin-resistant marker strain of *Eca* at three concentration levels (low, intermediate, and high) and planted in the field. The incidence of blackleg development was directly related to the level of seed tuber contamination. The higher the level of seed tuber contamination, the earlier was the appearance of disease in the season. High and low levels of seed tuber contamination were related to high and low disease incidence and progeny tuber contamination in potato cultivars Hermes and Moreue at all sampling times. The incidence of progeny tuber contamination during the growing season was related to *Eca* population in seed tubers and their transmission from seed tuber to progeny tubers. This process may be influenced by differences in soil moisture during cycles of wet and dry spells (Tóth et al. 2003).

Erwinia carotovora subspecies, causing soft rot diseases, produce a wide range of enzymes capable of degrading plant cell-wall components, compared with most pectolytic saprophytic bacteria. These enzymes include pectinases, cellulases, proteases, and xylanases with different properties. Pectinases are considered to be more important in pathogenesis for effecting tissue maceration and indirectly cell collapse. The sec-dependent two-step, type II controls secretion of pectinases and cellulases and it is also known as the general secretory pathway (GSP). Some of these enzymes known to be virulence factors were secreted through GSP by *Erwinia chrysanthemi* and *E. carotovora* subsp. *carotovora* (*Ecc*). The pectate lyase, polygalacturonase, and cellulase are exported to the periplasm, but are unable to move through the outer membrane and then into the extracellular environment. Secretion of major pectate lyases, polygalacturonases, and cellulases is controlled by the *out* gene cluster and the number of *out* genes may vary with *Erwinia* spp. (Lindberg and Collmer 1992; Reeves et al. 1993).

Erwinia chrysanthemi produces five major pectate lyases (Pels) grouped into two families (Pel A, D, E, and Pel B, C) and at least three minor Pels (I, L, Z) induced preferentially in plant tissues. In contrast, *E. carotovora* produces three major Pels belonging to the Pel B, C family and several minor plant-induced Pel (Hinton et al. 1989; Jafra et al. 1999). Pectic enzyme and isoenzyme expression in *Erwinia* is sequential in plants, implying that genes concerned are also regulated separately as well as by global regulatory systems such as quorum-sensing system. A possible biological function for the sequential enzyme production might be to maximize the activity of the principal enzyme responsible for tissue maceration. Pel requires optimum pH (c. 8) in plant tissue where the apoplastic fluid has a pH of c. 6. Operation of a pH-dependent, sequential regulation of pel A, D, and E genes was reported by Nachin and Barras (2000).

Cell-wall-degrading enzymes (CWDEs) are generally considered as virulence determinants required for development of symptoms after the initiation of pathogen growth. It may be difficult to differentiate the functions of some factors involved in pathogen development and others in actively overcoming host plant resistance. They may have functions as pathogenicity determinants as well as general housekeeping. The CWDEs form the principal virulence determinants of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and their synthesis is coordinately regulated by a complex network. The ExpS and ExpA proteins encoded by *expS* and *expA* genes were shown to be members of two-component sensor kinase and response regulator families, respectively (Eriksson et al. 1998).

The interaction between proteins controlling the virulence gene expression component regulator system, that influences endoPG production and virulence of *Ecc*, was studied. The genes coding for the main virulence determinants of *Ecc*, the CWDEs, were shown to be under the coordinate control of global regulator systems, including both positive and negative factors. Reduction in virulence and impaired production of one of these enzymes, an endoPG (PehA), were due to mutations in the *pehR* locus. The DNA sequence of the *pehR* locus was composed of two genes, namely *pehR* and *pehS*, present in an operon. Both genes are needed for transcriptional activation of the endoPG gene *pehA* as well as restoration of virulence, as revealed by complementation experiments (Flego et al. 2000). Motility of *Erwinia* spp. was found to be necessary for the successful invasion and infection of potato plants and was therefore suggested as a pathogenicity determinant. *Tn5*-generated *E. carotovora* subsp. *atroseptica* (*Eca*) mutants defective in flagella assembly protein was not motile and had reduced virulence (Mulholland et al. 1993). Another *Eca* mutant with multiple cell-surface defects, including alterations in synthesis of outer membrane proteins, lipopolysaccharide (LPS), enterobacterial common antigen (ECA), and flagella, as well as reduced synthetic ability of Pel and cellulose, was generated. These mutants were less virulent on potato plants (Tóth et al. 1999). Adhesion of bacteria to plant surface is a vital step for the initiation of pathogenesis. Adhesion is generally attributed to fimbriae or pili. *E. carotovora* cells adhered to different potato tissues to varying degrees. This activity appeared to be independent of the presence or absence of known adhesions. Strains of *Escherichia coli*, pathogenic to human beings, produce intimin. An outer membrane protein immunologically similar to the intimin protein was also shown to be produced by *E. chrysanthemi* (Wallace and Pérombelon 1993; Duarte et al. 2000).

The bacteria-to-bacteria communication is associated with a quorum-sensing (QS) process, based on the production of *N*-acylhomoserine lactones (AHL). The AHL produced by a specific *Erwinia carotovora* subsp. *atroseptica* (*Eca*) virulent on potato was identified to investigate its role in infection by *Eca*. A derivative of this strain that expressed an AHL lactonase gene and was capable of producing only low concentrations of AHL was generated. AHL production and QS were shown to regulate only those traits involved in the second stage of infection, tissue maceration and hypersensitive response (HR) in nonhost tobacco plants (Smadja et al. 2004). In a later investigation, a low-abundance protein was detected among the secreted proteins of *Eca*, and identified by two-dimensional gel electrophoresis and mass spectrophotometry. This protein, designated Sv_x, was found to be a homolog of the avirulence protein of *Xanthomonas campestris* with unknown function. Sv_x protein was secreted by the type II (out) secretion apparatus which governs the secretion of major known virulence factors PelC and CellV. Transcription of the *svx* gene was under *N*-acylhomoserine lactone (AHL-) mediated quorum sensing control. The mutant of *svx* generated by transposon insertion showed decreased virulence in potato plant assays, indicating a definite role for Sv_x in the pathogenicity of *Eca*. The type II secretory system functioned as a conduit for virulence factors, other than the principal pectinases and cellulases in *E. carotovora* subsp. *atroseptica* (Corbett et al. 2005).

The QS signal molecule *N*-(3-oxohexanoyl)-1-homoserine lactone (OHHL) produced by *Erwinia carotovora* subsp. *carotovora* (*Ecc*) controlled seven genes of *Ecc*. The mutants defective in proteins that could play a role in the interaction between *Ecc* and its plant hosts were enriched using TrophoA as a mutagen. Nip (*Ecc*) and its counterpart

in *E. carotovora* subsp. *atroseptica* (*Eca*) were identified. Necrosis induced in tobacco and stem rot induced in potato were due to Nip(*Ecc*) and Nip(*Eca*), respectively. Both proteins affected virulence of the pathogens on potato tubers. These proteins were placed in the family of proteins related to Nep1 from *Fusarium oxysporum* which induced necrotic responses in several dicotyledonous plants. In *Ecc* nip was repressed weakly by the LuxR family regulator *EccR*, and might be regulated by the negative global regulator *RsmA* (Pemberton et al. 2005).

The principal virulence determinants in *Erwinia carotovora* subsp. *carotovora* (*Ecc*) are controlled by a complex regulatory network. One of the regulators *ExpA*, a GacA homolog, determines transcriptional activation of the extracellular enzyme genes of *Ecc*. A key role of the *RsmA-rsmB* regulatory system during pathogenesis was demonstrated to be the integration of signals from the *ExpA* (*GacA*) and *KdgR* global regulators of extracellular enzyme production in *Ecc* (Hyytiäinen et al. 2001). In addition, many regulatory factors were found to be involved in the production of pectinase. The main regulatory proteins *KdgR*, *Crp*, *Pir*, and *PecS* primarily from *E. chrysanthemi* were characterized. By applying Southern blotting, homologs of *KdgR* and *Crp*, but not *Pir* and *PecS*, were detected in *Ecc* by introducing plasmids containing the regulatory genes from *E. chrysanthemi* (Matsumoto et al. 2003b). A new regulatory factor involved in the virulence of *Ecc* was identified in another study. The (Δ) *cytR* mutant was able to produce only low levels of polygalacturonase (*Peh*) and was nonmotile. The virulence of the mutant was significantly reduced in potato and Chinese cabbage when compared to the wild-type strain. The *CytR* homolog of *Ecc* appeared to control *Peh* production and synthesis of flagella, in addition to having an important role in its pathogenicity (Matsumoto et al. 2003a).

In a later study the protein *ExpR*, a *luxR* homolog, was shown to be an AHL receptor and activated transcription of *rsmA*, the gene encoding an RNA binding protein, which is a global negative regulator of exoproteins and secondary metabolites. AHL counteracted the transcriptional activity of *ExpR*. Most of the strains of *E. carotovora* subspecies contained two copies of the *expR* gene in their genomes, namely *expR1* and *expR2*. Both variants were able to activate *rsmA* transcription with significant differences in the pattern of their AHL interactions, the *rsmA* sequences to which they were bound, and their relative efficiencies of activation of *rsmA* transcription (Cui et al. 2006).

Bacterial pathogens possess flagella which are considered to be helpful to reach favorable environment (cells) or to escape from adverse conditions. The flagellum has a long helical filament composed of a single protein known as flagellin encoded by *fliC*. Two integral membrane proteins *MotA* and *MotB* are needed for flagellar rotation. Flagellar motility is an important virulence factor during infection and may also induce host defense responses in nonhost plant species (Young et al. 1999). The role of flagella in pathogenesis of *E. carotovora* subsp. *carotovora* (*Ecc*) was investigated by employing nonpolar in-frame deletion mutants of *fliC* and *motA* homologs. Both Δ *fliC* and Δ *motA* mutants were nonmotile on semisolid medium. Electron microscopic examination revealed that *motA* mutant was peritrichously flagellated like the wild-type strain. The Δ *fliC* mutant did not have flagella and was defective in flagellin synthesis. Both mutants were less efficient in inducing soft rot symptoms on Chinese cabbage, compared with the wild-type strain. However, the mutants were able to produce major extracellular enzymes effectively for their survival. The results suggested that motility, and not flagellar component(s), was the vital factor in successful induction of disease symptoms (Hossain et al. 2005).

In a later investigation, a nonflagellate, nonmotile mutant ($\Delta fliC$) and a flagellated nonmotile ($\Delta motA$) mutant of *Ecc* were compared for their ability to form biofilm. The mutants showed a similar reaction in biofilm formation on the surface of polyvinyl-chloride (PVC) microtitre plates. The results indicated that functional flagella were essential for biofilm formation which might help bacterial pathogens in host colonization, and flagellar motility could be indirectly involved in pathogenicity (Hossain and Tsuyumu 2006).

6.3.2 *Clavibacter* spp.

Clavibacter michiganensis subsp. *sepedonicus* (*Cms*), causal agent of potato ring rot disease, a Gram-positive bacterium, continues to be a major limiting factor affecting potato yield and quality in the field and in storage. The pathogen infects tubers through wounds and dispersal of the pathogen occurs during seed tuber cutting and handling. The disease is introduced into the field and carried to the subsequent generation by planting infected seed tubers, thereby completing the disease cycle. As the disease primarily spreads through contaminated seed tubers, the certification agencies enforce a zero tolerance for ring rot disease (Gutbrod 1987). Potato plants may not exhibit any visible symptoms. Latently infected plants and tubers carrying the pathogen escape and serve as sources of inoculum for the next generation. Persistence of *Cms* in seed tubers for several generations was investigated. Whole seed tubers were inoculated with different inoculum doses of *Cms* and planted in the field. The persistence of *Cms* in the absence of symptoms was determined for four generations, following introduction of inoculum. All inoculum doses were introduced through stolon end of seed tubers. Seed tubers were cut during propagation to prevent redistribution of inoculum. Infection by *Cms* was detected by ring rot symptoms in potato foliage or tubers or by eggplant bioassay. Detection of *Cms* was positively related ($r=0.97$) to inoculum doses (10^2 – 10^{10} cells mL⁻¹) and rapidly declined with increasing generations (G0–G3). The eggplant bioassay was found to be sensitive for *Cms*, as living pathogen cells were detected. Latency for three generations was the longest interval tested, indicating that detection of *Cms* based on symptom expression was not suitable to eradicate ring rot disease in seed tubers (Franc 1999).

The role of extracellular polysaccharides (EPS) produced by *C. michiganensis* subsp. *sepedonicus* in the process of infection of potato was studied. The interaction of *Cms* EPS with protoplasts isolated from leaf cells of plants grown in vitro and microsomal fractions obtained from cell suspension cultures of two potato cultivars susceptible and resistant to ring rot disease were investigated. The EPS intensively bound to protoplast surfaces and microsomal membrane fractions of susceptible cultivar, but not to those of resistant cultivar. Treatment with protease, excess of unlabeled EPS, and with dextran did not lead to the binding of fluorochrome-labeled EPS protoplasts and microsomal membranes of both susceptible and resistant cultivars. The results suggested that susceptible potato cultivars might have a greater number of receptors in the plasma membrane for binding with EPS produced by *Cms*, while the resistant cultivar might contain a small but sufficient number of receptors to EPS to facilitate induction of defense responses (Romanenko et al. 2003).

6.3.3 *Ralstonia* sp.

Ralstonia (*Pseudomonas*) *solanacearum* (*Rs*) has a wide host range including potato and solanaceous crop plants. *R. solanacearum* causes bacterial wilt and brown rot

disease in potato plants and tubers. Sudden wilting of plants, browning of vascular tissues of stem and roots, and rotting of tuber tissues that turn brown are the common symptoms of infection by this pathogen. Vascular bundles show bacterial pockets and disintegration of the affected tissues results in the collapse of affected plants. *R. solanacearum* can overwinter in diseased plants or debris, tubers, and probably in the soil. Bacteria released into the soil from plant tissues are spread through irrigation (soil) water, transplants, and contaminated knives. Infection may be initiated after entry of the pathogen through wounds in roots made by implements or at cracks where secondary roots emerge. *R. solanacearum* shows extensive genetic diversity among strains. Based on multilocus DNA sequence typing, *R. solanacearum* was considered to be a species complex divided into four groups called phylotypes. Phylotypes are subdivided into sequevars based on the sequences of the endoglucanase (*egl*) gene (Villa et al. 2005; Cellier and Prior 2010). *R. solanacearum* capable of infecting a large number of plant species produces extracellular polysaccharides (EPS) in culture, and a positive correlation between EPS production and virulence of the pathogen strains was observed (Buddenhagen and Kelman 1964). The crude cell-free extracts of the pathogen culture induced wilting in tomato cuttings. Using specific EPS mutants of *R. solanacearum* the ability of acidic EPS to induce wilting was demonstrated; this form of EPS was considered as the wilting factor of *R. solanacearum*. Inactivation of the *eps* operon by transposon tagging resulted in reduction of acidic EPS in planta, and consequently reduced virulence in the EPS mutants (Denny and Schell 1994).

Strains of *R. solanacearum* have been classified into five races based on their differential ability to produce acid from different carbohydrate substrates. There appears to be no general correlation between races and biovars, except that race 3 and race 5 strains are usually placed in biovar 2 and biovar 5, respectively. Race 3 biovar 2 (R3 Bv2), existing in cooler temperatures, could survive in latent infections of potato tubers which are responsible for the worldwide distribution of potato brown rot disease. Once *R. solanacearum* is introduced into a cropping system, it is difficult to contain the incidence and subsequent spread of the pathogen because of its wide host range, facilitating the perpetuation of the pathogen for long time in the absence of potato crops (Champoiseau et al. 2009). *Ralstonia solanacearum* (biovar 2A) is highly virulent, inflicting serious losses in tropical and subtropical countries (Elphinstone 2005). Transmission of the pathogen from mother plants to tubers occurs, irrespective of development of external symptoms. The progeny tubers remain asymptomatic and they may serve as potential sources of inoculum to new areas and subsequent generation. Seed tuber certification agencies therefore emphasize zero tolerance for this pathogen (Priou et al. 2010).

R. solanacearum has been reported to infect hundreds of plant species encompassing 44 families. The pathogen is disseminated via potato and banana propagative materials, causing extensive damages to these crops as well as to ginger and pepper (Norman et al. 2009). *Ralstonia solanacearum* (*Rs*) race 4 causes bacterial wilt of edible ginger (*Zingiber officinale*). The survival of *Rs* in plant-free soil and potting medium in the presence of inoculated plants was assessed. The presence of *Rs* in drainage water from soil and potting medium containing known concentrations of *Rs* was estimated by enzyme-linked immunosorbent assay (ELISA), immunostrip assay, and polymerase chain reaction (PCR) assay. In the absence of a plant or in the presence of nonwounded plants, pathogen populations declined rapidly in drainage water from potting medium during the first 9 days and were undetectable after 81 days. Immunostrip assay was more efficient in detecting *Rs* in all samples (100%) than the other two assays (Paret et al. 2010).

Banana Moko disease is caused by *Ralstonia solanacearum* race 2 and banana blood disease is due to *R. solanacearum* phylotypes IV. Moko disease is spread by insects from male buds with bacterial ooze to healthy inflorescences and also by contaminated cutting tools. *R. solanacearum* could survive in the soils in banana fields (Tinzaara et al. 2006). *Ralstonia solanacearum* (*Rs*) cells, as in many soilborne phytopathogenic bacteria, have swimming motility which makes a substantial quantitative contribution to virulence in the early stages of pathogenesis (disease development). The virulence of nonmotile *fliC* mutants of *Rs* was found to be significantly reduced (Trans-Kersten et al. 2001). The chemotaxis assays indicated that *Rs* was attracted to diverse amino acids and organic acids, and especially root exudates of host plant species (Yao and Allen 2006). The regulatory locus *pehSR* in *Ralstonia solanacearum* governs the production of polygalacturonase (PG) and other virulence functions. The *pehSR* mutant had negligible endoPG activity, while the exoPG activity showed reduction by 50%. The growth of the mutants, however, remained at the same level of wild-type strain, despite its reduced virulence. The expression of *pehSR* in plant increased as the proliferation of the pathogen was maintained. Involvement of the global virulence gene regulator PhcA in the negative regulation of the *pehSR* locus was inferred from studies on reporter gene (Allen et al. 1997). The extracellular plant cell-wall-degrading enzymes (CWDEs) and other proteins are secreted via the highly conserved type II protein secretion system (T2SS). *R. solanacearum* with a defective T2SS was found to be weakly virulent. Virulence of mutants, lacking only pectic enzymes (PehA, PehB, PehC, and Pme), was not significantly affected. However, all mutants lacking one or both cellulolytic enzymes (Egl or CbhA) induced wilting symptoms at a slower pace compared with wild-type strain (Liu et al. 2005).

A 70-mer oligonucleotide-based DNA microarray system was applied to investigate the role of genes under the control of the transcription activator HrpB that governs the pathogenesis of *Ralstonia solanacearum*. Among the 5074 genes generated, 243 *hrpB* upregulated genes and 50 *hrpB* downregulated genes were identified. The *hrpB* regulon was shown to extend beyond type III secretion system-related functions to include a number of genes governing chemotaxy, biosynthesis, and catabolism of various low-molecular-weight compounds and siderophore production and uptake. The results indicated that *hrpB* could act as a master regulatory gene governing a physiological swing associated with the shift from saprophytic to parasitic life (Occhialini et al. 2005). Two novel Hrp-secreted proteins PopF1 and PopF2 from *R. solanacearum* showed similarity to one another and to putative translocators, HrpF and NopX, from *Xanthomonas* spp. and rhizobia, respectively. Both *popF1* and *popF2* belonging to the HrpB regulon are required for interaction with plants. Although they were not needed for secretion of effector proteins, they were found to be essential for translocation of AvrA proteins into host plant cells. The results suggested that translocation of *R. solanacearum* and *Xanthomonas* spp. might be functionally conserved (Meyer et al. 2006). *R. solanacearum* encodes a large number of putative TTSS effectors. A group of seven genes having homologies with plant-specific leucine-rich repeats (LRR) has been identified. The proteins encoded by them were designated "GALA" proteins after a conserved GAXALA sequence in their LRR. GALA6 was translocated into plant cells (Cunnac et al. 2004; Occhialini et al. 2005). All of the seven GALA effector genes have been detected in a strain of *R. solanacearum* (Angot et al. 2006).

6.3.4 *Xanthomonas* spp.

When infected by microbial pathogens (fungi, bacteria, and viruses), banana suckers form the primary sources of inoculum that introduce the pathogens to new locations and also subsequent generations. In addition, the pathogens may survive in the propagules without exhibiting any visible symptoms. Banana *Xanthomonas* wilt (BXW), also known as bacterial wilt (BBW), is caused by *Xanthomonas campestris* (*vasicola*) pv. *muscearum* (*Xcm*). Symptoms of infection by *Xcm* include yellowing of leaves, wilting, exudation of yellowish bacterial ooze, premature ripening and rotting of fruits, and internal discoloration of vascular bundles. At the preflowering stage, plants can become infected directly from diseased mother plants or from the inoculum in the contaminated tools. The first visible symptom of inflorescence infection is wilting of male buds, followed by decay of the rachis, and finally bunch rotting. The pathogen in ooze is spread by visiting insects to healthy inflorescence (Tinzaara et al. 2006). *Xcm* in inflorescence-infected Pisang Awak plants with wilting male bud bracts was restricted to upper parts of the stem. Following inflorescence infection, *X. campestris* pv. *musacearum* moved along the stem, into the youngest leaf sheaths inserted on the stem, down into the corm, and into the older leaf sheaths. At the early stages of inflorescence infection, *Xcm* was restricted to the upper parts of the stem in the cultivar Pisang Awak, but it had moved further down the stem in cultivar Matooke. The pathogen was recovered more frequently from the younger, innermost leaf sheaths than from older, outer leaf sheaths. Older leaves originated from the corm and were therefore invaded later, following inflorescence infection (Ssekiwoki et al. 2010).

Several insects including honey bees visit the male flowers of both symptomatic and asymptomatic banana plants. *Xcm* could be isolated from honey bees and other insects such as stingless bees, fruit flies, and grass flies visiting male flowers. Transmission of the pathogen seemed to occur primarily through the male flower or neuter part of the floral raceme (Tinzaara et al. 2006).

The movement of *Xanthomonas campestris* pv. *muscearum* from inoculation point was investigated by inoculating the pathogen through abscission wounds of female bracts and male bud bracts with a contaminated machete. Inoculation through all floral entry points resulted in infection, with the highest disease incidence occurring in the combined male bract and male flower abscission wound inoculations. The systemicity of *Xcm* was revealed by the ability of the pathogen to live within the mat for long periods (5–16 months) without producing disease symptoms (Ocimati et al. 2013).

The efficiency of infection of *Xanthomonas campestris* pv. *musacearum* (*Xcm*), causing banana *Xanthomonas* wilt (XW) disease, through the corm, pseudostem inoculation at bunch harvest, leaf, and female and male bud bracts was evaluated. The male and female bud bract inoculations resulted in the highest incidence of XW (81% and 93%, respectively) compared with 0–44% infection for harvest and corm inoculations. Natural insect-mediated transmission in cultivar Pisang Awak led to up to 99% disease incidence. Floral inoculations and insect-mediated infections only caused floral symptoms. Symptom development in insect-mediated infections was similar following artificial male bud inoculations, confirming the male bud bract wounds form the main entry points for insect-mediated transmission. This situation reaffirms the importance of continuous and timely debudding to limit insect spread. Leaf and harvest inoculations caused only leaf symptoms and harvest inoculations caused only leaf symptoms, while corm inoculations led to late floral symptoms. Floral inoculations

were the principal mode of infection by *Xcm*. Single leaf inoculations resulted in 30% plant mortality, despite 100% incidence of disease, with 70% of plants recovering and bearing apparently healthy bunches and producing healthy suckers. A significant difference in the population of *X. campestris* pv. *musacearum* (CFU g⁻¹) was recorded between symptomatic and asymptomatic leaves, suggesting cultural practices that might reduce the pathogen population below the inoculum threshold could benefit the plant (Nakato et al. 2014).

Banana Xanthomonas wilt (XW) disease caused by *Xanthomonas campestris* pv. *musacearum* (*Xcm*) is transmitted via infected propagules, contaminated tools, and natural biological insect vectors. Different aspects of host–pathogen interactions such as pathogen survival, latently infected suckers, incidence of latent infections in symptomless plants in mats having diseased plants, and the incidence of XW disease across farms were investigated. Latent bacterial populations were detected in up to 20% of the third-generation suckers, with a significant reduction (20–43%) in subsequent generations. Low percentages of latently infected suckers (3–6%) were killed by *Xcm* (Fig. 6.4). In commercial fields, the incidence of symptomless infection by *Xcm* was up to 70%. The latent *Xcm* population generally declined from generation to generation. If fresh infection could be prevented, the bacterial population in the mats might decline in subsequent generations. The latency was considered to be due to possible accumulation of resistance and fitness genes within the infected plants. Latent infection was regarded as a type of host resistance or tolerance to some pathogens. Critical examination of symptomless suckers from mats in which parent plants were inoculated with *Xcm* earlier indicated incomplete movement of *Xcm* within the mats. Not all suckers from infected mats were found to be infected and some suckers remained free of XW pathogen, indicating the possibility of selecting pathogen-free suckers from such mats (Ocimati et al. 2015).

Asian citrus canker disease (Acc) caused by *Xanthomonas citri* (*axonopodis*) subsp. *citri* is one of the most important diseases incited by *Xanthomonas* spp. The pathogen occurs in the form of several strains that have been grouped into pathotypes. Pathotype A is widely distributed and endemic in many tropical and subtropical countries growing *Citrus* spp. Infection by *X. citri* pv. *citri* induces the formation of crateriform lesions surrounded by a water-soaked margin on leaves, fruits, and stems. Early infection on immature fruits may lead to cracking and malformation of fruits, making them unacceptable for fresh market. Levels of susceptibility/resistance to canker disease vary among *Citrus* spp. and cultivars. Optimum temperature for disease development may be 28–30°C (Koizumi 1985). The pathogen may enter into the plant through stomata or wounds. A concentration of 10⁴–10⁵ CFU mL⁻¹ and 10² CFU mL⁻¹ may be required for successful infection respectively for entry through stomata or wounds (Goodman 1982; Zubrzycki and de Zubrzycki 1987). The canker pathogen is spread by splashing rain and wind and also by larvae of leaf miner (*Phyllocnistis citrella*) through wounds made while feeding (Gottwald et al. 1997). In order to assess the risk of introducing Asian citrus canker pathogen through pollen, selections of citrus genotypes exposed to *Xcc* were used as pollen parents with pollen applied to disease-free trees of *Citrus maxima* and hybrids (susceptible) and *C. reticulata* and hybrids (resistant). *X. citri* pv. *citri* (*Xcc*) DNA was detected in 30% of flower samples and 23% of anther samples. No significant differences in *Xcc* populations were obtained between tissue type or genotypic group. However, regression between *Xcc* levels in flowers and percent of plant pedigree derived from mandarin had a negative correlation. The pathogen was detected inside flowers and

might be the result of contamination. The results suggested that there could be a risk of spreading *Xcc* through pollen from trees in infected farms (Stover and McCollum 2011).

The initial stage in the process of infection by bacterial pathogens is biofilm formation. *Xanthomonas citri* pv. *citri* (*Xcc*) develops a characteristic biofilm on both biotic and abiotic surfaces. A biofilm-deficient mutant was selected through screening of transposon mutagenesis library of *Xcc* 306 strain constructed using the commercial *Tn5* transposon EZ-Tn5 < KAN-1 > Tnp Transposome (Epicentre). Sequence analysis of a mutant obtained in the screening revealed that a single copy of the EZ-Tn5 was inserted at position 446 of *hrpM*, a gene encoding a putative enzyme involved in glucans synthesis. The product encoded by the *hrpM* gene was shown to be involved in β -1,2-glucan synthesis in *Xcc*. Mutation in *hrpM* resulted in a lack of visible symptoms after 4 weeks of inoculation in lemon and grapefruit plants. Further, the mutant had reduced ability to swim in soft agar, as well as reduction in resistance to H₂O₂, compared with wild-type strain. Complementation with plasmid pBR1-MCS containing an initial copy of the *hrpM* gene and its promoter restored the levels of wild-type strain in all defective phenotypes of *Xcc*. The results revealed that the *hrpM* gene contributed to the growth of *X. citri* pv. *citri* and its adaptation to different citrus genotypes (Malamud et al. 2012).

Survival of the bacterial pathogens in the absence of susceptible host plants is a critical factor for their perpetuation in different ecosystems. The ability of *Xanthomonas citri* pv. *citri* (*Xcc*) to survive in mandarin fruit was investigated. Asymptomatic fruit harvested from severely diseased Satsuma mandarin trees was tested for the presence of *Xcc*. The pathogen was not detected by polymerase chain reaction (PCR) assay in sonicates from 3491 mature asymptomatic fruit, indicating that healthy, asymptomatic Satsuma mandarin fruits were unlikely to harbor detectable concentrations of *Xcc*. The survival of *Xcc* on Satsuma mandarin fruits discarded in the orchard was examined in two consecutive years (2005, 2006). Bacterial populations on contaminated fruits decreased significantly in the first 3 days after fruit were discarded. The spread of *Xcc* from contaminated fruit into rainwater collected beneath the fruit was investigated by employing the PCR assay. No detectable population of *X. citri* pv. *citri* was present in the rainwater, indicating the absence of chances of the pathogen being released into the rainwater from canker lesions on fruit placed in close proximity to host plants that were in a receptive stage (Shiotani et al. 2009).

As for other *Xanthomonas* spp., *Xanthomonas citri* pv. *citri* (*Xcc*) contains the type III secretion system (T3SS) encoded by the *hrp* cluster governing both phenomena of hypersensitive response and pathogenicity. The protein–protein interactions involving subunits, regulators, and substrates of T3SS of *Xcc* were identified using a yeast two-hybrid system (Algeria et al. 2004). The *hrp* cluster of *Xcc* is composed of 25 genes extending from *hpaZ* and *hpaF* (da Silva et al. 2002). A region of the *hrp* cluster of *Xcc* was isolated and the interaction of mutants for the type T3SS with host and nonhost plants was characterized. Mutants for the operons *hrpB*, *hrpD*, and *hrpF* were constructed. These mutants were apathogenic and could not induce canker lesions in citrus plants or HR in cotton plants (a nonhost). In contrast, the wild-type exhibited pathogenicity and also induced HR in cotton. Furthermore, the mutants were unable to induce HR in bean, tomato, pepper, and *Nicotiana benthamiana*, whereas wild-type strain could induce HR on these plant species. It is possible that the gene *pthA* is required for elicitation of HR in nonhosts. The *pthA* is secreted by T3SS and the loss of HR in the nonhost cotton may be due to the requirement of a functional secretion system that translocates PthA into plant cells. The results indicated that genes present in

operons *hrpB*, *hrpD*, and *hrpF* genes are required for pathogenicity in susceptible plant species and also for induction of HR in nonhost plant species (Dunger et al. 2005).

Xanthomonas citri subsp. *citri* (*Xcc*) contains a gene in the *hrp* cluster that encodes a harpin protein designated Hpa1. Hypersensitive response (HR) was induced by Hpa1 in nonhost plants tobacco, pepper, and *Arabidopsis*. In contrast, Hpa1 elicited a weak defense response with no visible phenotype in susceptible citrus. Coinfiltration of *Xcc* with or without the recombinant Hpa1 protein in citrus leaves produced a larger number of cankers in the presence of the protein. Infiltration of the mutant Δ hpa1 of *Xcc* caused a smaller number of cankers. In addition, the lack of Hpa1 hindered bacterial aggregation both in solution and in planta. Analysis of citrus leaves infiltrated with Hpa1 revealed alterations in mesophyll morphology induced by the presence of cavitations and crystal idioblasts, suggesting the binding of the harpin to plant membranes and the elicitation of signaling cascades. Although Hpa1 elicited defense response in nonhosts and to a lesser extent in host plants, the main roles of Hpa1 seemed to be to alter leaf mesophyll structure and to aggregate bacterial cells facilitating enhancement of virulence and pathogen fitness. Expression of the N-terminal and C-terminal regions indicated that only the N-terminal region was involved in the enhancement of virulence and bacterial aggregation, supporting the role of the N-terminal region of protein as the main active domain (Sgro et al. 2012).

Host specificity genes of *X. citri* pv. *citri* (*Xcc*) was investigated using the unique A strain from Key lime (A^W) occurring in Wellington, Florida which induced hypersensitive reaction (HR) in grapefruit leaves. Using the heterologous tomato pathogen *X. perforans* as a recipient of the *Xcc*- A^W genomic library, a 1599 bp open reading frame (ORF) responsible for HR in grapefruit, but not in Key lime, was identified and designated *avrGf1*. The mutant Δ avrGf1 of *Xcc*- A^W produced typical, but reduced, canker symptoms (raised pustules) in grapefruit and typical canker symptoms in Key lime. The *X. perforans* transconjugant carrying an *Xcc*- A^W *hrpG* elicited HR in grapefruit and Key lime leaves. The *xopA* gene of *X. perforans* was partly responsible for HR. *Xcc*-A transconjugant carrying the *xopA* appeared to be a host-limiting factor (Rybak et al. 2009).

Xanthomonas citri pv. *citri* (*Xcc*) was investigated as a model of host specialization because of its pathogenic variants. Pathotype A strains could infect a wide range of members of Rutaceae. On the other hand, the host range of the pathotype A^*/A^W strains was restricted to Mexican lime (*Citrus aurantifolia*) and alemow (*Citrus macrophylla*). Worldwide diversity of a collection of 55 representative strains of *Xcc* was determined by amplified fragment length polymorphism (AFLP) analysis. The distribution of type III effectors (T3Es) was investigated in relation to host range of *Xcc*. The presence of 66 T3Es from xanthomonads in *Xcc* was examined. A repertoire of 28 effectors was identified and all *Xcc* strains shared 26 among them. On the other hand, two (*xopAG* and *xopC1*) were present only in some A^*/A^W strains. *xopAG* (=avrGf1) was detected in all A^W strains, but also in three A^* strains genetically distant from A^W strain. Further, all *xopAG*-containing strains induced HR on grapefruit and sweet orange. Analysis of *xopAD* and *xopAG* suggested horizontal transfer of these genes between *X. citri* pv. *bilavae*, another citrus pathogen and some *Xcc* strains. Pathotype A strains were genetically less diverse, induced identical phenotype responses, and possessed indistinguishable T3E repertoires. Conversely, A^*/A^W strains exhibited a wider genetic diversity in which clades correlated with geographical origin and T3E repertoires, but not with pathogenicity, as indicated by T3E deletion experiments (Escalon et al. 2013).

Development of resistance in bacterial pathogens to chemicals applied for the management of diseases caused by them is one of the survival mechanisms for their perpetuation in different ecosystems, where they may be confronted with other environmental stresses. Strains of *Xanthomonas citri* pv. *citri* (*Xcc*) were exposed to UV-irradiation and three amicarbazol-resistant mutants, namely *Xuv10*, *Xuv20*, and *Xuv40*, were generated. Minimal inhibitory concentration (MICs) of amicarbazol against growth of mutants and wild-type strain were 400 and 100 µg mL⁻¹, respectively. By using triphenyltetrazolium chloride (TTC), the inhibition by amicarbazol of succinate dehydrogenase (SDH) activities of wild-type strain and three resistant mutants of *Xcc* were assayed. Significant reduction in the activities of SDH in the resistant mutants, relative to the wild-type strain, was observed. The complete nucleotide sequences of four subunits (SdhA, SdhB, SdhC, and SdhD) of succinate-ubiquinone oxido-reductase (SQR) were cloned by PCR amplification. An amino acid mutation (His₂₂₉ → Leu₂₂₉) in *sdhB* conferred resistance in *Xcc* to amicarbazol. The results suggested that the amino acid mutation might alter the SDH complex in some way that prevented binding of amicarbazol (Li et al. 2006).

Resistance to copper in bacterial pathogens, especially in *Xanthomonas* spp. has been observed frequently. Multiple and independent introduction of *Xanthomonas citri* pv. *citri* (*Xcc*) strains with enhanced resistance to copper from Argentina and strains of *X. alfalfae* subsp. *citrumelonis* in Florida were observed. Partial nucleotide sequences of principal copper resistance genes, *copL*, *copA*, and *copB* from *Xcc* and *X. alfalfae* subsp. *citrumelonis*, were compared with strains of other *Xanthomonas* spp. resistant to copper isolated from 12 different countries; it was found that the *copLAB* gene cluster was present in many species of *Xanthomonas* from different countries. Alignment of partial nucleotide (nt) sequences of copper resistance genes among the copper-homology of ≥92, ≥96, and ≥91% for *copL*, *copA*, and *copB*, respectively, was made. Grouping of strains based on branching patterns of phylogenetic trees was similar for *copL* and *copA*, but differed for *copB*. High homology of the genes among strains indicated that copper resistance (Cu^R) genes might have a common origin. However, the slight differences in nucleotide sequences within groups of strains indicated that Cu^R genes could have been independently exchanged among species of *Xanthomonas* throughout the world (Behalu et al. 2013).

6.3.5 *Candidatus Liberibacter*

Huanglongbing (HLB), earlier known as citrus greening, is one of the most destructive diseases of citrus in Asian, African, and South and North American countries. The HLB is caused by a fastidious α-proteobacterium, “*Candidatus Liberibacter*” spp., which is phloem-limited. The causative agent has not been isolated in pure culture on cell-free artificial media. Three species involved in the HLB disease have been identified which differ in their vector specificity and requirement of environmental conditions for their development. “*Candidatus Liberibacter asiaticus*” is heat-tolerant and vectored by psyllids *Diaphorina citri* and *Trioza erytrea*. “*Ca. Liberibacter americanus*” is heat-tolerant and transmitted by *D. citri*. “*Ca. Liberibacter africanus*” is heat-sensitive and transmitted by *T. erytreae* and *D. citri* (Bové 2006). The pathogens reside in the phloem sieve elements in infected plants and inhibit the sugar transport. The symptoms of HLB include reduction in leaf size, pale yellowing, blotchy mottle or variegated type of chlorosis with small upright leaves, and die back of twigs at later stages. Dispersal of HLB

over long distances occurs through movement of infected citrus propagative materials, and short-distance spread is effected by psyllid species.

The genome of "*Ca. Liberibacter asiaticus*" has been sequenced, facilitating the availability of information on the genetic and derived biochemical and physiological characteristics of the pathogen. Further, different aspects of interaction between host and pathogen have been studied by applying different nucleic acid-based techniques. A combination of standard polymerase chain reaction (PCR) assay and real-time PCR format, targeting the putative DNA polymerase and 16S rDNA sequence of "*Ca. Liberibacter asiaticus*," respectively, were employed to monitor the distribution and movement of the HLB pathogen in the infected citrus tree. The presence of "*Ca. Liberibacter asiaticus*" was detected in the bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in endosperm and embryo of infected seeds in citrus trees. Quantification of pathogen populations in different tissues revealed the uneven distribution of HLB pathogen in citrus trees. The population of HLB pathogen ranged from 14 to 137,031 cells μg^{-1} of total DNA in different tissues, representing a 10,000-fold variation in bacterial population density in various tissues tested. The high HLB pathogen population in fruit peduncle makes it a target for sensitive detection of HLB in infected citrus trees. The detection of HLB pathogen inside the fruit and seed has potential significance for epidemiological investigations (Tatineni et al. 2008).

The distribution of "*Ca. Liberibacter asiaticus*" was quantified in a later investigation in tissues of six species of citrus in the field plants and plants propagated in the greenhouse in Florida and Beltsville, respectively. The populations of "*Ca. Liberibacter asiaticus*," as reflected by the distributions of 16S rDNA sequences specific for the pathogen in leaf midribs, leaf blades, and bark samples, varied by a factor of 1000 among samples from *Citrus* spp. and by a factor of 100 between two sweet orange trees tested. "*Ca. Liberibacter asiaticus*" was also readily detected in the roots of infected trees. Branches confirmed to be infected by HLB pathogen were selected and fruits were collected from those branches grown in the field. The pathogen was detected in all tissue types of symptomatic fruits of lemon and Palestine sweet lime with pathogen population levels varying from 10^5 to 10^9 genomes of "*Ca. Liberibacter asiaticus*" per gram of tissue and averaging 10^7 genomes g^{-1} (Table 6.1). Relatively high populations of the pathogen genomes were detected in the locular membranes. Assessment of populations of "*Ca. Liberibacter asiaticus*" in asymptomatic sweet orange fruit showed that the peduncle (1.4×10^6 genomes g^{-1}) and central axis (1.6×10^7 genomes g^{-1}) had high populations. However, the pathogen was able to multiply preferentially in the fruit septum reaching a higher concentration of 1.8×10^9 genomes g^{-1} . "*Ca. Liberibacter asiaticus*" could not be detected in the pericarp or mesocarp, endocarp, or locular membranes of the pre-symptomatic sweet orange fruit. The real-time qPCR procedure developed in this investigation was found to be highly sensitive and robust for detection of pathogen in citrus tissues (Li et al. 2009a).

In a later investigation, distribution of "*Ca. Liberibacter asiaticus*" (CLas) in citrus trees was monitored. Pathogen populations in cells in different plant parts, namely the peduncle, columella, leaves, seeds and young shoots, flower buds, flowers, and bark, of 6 year old infected grapefruit and sweet orange trees were assessed. The pathogen population was estimated using a previously described grand universal regression equation $Y = 13.82 - 0.2866X$, where Y is the log of the target copy number and X is the C_t (threshold cycle) of the assay. No significant difference in the pathogen titer in cells of plant parts, except the bark, could be observed between the two cultivars. Within the cultivar,

Table 6.1 Quantification of “*Ca. Liberibacter asiaticus*” genomes in DNA extracts of fruit tissues of huanglongbing (HLB-) infected citrus plants using qPCR assay (Li et al. 2009a).

Fruit tissue	Mean of Ct values of qPCR*			Genomes g ⁻¹
	Lemon	Lime	Mean	
Locular membrane	24.50a	31.18ab	27.84a	3.45 × 10 ⁸
Septa	29.40b	28.38a	28.89a	1.75 × 10 ⁸
Endocarp	30.98b	30.15a	30.56ab	6.00 × 10 ⁷
Peduncle	30.28b	33.37b	31.82b	2.50 × 10 ⁷
Central axis	39.48c	29.29a	34.38c	4.65 × 10 ⁶
Pericarp/mesocarp	37.92c	34.61b	36.26d	1.35 × 10 ⁶
Mean	32.09a	31.16a	–	–
Genomes g ⁻¹	2.1 × 10 ⁷	3.89 × 10 ⁷	–	–

* Mean crossing threshold (Ct) values followed by the same letter are not significantly different ($P=0.05$)

the bacterial populations varied with the plant part, with peduncle, columella, and midrib having significantly higher titer of CL as compared to other plant parts. Bacterial population titer was the lowest in young shoots (Kunta et al. 2014).

Use of disease-free plants is a critical component of a comprehensive strategy to manage huanglongbing (HLB) disease caused by “*Candidatus Liberibacter asiaticus*.” As most of the seed source of citrus trees for rootstock propagation were grown under natural conditions and in unprotected conditions, it became necessary to check the seeds and plants for freedom from HLB disease. Seedlings produced from seeds from infected rootstock, seed source trees, and Valencia sweet orange were indexed for the presence of “*Candidatus Liberibacter asiaticus*.” Infection of trees and fruit by HLB pathogen significantly reduced seed weight, seed germination, and seedling height. No typical symptoms of HLB disease were observed on the seedlings throughout the period of experimentation. Polymerase chain reaction was applied to detect the presence of HLB pathogen. Two of 686 rootstock seedlings and three of 431 sweet orange seedlings were positive for the pathogen, when the seedlings were very young. However, none of these seedlings showed a positive reaction with PCR assay at older ages. In addition, these older plants did not exhibit any of the HLB symptoms. HLB pathogen may possibly be translocated into some part of the embryo during seed development and might have been eliminated from the seed tissues, resulting in PCR-negative older plants (Albrecht and Bowman 2009).

In a later investigation, rootstocks used to produce HLB-free plants were raised from seeds obtained from fruit produced on trees that were not protected against citrus psyllids, the natural vectors of HLB pathogen. It was considered essential to ensure whether transmission of “*Ca. Liberibacter asiaticus*” from seeds to seedlings occurred or not. Further, the possible transmission of HLB pathogen through seed during germ-plasm exchange has to be prevented. Two groups of 360 or more seedlings each of different *Citrus* spp. were tested using the seeds from fruit on symptomatic trees in which infection of HLB pathogen was confirmed by PCR assays. These seedlings were indexed many times over a period of 3 years for the presence of “*Ca. Liberibacter*

asiaticus.” Leaf samples were subjected to qPCR assay. No HLB symptoms were observed on any of the test plants and none of the samples assayed revealed the presence of DNA of HLB pathogen. The results indicated that transmission of “*Ca. Liberibacter asiaticus*” did not occur through seeds of citrus cultivars tested during the period of 3 years (Hartung et al. 2010).

The possibility of transmission of “*Candidatus Liberibacter asiaticus*” in citrus seed was examined in another investigation. The frequency of detection of “*Ca. Liberibacter asiaticus*” DNA in individual fruit peduncles, seed coats, seeds, and in germinated seedlings from Sanguenelli sweet orange and Connors grapefruit sampled from infected trees was compared. The DNA extracts showed the presence of HLB pathogen in 36% and 100% in peduncles of Sanguenelli and Connors fruit, respectively, as revealed by the real-time quantitative PCR (qPCR) format. Likewise, the qPCR assay detected the pathogen in DNA extracts of 37% and 98% of seed coats and 1.6% and 4.0% of extracts from corresponding seeds of Sanguenelli and Connors, respectively. Apparent colonization of Connors seeds by HLB pathogen was more extensive and uniform compared with Sanguenelli seeds. However, no pathogen DNA could be detected either in Connors or Sanguenelli seedlings by the qPCR assay. The presence of pathogen DNA in fruit peduncle and seed coats did not facilitate the transmission of the pathogen to the seedlings (Hilf 2011).

Histological changes induced by “*Candidatus Liberibacter asiaticus*” in citrus accessions susceptible and tolerant to huanglongbing (HLB) disease were investigated. Anatomical changes induced in tolerant rough lemon and sensitive sweet orange seedlings in response to HLB pathogen were analyzed in leaf, stem, and root tissues by employing light microscopy and transmission electron microscopy (TEM). Both HLB-infected rough lemon and sweet orange plants showed phloem collapse, plugged sieve elements, and the accumulation of starch in leaf petioles of symptomatic leaves. Asymptomatic sweet orange leaves showed phloem cell collapse and starch accumulation, whereas rough lemon asymptomatic leaves did not exhibit any change. In addition, starch depletion, phloem cell collapse, and the absence of phloem fibers were observed in secondary roots of diseased sweet orange plants. Overall, the tolerant rough lemon plants were not markedly stressed as revealed by anatomical observations, compared with susceptible sweet orange plants. It was suggested that the absence of distinct changes in the rough lemon root system might be a critical factor preventing the development of HLB pathogen, contributing to its tolerance to the disease (Fan et al. 2013).

The systemic movement of phloem-limited bacterial pathogen “*Candidatus Liberibacter asiaticus*” (*Las*) in citrus and initiation of infection process in the citrus plant tissues were investigated. Movement of *Las* after initial infection was monitored in the leaves and roots of greenhouse trees. The importance of root infection by *Las* was revealed in root density, storage starch content, and vascular system anatomy in relation to *Las* presence in both symptomatic and asymptomatic field- and greenhouse-grown trees. The pathogen colonized the roots preferentially before leaves, where it multiplied and rapidly invaded leaves when new foliar flesh became the sink tissue for phloem flow. This showed that the roots were damaged by root infection prior to development of visible foliar symptoms, and was not associated with carbohydrate starvation caused by phloem-plugging as previously hypothesized. The results clearly revealed the significance of early root infection to tree health and suggested a model for the phloem-limited bacterial movement from the initial insect feeding site to the roots where the bacterial pathogen multiplies, damages the host root system, and then spreads

to the rest of the canopy during subsequent leaf flushes. This framework, suggested by the results, might be useful for testing the movement of phloem-limited bacteria to gather an insight into the patterns of pathogen movement and disease spread in other host–pathogen combinations (Johnson et al. 2014).

Potato zebra chip disease caused by “*Candidatus Liberibacter solanacearum*” poses a serious threat to potato production. The pathogen is transmitted by the psyllids *Bactericerca cockerelli* to potato and tomato. Disease symptoms include stunting, leaf curling, chlorosis, swollen internodes, and the formation of aerial tubers. Darkening of medullary rays in tubers may be clearly visible when tubers are fried for chip production. As the ZC disease control depends largely on early detection of infected plants and tubers, it was considered necessary to investigate the patterns of pathogen translocation through phloem in potato and tomato plants. Plants were inoculated using psyllid vectors. Polymerase chain reaction (PCR) assay was employed to detect the pathogen in leaves at different positions and at different periods after inoculation. The rates and direction of translocation of ZC pathogen in potato and tomato were similar to patterns of virus movement observed in plants. The pathogen could move from the site of inoculation to the main stem in as little as 7 days. In potato, ZC pathogen was detected in leaves on a second, noninfested stem when the stems joined via the tuber. Symptoms developed earlier in susceptible potato cultivars compared with tolerant cultivars. Pathogen population was very low in both potato and tomato. False-negative results need not indicate the absence of infection by ZC pathogen, and improved detection methods have to be developed for effective disease management (Levy et al. 2011).

The effect of zebra chip (ZC) disease of potato caused by “*Candidatus Liberibacter solanacearum*” on seed potato sprouting was assessed. The rate of ZC-affected seed potato emergence following cold storage was not affected by time of infection in the field and the majority of ZC-infected tubers did not sprout. Time required for emergence of potato seedlings from infected tubers was significantly longer in seed tubers from plants infected ≥ 2 weeks before harvest. The plants which emerged from ZC-infected tubers were stunted, nonvigorous, and often killed after a few weeks. The rate of successful detection of ZC pathogen increased during cold storage, suggesting a continued increase in pathogen population after harvest. The transfer of seed tubers from cold storage to room temperature resulted in an increase of “*Ca. L. solanacearum*” concentration. Up to 38% of tubers tested positive following exposure to room temperature after cold storage. The results indicated that seedborne ZC had an important role in disease development and improved detection methods should be applied for the detection of ZC pathogen at harvest, especially in tubers infected late in the growing season (Rashed et al. 2015).

The patterns of phloem colonization by potato zebra chip disease pathogen “*Candidatus Liberibacter solanacearum*” (CLS) and other endophytic bacteria were studied to have a better understanding of pathogen biology and interaction with endophytic bacteria in potato tubers. Endophytic bacteria in potato tubers affected by ZC disease were examined by scanning electron microscopy (SEM), in vitro cultivation, and PCR analyses. Bacillus, coccus, and filamentous bacterial cells were observed in phloem tissue of ZC diseased tubers using SEM, but were not detected in healthy tubers. Fifty-one bacterial isolates were obtained from ZC-diseased tubers and 34 isolates were isolated from non-ZC-diseased tubers. Comparison of 16S rDNA sequences of the isolates led to the identification of bacteria and placement in 16 genera. Bacteria belonging to seven genera, namely *Brachybacterium*, *Enterobacter*, *Microbacterium*, *Paenibacillus*,

Table 6.2 Influence of temperature on development of ZC symptom and pathogen growth (potato plants inoculated with ZC pathogen and maintained in growth chamber at different temperatures) (Munyaneza et al. 2012).

Temperature range (°C)	No. of plants exposed/ no. of plants with ZC symptoms	No. of PCR-positive plants with ZC symptoms ^a	Time in days to onset of death	Time in days to plant
12–17	18/18	18	73.7 ± 5.6a	101.6 ± 6.7a
20–25	39/39	39	30.0 ± 0.5b	33.3 ± 0.7b
27–32	39/39	39	20.7 ± 0.2c	24.5 ± 0.2c
32–35	30/0	0	–	43.8 ± 2.4 ^b
35–40	39/0	0	–	45.0 ± 3.2

^a Means followed by different letters in the same column are significantly different; ^b Plants at temperatures above 32°C died before symptom development.

Staphylococcus, *Stentrophomonas*, and *Varivorax*, were isolated only from diseased tubers, while four genera, namely *Bacillus*, *Nocardia*, *Sphingomonas*, and *Spingophyxis*, were present only in healthy tubers. The results indicated the associative effects of “*Ca. Liberibacter solanacearum*” with other endophytic bacteria in potato tubers (Liu et al. 2013). Further investigations to assess the biocontrol potential of the bacterial species present in potato tubers may shed light on the feasibility of employing the most potent bacteria for the management of ZC disease.

The development of huanglongbing (HLB-) associated “*Candidatus Liberibacter asiaticus*” and the expression of symptoms have been reported to be adversely affected by temperature (Bové 2006). The effects of temperature on development of “*Ca. L. solanacearum*” and ZC disease were evaluated. Potato plants were inoculated with ZC pathogen, using infective psyllids at various temperatures, under laboratory conditions. All plants maintained at 12–17°C, 20–25°C, and 27–32°C developed ZC symptoms (Table 6.2). The symptomatic plants indexed using PCR assay showed positive amplification of pathogen-specific DNA fragment (Fig. 6.5). Plants maintained at 32–35°C and 35–40°C did not develop disease symptoms and ZC pathogen could not be detected in these plants by PCR assay. Temperatures above 32°C were detrimental to the development of ZC pathogen. The disease incidence and severity might therefore be significantly affected by the range of temperatures existing in different geographical regions (Munyaneza et al. 2012).

The biochemical responses of potato plants to infection by “*Ca. Liberibacter solanacearum*” were investigated. Levels of phenolics, peroxidases, polyphenol oxidases, and reducing sugars changed during infection, with higher levels occurring in tubers infected at least 5 weeks before harvest than in those infected only 1 week before harvest. Concentrations of phenolics alone appeared to be associated with ZC pathogen titer. Shifts in metabolic profiles might be independent of variations in the titer of the ZC pathogen (Rashed et al. 2013).

6.3.6 *Streptomyces* spp.

Streptomyces spp. are mostly soil-inhabiting bacteria and a few of them cause diseases of underground structures of plants such as tubers of potato. *S. scabies* causes the



Figure 6.5 Detection of “*Candidatus Liberibacter solanacearum*” in potato plants exposed to liberibacter-infective psyllids at 24–28°C by PCR assay. Lane 1: DNA standards; lanes 2–8: liberibacter-inoculated plants maintained at 12–17°C; lanes 9–15: liberibacter-inoculated plants maintained at 0–25°C; lanes 16–21: liberibacter-inoculated plants maintained at 27–32°C; lanes 22–29: liberibacter-inoculated plants maintained at 32–35°C; lanes 31–37: liberibacter-inoculated plants maintained at 35–40°C; lane 38: negative control; and lanes 30 and 39: pathogen DNA (positive controls). Source: Munyaneza et al. (2012). Reproduced with permission of the American Phytopathological Society, MN, USA.

common scab disease of potato tubers which occurs in all countries growing potato crops. In addition, carrot, radish, sugar beet, and parsnip are also infected by *S. scabies* (Stevens et al. 2001). Symptoms induced by *S. scabies* include raised, pitted, or superficial lesions that are usually darker in color. The disease severity depends on virulence of the pathogen strain and susceptibility levels of potato cultivar. *S. scabies* is Gram-positive, produces gray spores borne in spiral chains, and is characterized by melanin production on tyrosine-containing medium and utilization of nine diagnostic sugars. *S. scabies* ATCC49173 type strain is the most widely occurring strain in most potato-growing countries. Another species *S. turgidiscabies* ATCC700248 strain has been reported on potato tubers in Japan and Finland. *S. turgidiscabies* is morphologically and genetically distinct from *S. scabies* (Takeuchi et al. 1996; Lindholm et al. 1997).

The soilborne inoculum of *S. scabies* is predominant and seed tuber-borne inoculum is important for pathogen dispersal over long distances and the introduction into new locations where the strains or species of *Streptomyces* may be absent. The possibility of cooccurrence of *Streptomyces scabies* and *S. turgidiscabies* in potato fields and their survival on potato tubers for 6 months (the minimum time seed potatoes are stored) was examined. A time-saving and cost-effective PCR-based procedure was developed for species-specific detection of *S. scabies* and *S. turgidiscabies* in seed tubers. The occurrence of *S. scabies* and *S. turgidiscabies* in the same field, potato cultivars, tubers, and even in the scab lesion was revealed by the PCR format developed in this investigation. Repeated testing of a large number of different characterized *Streptomyces* strains originally isolated from potato tubers indicated that the primers based on variable regions of 16S rRNA gene sequences provided a highly species-specific PCR assay. Under glasshouse conditions, *S. scabies* and *S. turgidiscabies* were found to be transmissible via seed tubers after 6 months of storage. The incidence of *S. scabies* was almost twice the incidence of *S. turgidiscabies* on tubers tested fresh from the soil. Both pathogens were transmitted through seed tubers of Matilda and Sabina cultivars after 24 weeks of storage at 4°C (Lehtonen et al. 2004).

A new strain of *Streptomyces scabies* was isolated from scabby potatoes in southern Idaho, USA. The isolates (5) of this strain were consistently more virulent than the type strain ATCC49173 on radish, as indicated by disease severity scores, reduced seedling emergence, and reduced long-term plant survival. This strain had hallmarks of *Streptomyces* pathogenicity island (PAI) and possessed genes encoding the synthetase for the pathogenicity determinant thaxtomin and for a second pathogenicity factor

tomatinase. However, it lacked a third gene characteristic of the *Streptomyces* PAI, the *nec1* gene. The absence of a gene homologous to *nec1* was confirmed in two isolates by Southern blotting using the 700bp *nec1* PCR fragment as a probe. The Idaho isolates appeared to share a version of the PAI that differed from the PAI of other plant pathogenic *Streptomyces* spp. Based on morphological characteristics and the unique sequence of the 16S rRNA gene and combination of markers of the PAI, the Idaho strain was considered as a new genotype of plant pathogenic *Streptomyces*. Lack of PCR amplification in the Idaho isolates with *S. scabies*, *S. europaeiscabies*, or *S. acidiscabies*-specific primers was revealed by the PCR assay (Fig. 6.6). Isolates of Idaho strain were pathogenic to potato and radish and produced scab lesions on underground stems and stolons, in addition to potato tubers (Wanner 2007).

As the infection by *Streptomyces scabiei* occurs during early tuber development, it is not possible to follow disease development in pot-based system or in the field. A novel method involving the use of a hydroponic system was developed for precise monitoring of tuber development and successful infection of tubers in a nondestructive manner. Three potato cultivars – Desirée, Russet Burbank, and Shepody – were inoculated either by spraying the spore suspension as a fine spray mist or by applying the suspension directly on the tubers as a droplet (110 µL), using a micropipette. Spray inoculation produced about twice as many lesions than droplet inoculation method. Among the potato cultivars, Desirée and Shepody showed double the number of lesions that were produced on Russet Burbank potato (Fig. 6.6). Tubers were more susceptible to infection by *S. scabies* between 3 and 20 days after tuber initiation, confirming the earlier suggestion that the early period of tuber formation was critical to common scab disease development in potato tubers (Khatri et al. 2010).



Figure 6.6 Symptoms of common scab disease caused by *Streptomyces scabiei* on potato tubers of cultivar Desirée at harvest, following inoculation at 13 and 18 days after tuber initiation. Source: Khatri et al. (2010). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

A mechanism for the spread of plant pathogenicity determinants in soil *Streptomyces* by horizontal transfer of large chromosomal region containing genes for pathogenicity was suggested by Kers et al. (2005). Phylogenetically diverse soil streptomycetes have possibly acquired a set of genes for pathogenicity harbored in a pathogenicity island (PAI) to become pathogenic to plants. *Streptomyces turgidiscabies* produces the phytotoxin thaxtomin A. The biosynthetic genes for thaxtomin A are present on the large mobile pathogenicity island. The Nec1 protein induces necrosis on excised potato tuber tissue. The G + C content of *nec1* indicated a lateral transfer from unrelated taxon. An active 16kDa form Nec1 was shown to be secreted by *S. turgidiscabies*. The 151-amino acid C-terminal region of the Nec1 was shown to be secreted by *S. turgidiscabies* and this region conferred the necrogenic activity, as revealed by deletion analysis of *nec1* gene. The *nec1* deletion mutant exhibited significantly compromised virulence on *Arabidopsis thaliana*, *Nicotiana benthamiana*, and radish (*Raphanus sativus*). Aggressive colonization and infection of root meristem of radish by the wild-type Car 8 strain was observed, whereas the Δ *nec1* mutant Car 811 did not infect the radish root tissues (Joshi et al. 2007).

Global gene expression responses in common scab (CS-) *Streptomyces scabies*–potato tuber interaction was studied. Expression of 8746 genes in the type strain of *S. scabies* (ATCC49173) was analyzed using a custom-designed, gene-specific oligonucleotide microarray. The pathogen was grown in liquid yeast malt extract (YME) medium and 480 genes were upregulated at 24 h after supplementing the cultures with potato microtubers. Quantitative PCR analysis of eight putative or known virulence genes was consistent with the microarray data. Elevated expression of the thaxtomin A synthetase gene *txtA* and *txtR* genes regulating biosynthesis of thaxtomin A, the principal pathogenicity factor of *S. scabies*, and production of thaxtomin A were detected in cultures containing microtubers. Thaxtomin was also produced in cultures lacking microtubers, indicating that growth in YME induced thaxtomin production in *S. scabies*. All microtubers produced in vitro did not show symptoms on inoculation with the pathogen, indicating differences in the susceptibility of microtubers grown under uniform in vitro growth conditions. This condition might have limited the differences in gene expression observed between microtubers exposed to *S. scabies*, compared to the uninoculated controls (Dees et al. 2014).

6.4 Biology of Phytoplasmal Pathogens

Plant diseases assumed to be caused by viruses were previously broadly divided into two groups, namely yellows type and mosaic type diseases. Later, numerous yellows type diseases were demonstrated to be due to wall-less prokaryotes, but not due to viruses. Similarity in symptomatology and transmission by insects, often by the same taxonomic groups, were the primary reasons for the failure of earlier researchers to recognize that some of the yellows-type diseases were caused by organisms related to bacteria. The term mycoplasma-like organisms (MLOs) was first applied because of their morphological and ultrastructural similarity to mycoplasmas pathogenic to humans and animals. MLOs and mycoplasmas were placed in the Class Mollicutes which included prokaryotes without cell walls. However, the principal difference between the MLOs and mycoplasmas was the nonculturability of MLOs on any cell-free media, while mycoplasmas could be obtained in pure cultures (Lee and Davis 1984). The development of various molecular

techniques resulted in the separation of MLOs pathogenic to plants from the mycoplasmas infecting humans and animals, and facilitated their recognition as a separate group of plant pathogens. The trivial name “phytoplasmas” was officially adopted for the plant pathogenic mollicutes.

6.4.1 General Characteristics of Phytoplasmal Pathogens

Phytoplasmas differ from bacteria in the absence of cell walls and possession of a triple-layered membrane in place of a cell wall. Lack of a rigid cell wall allows them to be pleomorphic, assuming a variety of shapes and sizes ranging from 300 nm to 1.0 μ m in diameter. Phytoplasmas therefore have smallest known cells capable of independent multiplication. Due to the absence of a cell wall and consequently penicillin-binding sites, hytoplasmas are resistant to penicillin while bacteria are sensitive to penicillin. Phytoplasmas are absolutely dependent on sterols for growth, whereas bacteria do not require sterols for their growth. Two groups of Japanese researchers – Doi et al. (1967) and Ishiie et al. (1967) – demonstrated the presence of the causative agents in the phloem of yellows diseases affecting mulberry, potato, China aster, and pawlonia and their susceptibility of tetracycline antibiotics. None of the phytoplasmas has been cultured successfully in a cell-free medium so far, so information on their cultural characteristics is not available. Because of the absence of a rigid cell wall, phytoplasmas are able to pass through pores of 220 nm diameter even though the diameter of a viable cell may be more than 300 nm (Musetti and Favalli 2004). Precise identities of phytoplasmas have not been established depending on the morphological and biochemical characteristics, as in the case of bacterial pathogens.

Molecular analyses of phytoplasma genomes have provided significant insight into their molecular diversity and genetic interrelationships, which have served as the basis for several investigations on phytoplasma phylogeny and taxonomy. The first comprehensive classification scheme based on restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA was presented by Lee et al. (1998, 2000). According to this classification, the phytoplasmas are placed into 28 groups (16S rRNA group I to group XXVIII) and this system has been widely adopted. In the absence of descriptions of organisms cultured in vitro for naming the species in the class Mollicutes, phytoplasma lineages are referred to as “*Candidatus* Phytoplasma species.” The term “*Candidatus*” in scientific classification is a formal word that is placed before the genus and species name of bacteria that cannot be maintained in Bacteriology Culture Collection. “*Candidatus*” status may be used when a species or genus is well characterized, but unculturable (IRPCM 2004). For example, in Apple proliferation group (16Sr X), the subgroup X-A contains apple proliferation agent designated “*Candidatus* Phytoplasma mali.” Among the members of class Mollicutes, pathogens included under the genus *Spiroplasma* have been isolated in pure culture. *Spiroplasma citri*, causing corn stunt and citrus stubborn diseases, has been characterized. *S. citri* is a facultative anaerobe, wall-less pleomorphic cell with spiral morphology formed generally in young actively growing cultures, with a minimum viable length of helix of two turns (about 2.0 μ m $\times$ 0.1–0.2 μ m). The helices exhibit flexing and twitching movements and often rotate in a corkscrew fashion. *S. citri* lacks flagella and forms characteristic “fried egg” colonies on media containing 20% horse serum (Bradbury 1991).

6.5 Disease Cycles of Phytoplasmal Pathogens

Phytoplasmas induce certain distinct symptoms, though some symptoms may be similar to those caused by viral pathogens. Phytoplasmas cause general stunting or dwarfing of whole plants or plant parts. Chlorosis and smalling of leaves are observed frequently in infected plants in the early stages of plant growth. Antholysis of floral parts is the most characteristic feature of many phytoplasmal diseases, resulting in virescence, phyllody, and proliferation of floral parts that are transformed into leaf-like structures. Partial or total sterility of diseased plants may be seen, depending on the stage of plant growth at which infection occurs. Proliferation of axillary buds and formation of a large number of thin shoots may be evident. Periwinkle is susceptible to several phytoplasmas, and the type of symptoms induced in this plant species may help in tentative identification of the phytoplasma concerned. The phytoplasmas are spread by leafhoppers, planthoppers, and psyllids from plant to plant and their host range is limited by the preference and host range of the vector insects. Phytoplasmas are obligate parasites occurring in the plant phloem tissues and insects. The genomes of phytoplasmas are very small when compared to those of their ancestors (walled bacteria in the *Bacillus/Clostridium* group). They lack several pathways for synthesis of compounds necessary for their survival (Bai et al. 2006). As the phytoplasmas cannot exist in the free-state outside living cells, they have to be carried by infected propagative plant materials or the vector insects for their spread to short or long distances.

Information available on the infection process of phytoplasmas is very much limited. All phytoplasmas have to be introduced into the susceptible host plant cells as the first step in pathogenesis by specific insect vector(s). The insects acquire the phytoplasmas from infected plants through the stylet, then move through the intestine and circulate in the haemolymph. Subsequently, the salivary glands of the insects are colonized by the phytoplasmas. At the end of the incubation or latent period in the body of the insect, the phytoplasmas are injected, along with the saliva, into the susceptible plants while the insects feed on them. The latent period may vary widely from a few days to several weeks in different phytoplasma-vector combinations. Phytoplasmas may be experimentally transmitted from infected plant to healthy plant through parasitic dodder (*Cuscuta* spp.). The possibility of transmission of phytoplasma by seed was indicated in the case of Coconut lethal yellowing phytoplasma (Cordova et al. 2003) and the phytoplasma infecting alfalfa and lime (Khan et al. 2002; Botti and Bertaccini 2006). Spread of phytoplasmas may occur in a larger scale via vegetative propagation such as grafting of infected materials, through cuttings, micropropagation, and any other methods of multiplying plant materials that do not involve sexual reproduction (Narayanasamy 2011).

Potato purple top phytoplasma, transmitted by sugar beet leafhoppers, infects potato crops, reducing the yield significantly. This phytoplasma belongs to the group 16Sr VI. Under field conditions, eight potato cultivars were allowed to be infested by native populations of the leafhoppers during 2006, 2007, and 2008. A polymerase chain reaction (PCR) assay was applied to detect the presence of purple top phytoplasma, and the PCR-positive plants were marked with flags. Tubers were harvested from PCR-positive plants and tested for the presence of the pathogen. A subset of phytoplasma-positive tubers was planted in the greenhouse and the daughter plants growing from infected tubers were indexed for the phytoplasma. The frequency of tuber transmission ranged over 4–96% among the eight cultivars tested for a period of 3 years. Transmission of the

pathogen from infected tubers to daughter plants ranged over 0–50%, depending on the cultivar and year of testing. Russet Burbank showed the lowest rate of tuber transmission in 2 of 3 years among the eight cultivars. Across the eight cultivars, an average of 22%, 15%, and 16% of infected tubers produced infected daughter plants in 2006, 2007, and 2008 respectively. This investigation showed that adequate attention has to be paid to the selection of seed tubers to reduce the incidence and subsequent spread of potato purple top disease (Crosslin et al. 2011).

Adhesion of phytoplasma to host cells is required for successful colonization of host cells. This step is mediated by surface proteins and, among them, adhesins play an important role in pathogen invasion and pathogenicity (Rottem 2002). Abundant immunodominant proteins that are mostly membrane proteins at the surface of phytoplasmas have been identified. Major surface proteins of Mollicutes have been shown to have a role in recognition, adherence to plant or insect host cells, pathogenicity, and triggering host resistance responses. Major surface epitopes specific to each phytoplasma have been identified and these proteins are likely to be key participants in the specific interactions with host cells. An adhesin-like gene was identified in *Spiroplasma kunke-*lii**, the causal agent of corn stunt disease. Primers and fluorogenic probe based on the nucleotide sequence of the adhesin-like gene were prepared to develop a field-deployable real-time PCR assay for detection of the pathogen (Wei et al. 2006).

The interactions between the phytoplasma and plant host have been investigated in a few pathogens. Immunodominant membrane proteins and the genes encoding them were characterized in some phytoplasma groups. Three distinct types of immunodominant proteins were identified: (i) immunodominant membrane protein (Imp) was detected in sweet potato witches' broom (SPWB) (Yu et al. 1998) and apple proliferation (AP) (Berg et al. 1999); (ii) immunodominant membrane proteins A (IdpA) was located in the phytoplasma causing Western X (WX) disease (Blomquist et al. 2001); and (iii) antigenic membrane protein (Amp) was detected in the phytoplasmas causing clover phyllody (CPh) (Barbara et al. 2002) and onion yellows (OY) phytoplasma (Kakizawa et al. 2004). This protein was exported via the Sec protein secretion system accompanied by the cleavage of its N-terminal signal sequence (Kakizawa et al. 2004). Variations in amino acid sequence and antigenicity in these proteins have been observed. They may be secreted across phytoplasmal cell membrane, during protein localization. The genes of onion yellows (OY) phytoplasma that encode the SecA and SecY proteins forming essential components of the Sec protein translocation system were identified, indicating the existence of a Sec system in the phytoplasmas (Kakizawa et al. 2001). In a later investigation, a gene that encodes a putative Sec E protein, a component of the Sec protein translocation system was cloned from the OY phytoplasma. Further, a gene encoding an antigenic membrane protein (Amp) of OY was cloned and sequenced. The anti-Amp antibody generated using bacterially expressed part of OY Amp reacted specifically with an OY-infected plant extract in Western blot analysis. A partial OY Amp protein expressed in *Escherichia coli* was localized in the periplasm as a shorter, putatively processed from the protein. In addition, OY Amp protein expressed in OY and detected in OY-infected plants was also processed (Kakizawa et al. 2004).

Phytoplasmas are transmitted by insects in a circulative manner and specific insect species alone can acquire the phytoplasma from infected plants and transmit to healthy plants. The molecular basis of the interaction between *Spiroplasma citri* and the leafhopper *Circulifer haematiceps* was studied. In *S. citri*, an adhesion-related protein designated P89 was found to be directly involved in the *Spiroplasma*-vector

insect cell interaction (Yu et al. 2000). The proteins of *C. haematoceps* were screened by putative *S. citri*-binding molecules, using a spiroplasmas overlay assay of protein blots (Far-Western assay). Insect proteins were purified by SDS-PAGE technique, blotted and probed with *S. citri* proteins. *Spiroplasma* proteins showed affinity for even leafhopper proteins. The interaction between *S. citri* proteins and insect proteins with molecular masses of 50 and 60 kDa was investigated. The spiroplasmas protein with a molecular mass of 24 kDa and insect-binding capacity was the most abundant membrane protein of *S. citri*, designated spiralin. Experiments with purified spiralin and insect glycoprotein confirmed the binding of spiralin to insect glycoproteins of 50 and 60 kDa. The results indicated a key role for spiralin in the transmission of *S. citri* by mediating spiroplasmas adherence to epithelial cells of insect vector gut or salivary gland (Killiny et al. 2005).

Specific vector insects transfer the phytoplasmas, along with insect saliva, into the plant sieve elements from which the phytoplasma spreads systemically to other plant parts using the sieve tube system. However, they are unable to reach and settle in the apical meristems, making it difficult to become seedborne with a few exceptions. Because of their small size and pleomorphic form, phytoplasmas can pass freely through sieve pores so that they may be moved along with photosynthates from leaves to sugar-consuming plant organs. However, a high titer of phytoplasmas in source leaves and low colonization in sink tissues have also been reported, indicating that translocation of phytoplasmas cannot be explained only by assimilate flow (Christensen et al. 2004). The distribution of phytoplasmas in infected plants has been reported to be uneven, and seasonal fluctuations in phytoplasma titers in woody perennial plants are known. Generally phytoplasma populations were low in the roots (sink organ) and moderate in stems, while mature leaves (source organs) contained the highest titer of phytoplasmas (as high as 40-fold higher than that of roots). Variations in the amount of phytoplasma DNA between individual plants propagated from the same mother plant was also observed (Christensen et al. 2005).

Chlorosis of foliage, dwarfing, and malformation of floral parts or entire inflorescence are the symptoms frequently observed on plants infected by phytoplasmas. Reduction in the concentrations of photosynthetic pigments and total soluble proteins, impaired movement of carbohydrates, and alterations in the hormone balance and amino acid transport have been suggested as the possible factors involved in the appearance of disease symptoms (Bertamini and Nedunchezian 2001; Maust et al. 2003). Accumulation of carbohydrates in mature leaves and lower starch content in sink tissues, such as roots, might be corollary to impaired photosynthesis in phytoplasma-infected plants and a secondary effect following inhibition of phloem transport. Multiplication of phytoplasmas reaching high levels might result in clogging of sieve elements. However, reduced translocation of carbohydrates occurred in plants with low phytoplasma titer, indicating that the pathogens might have a more indirect influence on host metabolism and phloem function (Maust et al. 2003). Higher consumption of carbon sources might affect the growth rate of phytoplasma, and this may directly or indirectly produce more severe symptoms in onion infected by yellows phytoplasma which include severe yellowing, stunting, proliferation, and witches' broom symptoms (Oshima et al. 2001).

Very little is known about the modes of reproduction (asexual/sexual) of phytoplasmas which possess one circular double-stranded DNA chromosome as for other members of Mollicutes. In the phytoplasma, the triplet coding for tryptophan (trp) is UGG. On

the other hand, other prokaryotes (mycoplasmas and spiroplasmas) have the triplet UGA coding for trp. Further, phytoplasmas are genetically different since they have a spacer region (about 300bp) between the 16S and 23S ribosomal regions which codes for isoleucine tRNA (tRNA^{Ile}) and part of the sequences of alanine tRNA (tRNA^{Ala}). The genome of phytoplasmas has low G + C content and many predicted genes are present in multiple copies. Phytoplasma genomes contain large numbers of transposon genes and insertion sequences that are unique to them. They are responsible for variability and help the phytoplasmas in their survival in diverse environments of plants and insects. These sequences are referred to as variable mosaics (SVM) and potential mobile units (Bai et al. 2006; Jomanitiene et al. 2007).

Phytoplasmas contain extrachromosomal DNA (EC-DNA) such as plasmids, as in bacterial plant pathogens. Genes coded by EC-DNA (plasmids) have important roles in the pathogenicity and virulence of several bacterial plant pathogens. Similar roles for genes in plasmids of phytoplasmas are inferred as revealed by investigations on onion yellows (OY) strain of "*Candidatus Phytoplasma asteris*" (Ishii et al. 2009). The nutritional requirements of the phytoplasmas are indicated to some extent by the analysis of the genome sequences of the onion yellows (OY) phytoplasma. The OY genome is about 861kb in size and contains 754 open reading frames (ORFs), corresponding to 73% of coding capacity. The OY phytoplasma does not have genes for the biosynthesis of amino acids and fatty acids, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (production of ATP). Further, genes for the phosphotransferase system and for metabolizing UDP-galactose to glucose-1-phosphate are not present in the phytoplasmas, suggesting that these pathogens are endowed with a unique intake and metabolic system, yet to be identified. As the phytoplasmas lack several important functions that are carried out by bacteria and other microorganisms, the phytoplasmas represent a group of phytopathogens existing as a result of a reductive evolution as a consequence of life as intracellular parasites in nutrient-rich environments (Oshima et al. 2004). However, phytoplasmas contain many genes encoding transporter systems such as malate, metal-ion, and amino acid transporters, some of which have multiple copies. These transporter systems appear to be efficient in providing essential nutrients from the host plants which suffer because of the disturbed metabolic balance, leading to development of disease symptoms (Oshima et al. 2004).

Some of the phytoplasmal pathogens that have been studied in some detail are described in the following sections.

6.5.1 Apple Proliferation Phytoplasma

Apple proliferation (AP) is a severe disease of apple caused by AP group of phytoplasmas (Seemuller et al. 1998). The pathogen also infects stone fruit trees. This group includes European stone fruit yellows ("*Ca. Phytoplasma prunorum*") and pear decline ("*Ca. Phytoplasma pyri*"). The pathogen could be detected in the sieve tubes of the current season's phloem. It is highly pleomorphic, cell size ranging over 200–800 nm in diameter, enclosed by trilaminar cytoplasmic membrane. Nonribosomal DNA fragment of the pathogen has three putative ORFs. At least three different AP phytoplasma subtypes designated AT-1, AT-2, and AP-15 have been recognized (Jarausch et al. 2000). Molecular characterization of the genes coding the ribosomal proteins L22 and S3 indicated that the heterogeneity within isolates of "*Ca. Phytoplasma mali*" was at higher

level. Based on the analysis of ribosomal and nonribosomal DNA fragments of “*Ca. Phytoplasma mali*” in populations from northwestern Italy, two phytoplasmal genetic lineages, AT-1a and AT-1b, were differentiated. The molecular markers on 16S rDNA identified in this investigation might be helpful for epidemiological studies on apple proliferation disease (Casati et al. 2010).

Apple proliferation phytoplasma (AP) is reported to be transmitted via natural root fusions/root grafts and through psyllids and leafhoppers. *Cacopsylla picta* and *C. melanoneura* transmit AP phytoplasma in a persistent-propagative manner. Once the vectors become infective, they remain infective throughout their lifespan. The overwintering psyllids could transmit the AP phytoplasma (Tedeschi et al. 2003). AP phytoplasma distribution in apple trees was found to be irregular. During winter months, the AP phytoplasma population decreased markedly (Schaper and Seemüller 1982). The phenomenon of recovery (spontaneous remission) of AP phytoplasma-infected plants was observed following the cooler months. The recovered plants had high populations of the pathogen in the roots (Carraro et al. 2004). Loss of plant vigor and proliferation of terminal buds resulting in a rosette of leaves during autumn are the early symptoms. Later premature development of axillary buds, giving rise to formation of secondary shoots/shoot proliferation (witches’ broom), may confirm the infection by APP (Bovey 1963).

AP phytoplasma has been classified as a quarantine organism in Europe and North America, emphasizing the urgent need for development of a rapid, specific, and sensitive diagnostic procedure for large-scale application (Baric and Dall-Via 2004). Real-time PCR format, developed in a later investigation, was a high-throughput detection method to detect all destructive phytoplasmas belonging to the apple proliferation group that are recognized as quarantine organisms. This procedure provided highly sensitive detection due to the high level of specificity of the primers employed. It has the potential for wide application in a large-scale sanitary program in nursery selection (Torres et al. 2005).

6.5.2 Lime Witches’ Broom Phytoplasma

“*Candidatus Phytoplasma aurantifoliae*” is the causal agent of lime witches’ broom disease. This phytoplasma belongs to the Faba bean phyllody phytoplasma group (16Sr II) (Seemuller et al. 1998), 16Sr II subgroup B (Lee et al. 1998). “*Ca. Phytoplasma aurantifoliae*” infects the citrus cultivars acid lime, Indian Palestine sweet lime (*C. limettioides*), sweet limetta, and citron (*C. medica*). The infected trees show small pale green leaves and thin shoots developed from axillary buds with short internodes. The pathogen is localized in the phloem elements of diseased plants and is irregularly distributed. The midribs of the small leaves contain the highest pathogen population. “*Ca. Phytoplasma aurantifoliae*” could be experimentally transmitted to *Catharanthus roseus* using parasitic dodder (*Cuscuta campestris*) (EPPO 2006). The phytoplasma induced callus deposition in the sieve plates, accumulation of starch in chloroplasts, disorganization of chloroplasts, alterations in cell-wall thickness, and accumulation of polyphenols, in addition to phloem necrosis (Mardi et al. 2011). Several genes that were modulated in Mexican lime trees following infection by the phytoplasma were related to defense, cell walls, and response to stress. The expression of autophagy protein 5 was repressed. Autophagy is a survival mechanism that protects cells against unfavorable environmental conditions such as pathogen infection (Kwon and Park 2008; Zamharir and Salekdeh 2013).

6.5.3 Flavescence Dorée (FD) Phytoplasma

“*Candidatus* Phytoplasma vitis” is the causal agent of grapevine flavescence doree disease infecting grapevine plants in the Europe. “*Ca. Phytoplasma solani*” causes bois noir, another important phytoplasmal disease of grapevine. “*Ca. Phytoplasma vitis*” belongs to the group 16Sr V, subgroups C and D (16Sr V-C and 16Sr V-D). “*Ca. Phytoplasma vitis*” causes serious yellows disease and is a quarantine pathogen in Europe. Grape and wine production is significantly reduced due to progressive decline of plants and the susceptible plants are killed within a few years.

FD is caused by several strains which are classified into subgroups 16Sr V-C and 16Sr V-D. The strains of 16Sr V-D are most widespread in the grapevine-growing countries (Arnaud et al. 2007; Filippin et al. 2009). The pathogen is an obligate parasite found in the phloem sieve tubes of infected plants. The vector of “*Ca. Phytoplasma vitis*” is the American grapevine leafhopper *Scaphoideus titanus*. It can transmit the pathogen after an incubation period of 4 weeks (Papura et al. 2012). Long-distance dispersal of the pathogen is largely due to transport of infected propagative materials. Because of the irregular distribution of the pathogen in the infected grapevine plants, it is difficult to detect the phytoplasma in different plant parts. The symptoms include leafroll, discoloration of lamina and veins, partial or total lack of lignin accumulation (lignification) with flexuous canes, and decline of part of or an entire vine stock. Shoots fail to ripen and are thin, rubbery, and hang down. Buds may become necrotic. Flavescence dorée is listed as a harmful pathogen in 16 countries (Mackesy and Sullivan 2013).

6.5.4 Palm Lethal Yellowing (LY) Phytoplasma

“*Candidatus* Phytoplasma palmarum” is the incitant of palm (coconut) lethal yellowing disease. This disease has been recorded in Haiti, Dominican Republic, Cuba, and Jamaica, and in Florida and Texas in the USA. This phytoplasma, based on 16Sr rRNA gene sequence, was assigned to 16Sr IV Coconut yellows group, subgroup A (Lee et al. 2000). The disease was reported to occur in several locations throughout the Old World humid tropics. The presence of the pathogen was observed in immature tissues of palms by electron microscopy (Thomas and Norris 1980) and was detected by PCR assay by employing phytoplasma “universal” and/or group-specific rRNA operon primer pairs (Cordova et al. 2003). The only known vector of the phytoplasma is the neotropical planthopper *Hapalaxius* (= *Myndus*) *crudus* (American palm cixiid). Primary infection of the highly susceptible Atlantic tall coconut ecotype is followed by a prolonged latent phase, estimated as 112–262 days in young nonbearing palms and as long as 450 days in mature palms of large size.

Treatment of early infected palms with oxytetracycline-HCl through stem injection resulted in remission of disease symptoms to some extent. A wide variation in symptoms was observed and none had diagnostic value. Premature shedding of most of the fruits, regardless of developmental stages, blackening of the fruit, and necrosis of newly emerging inflorescence with partially or totally blackened flower spikelets were observed in the infected palms. All palm species are susceptible and *Thrinax radiata* (Florida thatch palm) is a symptomless host for the phytoplasma. Symptoms of lethal yellowing may overlap some of the symptoms of Ganoderma butt rot disease, boron deficiency, and potassium deficiency, resulting in confusion and inconclusive diagnosis of phytoplasma disease (Sullivan and Harrison 2013).

6.6 Appendix

6.6.1 Stab-Inoculation of *Xanthomonas campestris* pv. *vitians* on Lettuce Plants (Barak et al. 2002)

6.6.1.1 Isolation of the Pathogen

- i) Select healthy lettuce plants (8–11 weeks old) and stab the lower stems with a sterile dissecting needle at 0.5 cm above the soil line to a depth of 5 mm.
- ii) Place the cell suspension (20 µL) of the bacterial pathogen in the wound in 16 plants using a Pipetman (P20), and maintain suitable control (4 plants).
- iii) Take samples of inoculated plants (4) and control plant (1) at 4 h interval commencing at 4 h postinoculation, and assay the sample to determine pathogen populations in each sample.
- iv) For detection of the pathogen, after removal of leaves cut the stem into 2 cm sections with a sterile razor blade and surface-sterilize the sections for 10 min with detergent (5% Physan 20; Marin, CA, USA); rinse with sterile distilled water for 2 min.
- v) Place the sections individually in a Universal extraction bag (Bioreba Ag; Reinach, Switzerland); crush with a homogenizer; add sterile water (1 µL) to each bag and mix well.
- vi) Plate 100 µL suspension on suitable medium for isolation of the bacterial pathogen.

6.6.1.2 Determination of Pathogen Location in Lettuce Stems

- i) Cut surface of each stem piece from stab-inoculated plants and press them gently to squeeze out the vascular fluids for 10 s onto the medium kept in petridishes; mark the outlines of the stem sections on the under-surface of the plate with a marker.
- ii) Incubate for 3 days at 28°C.
- iii) Record the position and appearance of the bacterial colonies developing on the medium.
- iv) Establish the identity of the bacteria using a PCR-based technique.

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7

Detection and Identification of Viruses and Viroids

Viruses and viroids infect large numbers of plant species; some of them are transmitted through seeds of certain plant species only, and not in all plant species infected by them. Many seedborne viruses have been recognized as pathogens of quarantine importance. Infected seeds and propagules, forming the primary sources of inoculum, carry the viruses over short and/or long distances and also to subsequent generations of crop plants, and introduce more virulent strains into new areas where they are not prevalent or provide additional inoculum facilitating rapid spread of the disease(s). In addition, vertical spread of the viruses and viroids to subsequent generations occurs through multiplication of asymptomatic seeds and propagules that are not certified by appropriate agencies. As the management of viral pathogens through host resistance, vector control, and cultural practices has been shown to have limited effectiveness, use of virus-free seeds and propagules has been emphasized for restricting incidence and spread of the disease(s) as in the case of *Barley stripe mosaic virus*, *Lettuce mosaic virus*, *Bean common mosaic virus*, *Cowpea mosaic virus*, and *Soybean mosaic virus*. Certification programs to provide clean and virus-free seeds and propagules and quarantine programs to restrict/avoid the introduction, incidence, and spread of the virus and viroid diseases are in operation in several countries with their own rules and regulations. Various methods have been developed based on the biological, biochemical, immunological, and nucleic acid properties of viruses for reliable, sensitive, and specific detection, identification, and differentiation of viruses and their strains in seeds and propagules (Narayanasamy 2011). The relative usefulness and applicability of these diagnostic methods are discussed in this chapter.

7.1 Detection of Viruses in Seeds

Viruses are submicroscopic pathogens that cause several serious diseases in all known organisms from bacteria to highly evolved human beings. They are obligate parasites requiring the availability of living cells for their replication and development. Plants are infected by one or more viruses, most of them being spread by insects, mites, nematodes, fungi, and parasitic dodders as their natural vectors. The viruses have to be introduced into the susceptible cells through the aid of vectors while feeding on the susceptible host plant tissues, since they cannot penetrate plant cells through intact epidermis or natural openings as fungal or bacterial pathogens do. Experimental

transmission of plant viruses can be achieved through mechanical transmission or graft-inoculation. Plant viruses are intracellular, obligate pathogens and are devoid of any demonstrable enzymatic activity, facilitating parasitic activity. They have a simple constitution and differ distinctly from other cellular pathogens not only in their size but also in their shape, which varies from rigid rod-shape or bacilliform to spherical or polyhedral. The viruses primarily contain a protein coat which encloses either a ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecule(s) as their genome. Plant viruses have a unique method of replication which is not observed in any other known organisms. The protein coat and genomic nucleic acid are synthesized separately at different specific sites in susceptible cells and, as the concentration of the protein and nucleic acid components reach required levels, they are assembled together at the appropriate time to form progeny virus particles. Studies of molecular and biological characteristics of viruses have been instrumental in understanding the functions of different cistrons (genes) in the viral genomes, and the various strategies adopted by different viruses for replication that result in increase in virus populations under optimal conditions, leading to the development of symptoms characteristic of the virus infection.

7.1.1 Plant Virus Taxonomy

The name of the host plant and the type of major symptoms induced by the virus formed the criteria for naming viruses earlier (e.g., *Tobacco mosaic virus*). As the number of reported viruses infecting plants has increased rapidly, the need for a comprehensive system of nomenclature and classification of viruses was realized. The International Committee on Nomenclature of Viruses (ICNV) was established in 1966 to develop a universal taxonomy for viruses. With increasing knowledge of the properties of plant viruses, they have been classified into 20 families and 71 genera, with another 17 genera yet to be assigned to appropriate families. Viruses are divided into two groups based on the nature of viral genome as either RNA viruses and DNA viruses. The RNA viruses are divided into single-stranded (ss) + sense RNA viruses, double-stranded (ds) + sense RNA viruses, and single-stranded (ss)–sense RNA viruses. The number of DNA viruses infecting plants is much smaller, and they are divided into ss-DNA viruses and reverse-transcribing ds-DNA viruses. The virus particle morphology and presence of membranous envelope, immunological properties, nucleotide sequences of viral genomic nucleic acid and amino acid sequences of capsid protein, and the presence of virus-associated protein are the additional important properties used for the identification, classification, and differentiation of plant viruses and their strains (Table 7.1) (Mayo and Brunt 2007).

7.1.2 Biological Methods

7.1.2.1 Symptoms Induced by Viruses

The pathogenic potential (virulence) of the virus determines its ability to infect one or more plant species in which they may induce similar or a range of symptoms. The seed-borne viruses may or may not induce recognizable symptoms on the seeds and/or plants. However, many of them cause a reduction in size and weight and shrinkage resulting in lower seed germination percentage and/or weak seedlings. Distinctive symptoms of infection of seeds by viruses may be clearly visible in the young seedlings and may be useful for putative identification of viruses. The extent of seed transmission of viruses to plants growing from those seeds, and the severity of symptoms expressed,

Table 7.1 Plant viruses belonging to different families, genera, and type species transmitted through seeds/propagules of infected plants (Mayo and Brunt 2007).

Virus genome	Family	Genus	Type species
(+) sense ss-RNA viruses	Potyviridae	<i>Potyvirus</i>	<i>Potato virus Y</i>
	Comoviridae	<i>Comovirus</i>	<i>Cowpea mosaic virus</i>
		<i>Nepovirus</i>	<i>Tobacco ringspot virus</i>
	Luteoviridae	<i>Enamovirus</i>	<i>Pea enation mosaic virus</i>
	Tombusviridae	<i>Carmovirus</i>	<i>Carnation mottle virus</i>
	Bromoviridae	<i>Alfavirus</i>	<i>Alfalfa mosaic virus</i>
		<i>Cucumovirus</i>	<i>Cucumber mosaic virus</i>
	Closteroviridae	<i>Ampelovirus</i>	<i>Grapevine leafroll-associated virus 3</i>
	Flexiviridae	<i>Potexvirus</i>	<i>Potato virus X</i>
		<i>Trichovirus</i>	<i>Apple chlorotic leaf spot virus</i>
		<i>Mandarivirus</i>	<i>Indian citrus ringspot virus</i>
		<i>Tobamovirus</i>	<i>Tobacco mosaic virus</i>
	Unassigned genera	<i>Tobravirus</i>	<i>Tobacco rattle virus</i>
		<i>Hordeivirus</i>	<i>Barley stripe mosaic virus</i>
		<i>Pecluvirus</i>	<i>Peanut clump virus</i>
		<i>Pomovirus</i>	<i>Potato mop-top virus</i>
		<i>Sobemovirus</i>	<i>Southern bean mosaic virus</i>
		<i>Ophiovirus</i>	<i>Citrus psorosis virus</i>
(-) sense ss-RNA viruses	Unassigned genera		
ds-RNA viruses	Reoviridae	<i>Fijivirus</i>	<i>Fiji disease virus</i>
ss-DNA viruses	Nanoviridae	<i>Babuvirus</i>	<i>Banana bunchy top virus</i>

depend on the level of susceptibility/resistance of cultivars of the crop plant species. The incubation period required for symptom expression may vary depending on the plant species/cultivar. If the incubation period is long in the primary host (crop), some plant species designated as diagnostic, indicator, or local lesion hosts are used for detecting these viruses, especially those viruses infecting fruit trees or woody plants. The virus may be localized in the inoculated leaves, which may exhibit chlorotic or necrotic local lesions (primary symptoms). As the local lesions are considered to be initiated by a single virus particle, the plant species responding with the production of countable local lesions may be used for the detection and quantification of concentration of the target virus(es). Plant species commonly used as indicator/diagnostic hosts are *Nicotiana glutinosa*, *N. clevelandii*, *Gomphrena globosa*, *Phaseolus vulgaris*, *Vigna unguiculata*, *Chenopodium amaranticolor*, *C. quinoa*, and *Cucumis sativus* (Chitra et al. 1999; Narayanasamy and Doraiswamy 2003; Rowhani et al. 2005). *Wheat streak mosaic virus* (WSMV) infects pearl millet (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor*), causing virus-like symptoms. WSMV was detected by mechanically inoculating 10 day old seedlings raised on soil-filled metal flats of foxtail millet (*Setaria italica*), porosomillet (*Panicum milliaceum*), Westford barley, corn, and sorghum plants.

The inoculated plants were maintained in a greenhouse for 3 weeks under natural light conditions at 18–27°C. These assay plants produced disease symptoms and the infection by WSMV was confirmed by ELISA tests (Seifers et al. 1996).

7.1.2.2 Modes of Transmission of Viruses

Information on the modes of transmission of virus(es) under investigation may be useful as one of the criteria to identify the viruses concerned.

7.1.2.2.1 Seed Transmission

It has not yet been possible to isolate viral pathogens in any cell-free medium, so the extracts of seeds or seed parts are inoculated onto healthy plants of the same species or a diagnostic host plant for detecting the presence of the virus in seed tissues. The transmission of viruses via seeds occurs in the case of several viruses infecting different crops. Seed transmission is one of the characteristics of *Nepoviruses* and *Tobraviruses*, whereas this phenomenon occurs in the case of viruses belonging to *Comovirus*, *Cucumovirus*, *Ilarvirus*, *Potyvirus*, *Hordeivirus*, and *Tobamovirus* groups. On the other hand, seed transmission is rarely observed in the viruses belonging to *Bromovirus*, *Carlavirus*, *Potexvirus*, *Tombusvirus*, and *Tymovirus* groups (Matthews 1981). Seed transmission of viruses may be of two types. Seed transmission may occur due to contamination of seed coat as in *Tobacco mosaic virus* (TMV) in tomato seeds and the infection of developing seedling may result due to mechanical means. A small percentage of seeds carry the virus only in the endosperm where the virus can persist for several years. In other virus–host interactions, the virus infects the embryo either through the ovary or pollen. This type of seed transmission is more common and is widely prevalent (Broadbent 1965).

Grow-out tests are conducted in insect-proof greenhouses by planting the required number of seeds of samples in pots with soil amended with needed nutrients. The seedlings are observed at regular intervals for the development of visual symptoms characteristic of the virus under investigation. Mungbean plants were inoculated with *Blackgram leaf crinkle virus* at 5 day intervals from 5 to 50 days of age and the development of symptoms of the disease was recorded. The percentage of seed transmission of the virus was high (41.9%), when 5 day old plants were inoculated, and the percentage of infection decreased progressively as the age of plants at inoculation increased. Multiple correlations indicated a negative correlation between percent infection of plants and age of plants at inoculation, and also between percentages of seed transmission of the virus (Narayanasamy and Jaganathan 1975). The detection of *Lettuce mosaic virus* (LMV) in commercial lettuce seed stocks in the USA was based on growing large numbers of lettuce seedlings in aphid-proof glasshouses and inspecting the seedlings (indexing) for the development of mosaic disease symptoms. The importance of lettuce mosaic disease was reduced significantly by selecting disease-free plants (Grogan et al. 1952; Grogan 1983). The tolerance level of LMV was derived empirically by relating the seed infection (%) to numbers of infected plants at harvest. The tolerance limit of LMV varied with different countries. Lettuce seed consignment from the USA by UK could be obtained only from lettuce crops that had no LMV-infected seedling among 3000 seedlings. Alternatively, none of the 5000 seeds tested on a suitable indicator plant carried LMV (Anonymous 1980). Excellent control was achieved in California for more than 20 years by adopting a tolerance of 0 infected seeds in 30,000 seeds, an eye-opener for all involved in the effective management of seedborne virus diseases.

The importance of seed health testing to reduce the incidence of LMV in lettuce was clearly demonstrated, and similar benefits for crop production was also indicated for the fungal pathogen *Leptosphaeria maculans* in brassica and bacterial pathogen *Clavibacter michiganensis* subsp. *michiganensis* in tomato (Maddox 1998). Grow-out tests were performed for indexing cowpea seeds of 2112 germplasm accessions and pre-introduction seed lots of cowpea (*Vigna unguiculata*). The presence of 45 seed-borne isolates of *Blackeye cowpea mosaic virus* (BICMV) and 54 seedborne isolates of *Cowpea aphid-borne mosaic virus* (CABMV) potyviruses in cowpea seeds was detected by inspecting the developing seedlings (Bashir and Hampton 1996). Cowpea seed samples (136) were tested by growing-out for the presence of blackeye cowpea mosaic strain of *Bean common mosaic virus* (BCMV), *Cowpea aphid-borne mosaic virus*, *Cowpea mosaic virus* (CpMV), *Bean yellow mosaic virus* (BYMV), and *Cucumber mosaic virus* (CMV). Of the 136 seed lots tested, 43 cowpea seed lots were infected by BCMV-BICMV strain (Udaya Shankar et al. 2009).

The frequency of seed transmission of *Indian peanut clump virus* (IPCV) was determined by sowing seeds in wooden trays at 100 seeds/tray in a glasshouse at 25–30°C. Peanut plants were scored for disease symptoms at three weeks after emergence. In addition to assessment of IPCV infection by visual observation, enzyme-linked immunosorbent assay (ELISA) tests were also performed to detect the virus in the seedlings. The frequency of seed transmission of IPCV was highest in the genotype ICG3704 (28/275) and the lowest in ICG4790 (6/170), indicating the differences in the ease of seed transmission of the virus in some peanut genotypes (Reddy et al. 1998). The extent of seed transmission of *Cucumber mosaic virus* in spinach was determined by seed grow-out tests. Crosses were made between healthy and infected male and healthy and infected female plants in all combinations under controlled conditions in glasshouse. Approximately 50–100 seeds derived from each of 5–10 maternal plants per cross were harvested and plants from these seeds were grown separately in large flats. Virus infection was assessed by symptoms at 5-leaf stage of plant growth. Of the 536 seedling tested, 79 seedlings harbored CMV; the average infection incidence for five experiments was 14.8%. Since the spinach seedlings were well protected against outside sources of CMV and aphid vectors, the seedlings showing CMV symptoms indicated positive transmission of the virus through spinach seed. Infection of seedlings by CMV was confirmed by ELISA tests (Yang et al. 1997).

Several factors influence the seed transmission of viruses. Variations in the proportions of seeds carrying the virus have been observed. Seed transmission of some viruses may occur in high proportions as in the case of *Barley stripe mosaic virus* in barley seeds (15–100%), *Bean common mosaic virus* in bean (1–93%), *Tomato black ring virus* in soybean (91%), *Tobacco ringspot virus* in soybean (78–82%), and *Tomato ringspot virus* (76%) (Shepherd 1972). Furthermore, the frequency of seed transmission may depend on the strains of the virus. Strains of *Alfalfa mosaic virus* and *Peanut mottle virus* are transmitted in different proportions of seeds from infected plants (Frosheisher 1964; Adams and Kuhn 1977). Seed transmission of *Soybean mosaic virus* is significantly affected by resistance/susceptibility of soybean cultivars, as reflected by percentages of seed transmission in the range 0–68%. Likewise, *Bean common mosaic virus* was transmitted via seeds of different cultivars to different extents (<1% to >75%). Similar variations in seed transmission of *Lettuce mosaic virus*, *Barley stripe mosaic virus*, and *Peanut mottle virus* (ICRISAT 1984) have been reported. Seed transmission is at a higher rate when infection occurs in younger plants, before fertilization of the ovules.

The younger the plants at the time of infection, the greater the percentage of seed transmission. *Tobacco ringspot virus* may be transmitted through 100% of soybean seeds if plants are infected for a considerable period before flowering (Owusu et al. 1968). In blackgram (*Phaseolus mungo*), seed transmission of leaf crinkle virus was progressively reduced from 42% to 3% as the age of plants at the time of inoculation increased from 5 days to 45 days (Narayanasamy and Jaganathan 1975).

Some of the seedborne viruses are transmitted to ovules of healthy plants through pollen from infected plants, implying that seeds alone carry the virus in such plants that are otherwise virus-free. *Common bean mosaic virus* was transmitted to about 25% of the progeny of healthy bean plants which were fertilized with pollen from infected plants (Nelson and Down 1933). Infected pollen produced 60–76% infection of progeny by *Common bean mosaic virus*, while infection of female plants resulted in 67% seed infection. When both parents were infected, seed infection reached a higher level (86%), indicating that infections of male and female parents had an additive effect on seed transmission of *Common bean mosaic virus* (Medina and Grogan 1961). *Alfalfa mosaic virus* was found to be more efficiently transmitted through pollen than via ovaries (Frosheiser 1964). The presence of virus particles in pollen could be detected using an electron microscope. *Barley stripe mosaic virus* particles were observed in both the cytoplasm and nuclei of the sperm cells (Carroll 1980).

7.1.2.2.2 Transmission of Viruses by Vectors

The nature of the vector transmission characteristics of the viruses have been the basis of grouping some viruses. Except for *Tobacco mosaic virus*, all seed-transmitted viruses have one or more species of aphids, whiteflies, nematodes, or fungi as their natural biological vectors involved in the spread of diseases caused by them. In such cases, use of virus-free seed alone may not be sufficient for effective management of the virus diseases. Generally, the seedborne viruses have a nonpersistent or semipersistent relationship with their vectors. The type of relationship between virus and vector and the vector specificity may help in the putative identification of plant viruses. These procedures are however time-consuming and cumbersome, although they provide the basic information of the biological characteristics of plant viruses (Narayanasamy 2001, 2011).

7.1.2.2.3 Mechanical (sap) Transmission

The transmission of plant viruses by extracting the sap from infected tissues, using a buffer to protect the viruses against inactivation and rubbing the infective sap on the leaves of susceptible plants dusted with an abrasive, may be achieved in the case of most seed-transmitted viruses. Viruses such as *Tobacco mosaic virus* (TMV) and *Potato virus Y* (PVY) can be easily transmitted by mechanical inoculation. *Grapevine fan leaf virus* (GFLV) could be indexed on the indicator hosts such as *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana clelandii*, and *Cucumis sativus* (cucumber), which produce countable local lesions much earlier than the systemic host grapevine, facilitating early detection of the virus. Likewise, members of *Nepovirus* and *Ilarvirus* infecting *Prunus* spp. could be detected by sap inoculation to *C. quinoa*, *Nicotiana occidentalis*, *N. benthamiana*, and *C. sativus* (Rowhani et al. 2005). Isolates of *Zucchini yellow mosaic virus* (ZYMV) infecting cucurbits could be detected and differentiated based on the type of symptoms induced by them on nine host plant species which reacted with local lesions or systemic symptoms (Bananej et al. 2002). *Cucumber mosaic virus* (CMV) isolates

associated with Banana mosaic disease (BMD) were grouped into four pathotypes based on the symptoms induced in banana, *N. glutinosa*, and cowpea (Chou et al. 2009).

7.1.2.2.4 Grafting/Budding Methods

All viruses infecting dicotyledonous plants may be transmitted by appropriate grafting/budding procedures. Common horticultural methods of propagation may be enough for the transmission of most of the viruses by inserting the scion tissue or bud from infected plants into the healthy test plant. Indexing mother plants and planting materials for the presence of viruses is generally adopted for certification and quarantine programs. Grapevine viruses such as *Grapevine fan leaf virus* (GFLV) may be detected by graft-inoculating *Vitis rupestris* St George (Rowhani et al. 2005). Indexing of mother plants for the presence of viruses is routinely adopted for other fruit crops such as citrus, prune, and stone fruit crops to identify those infected and eradication any viruses before the planting materials are taken for propagation.

7.1.3 Immunoassays

Immunoassays have been applied effectively for the detection, identification, and differentiation of plant viruses and their strains. They have been used more frequently for the detection of viruses when compared to other microbial pathogens (fungi, bacteria, and phytoplasmas). Because of the structural simplicity of plant viruses, antisera specific to numerous plant viruses could be prepared more easily. On the other hand, fungal and bacterial pathogens have complex antigens whose nature may vary depending on the stage of development in their life cycle. The molecular viral protein used to induce the production of antibodies is referred to as immunogen and the molecules recognized by the antibodies are designated antigens. The lymphocytes circulating in the blood secrete antibodies in response to the presence of an immunogen, and the cell-free component of blood containing antibodies is the antiserum. A number of antibodies may be produced when a purified virus preparation is injected into an animal system and these antibodies may recognize different binding sites (epitopes) on the same virus protein molecules. These antibodies are known as polyclonal antibodies (PABs) which may show cross-reaction with closely related virus or strain, indicating the lower level of specificity of serological reaction.

The hybridoma technology introduced by Kohler and Milstein (1975) was useful in overcoming many problems associated with PABs. Hybridomas are somatic cell hybrids formed by the fusion of β -lymphocytes (antibody-producing cells) with myeloma cells (capable of indefinite multiplication). The hybridoma has the ability to produce a specific antibody from the lymphocyte and to be grown on synthetic medium indefinitely in vitro from the myeloma cells. The monoclonal antibodies (MAbs) produced by a hybridoma are identical and specific to a single epitope of the immunogen. Details of production of PABs and MAbs are available in the earlier publications (Narayanasamy 2005, 2011).

The positive reactions of immunoassays may be directly visualized or recognized by the binding of a specific antibody to its homologous antigen. The immunoassays depending on the immunoprecipitation of antibody/antigen complex are performed in narrow tubes. Some of the immunoassays depend on the ability of antigen/antibody to diffuse through agar and the formation of visible precipitin lines, indicating the positive interaction between the reactants. Electrophoresis of antigen mixtures followed by

immunodiffusion of the antibodies may be used for identification of virus strains. Agglutination of antigens with related antibodies adsorbed onto a protein A-coated surface of cells of the bacterium *Staphylococcus aureus*, or covalently bound to protein A-coated Sepharose beads, formed the basis of establishing positive results of virobacterial agglutination (VBA) test for the detection of *Potato virus Y* (Walkey et al. 1992) and *Cocoa swollen shoot virus* (Hughes and Ollennu 1993). As these immunoassays require large volumes of antiserum and high concentrations of virus in plant tissues, in addition to being time-consuming and less consistent in providing the results, they are not employed where more precise and sensitive detection of viruses are needed.

Labeled antibody techniques have been developed by attaching labels or markers such as enzymes, fluorescent dyes, or radioactive materials to either antigens or antibodies. These methods have higher levels of specificity, sensitivity, reliability, and rapidity in detection and identification of viruses, particularly in asymptomatic plant tissues in which the virus titer is likely to be low and uneven. Further, the possibility of automation for handling a large number of samples has attracted the attention of the personnel of quarantine and certification programs. Among the labeled antibody techniques, enzyme-linked immunosorbent assay (ELISA) and its variants have been shown to be effective for the detection, identification, and differentiation of plant viruses transmitted via seeds and propagules. The immunodiagnostic assays were initially developed for the detection of viruses in plant tissues and were later adapted for the detection of fungal and bacterial pathogens.

Extracts of seeds for detection of viruses are prepared by grinding the seed samples and suspending in a suitable buffer to protect the virus particles against denaturation, followed by centrifugation to sediment heavier fragments which may be pelleted. The supernatant containing the target virus(es) is assayed for their detection and quantification. Alternatively, the seeds are incubated for the required period at optimal temperature and moisture conditions. The extracts are prepared from emerging seedlings and seed remnants using a suitable buffer. This procedure allows virus replication during the incubation period, facilitating successful detection of the target virus(es) and improving the sensitivity of virus detection by the immunoassay. The standards have been prescribed for each seed-transmitted virus and the immunoassay which is sensitive enough to meet the tolerance limits has to be selected for virus detection. The presence of even a single lettuce seed infected by *Lettuce mosaic virus* (LMV) in a lot of 5000 seeds is not permitted in the UK (Maude 1996). The tolerance limit for each pathogen is determined based on the potential of the target pathogen for rapid spread, the economic importance of the crop, and the extent of damage that is likely to be caused if the proper detection procedure is not followed to eliminate the target virus(es). The seed consignments are accepted or rejected based on the results of the precise, reliable, sensitive, and specific diagnostic assay employed. Immunoassays employed for the detection of different plant viruses are listed in Table 7.2.

7.1.3.1 Enzyme-Linked Immunosorbent Assay

Double-antibody sandwich (DAS-) enzyme-linked immunosorbent assay (ELISA) has been more frequently employed than other ELISA formats for detection and identification of viruses and proteinaceous compounds. Other variants of ELISA test are direct antigen coating (DAC-) ELISA, protein A (extracted from the cells of *Staphylococcus aureus*) coating (PAC-) ELISA, and indirect virus antibody, in addition to labeled antiglobulin conjugate. The DAS-ELISA method is highly strain-specific and requires

Table 7.2 Seed transmission of plant viruses in different host plant species, as revealed by immunoassays.

Immuno assay/virus	Host plant species	References
Ouchterlony double diffusion test		
<i>Pea seedborne mosaic virus</i>	Pea	Zimmer (1979)
Radial immuno-diffusion test		
<i>Barley stripe mosaic virus</i>	Barley	Slack and Shepherd (1975)
Virobacterial agglutination test		
<i>Bean common mosaic virus</i>	Bean	Walkey et al. (1992)
<i>Cocoa swollen shoot virus</i>	Cocoa	Hughes and Ollennu (1993)
<i>Lettuce mosaic virus</i>	Lettuce	Walkey et al. (1992)
Cytofluorimetric method		
<i>Tomato mosaic virus</i>	Tomato	Iannelli et al. (1997)
Enzyme-linked immunosorbent assay (ELISA) formats		
<i>Apple latent spherical virus</i>	Apple	Nakamura et al. (2011)
<i>Bean common mosaic virus</i>	Bean	Klein et al. (1992); Larsen et al. (2005)
<i>Bean common mosaic necrosis virus</i>	Bean	Larsen et al. (2005)
<i>Bean pod mottle virus</i>	Soybean	Hobbs et al. (2003)
<i>Cowpea aphid-borne mosaic virus</i>	Cowpea	Ojuederie et al. (2009)
<i>Cucumber mosaic virus</i>	Bean, pumpkin, lupin	Doolittle and Walker (1925); Bos and Maat (1974); Yang et al. (1997); O'Keefe et al. (2007)
<i>High Plains virus</i>	Corn (maize)	Forster et al. (2001)
<i>Indian citrus ringspot virus</i>	Kinnow mandarin	Prabha and Barnwal (2011)
<i>Lettuce mosaic virus</i>	Lettuce	Dinant and Lot (1992)
<i>Papaya lethal yellowing virus</i>	Papaya	Cammaraco et al. (1998)
<i>Papaya ringspot virus</i>	Tobacco	Zein and Miyatake (2009)
<i>Pea seedborne mosaic virus</i>	Field pea	Kohnen et al. (1995); Coutts et al. (2009)
<i>Rice yellow mottle virus</i>	Rice	Konate et al. (2001)
<i>Soybean mosaic virus</i>	Soybean	Maury et al. (1985); Koning and Tekrony (2003); Hobbs et al. (2003); Ahangaran et al. (2009)
<i>Squash mosaic virus</i>	Cucurbit	Nolan and Campbell (1984)
<i>Zucchini yellow mosaic virus</i>	Melon	Lecoq et al. (2003)

(Continued)

Table 7.2 (Continued)

Immuno assay/virus	Host plant species	References
Lateral flow system		
<i>Pepino mosaic virus</i>	Tomato	Salomone and Roggero (2002)
Dot-immunobinding assay		
<i>Barley stripe mosaic virus</i>	Barley	Lange and Heide (1986)
<i>Bean common mosaic virus</i>	Bean	Lange and Heide (1986)
<i>Peanut mottle virus</i>	Peanut (groundnut)	Sherwood et al. (1987)
<i>Pea early browning virus</i>	Pea	Lange et al. (1991)
<i>Squash mosaic virus</i>	Squash	Lange and Heide (1986)
Fluorescent antibody technique		
<i>Lettuce mosaic virus</i>	Lettuce	Dolares-Talens et al. (1989)
<i>Soybean mosaic virus</i>	Soybean	Dinant and Lot (1992)
Petridish-agar immunomagnetic assay		
<i>Tomato mosaic virus</i>	Tomato	Abdalla and Albrechsten (2001)
<i>Blackeye cowpea mosaic virus</i>	Cowpea	Abdalla and Albrechsten (2001)
Immunosorbent electron microscopy		
<i>Alfalfa mosaic virus</i>	Alfalfa	Pesic et al. (1988)
<i>Barley stripe mosaic virus</i>	Barley	Brlansky and Derrick (1979)
<i>Cucumber mosaic virus</i>	Spinach	Yang et al. (1997)
<i>Fig latent virus 1</i>	Fig	Castellano et al. (2009)
<i>Soybean mosaic virus</i>	Soybean	Brlansky and Derrick (1979)
<i>Tobacco ringspot virus</i>	Tobacco	Bralansky and Derrick (1979)

the preparation of different antibody conjugate for each virus/strain to be detected. On the other hand, the indirect ELISA format provides an important advantage in that a single conjugate may be employed for different viruses and the antirabbit globulin conjugate or antigoat globulin conjugate, commercially available, may be used. When the PAC-ELISA procedure is applied, necessary care has to be taken to use the optimal concentration of protein A since higher concentrations may result in nonspecific reactions and lower concentrations may lead to negative results. The sensitivity of different ELISA formats may vary depending on several factors such as host–virus combinations, tissue tested, and level of susceptibility/resistance of the cultivar to the virus to be detected.

In order to reduce the cost of ELISA tests, less expensive chemicals were tested without compromising the sensitivity of the alkaline phosphatase (ALP) system. An ELISA test conducted with horseradish peroxidase was found to be as specific and sensitive as the ALP system for the detection of *Potato virus Y* (PVY) in the infected

plants (Polak and Kristek 1988). β -galactose isolated from *Escherichia coli* was shown to be more effective in labeling antibodies specific for *Potato virus X* (PVX) than horseradish peroxidase, since it decreased the background reaction of plant sap (Neustroeva et al. 1989). Another modification in the application of enzyme used for labeling the antibodies is the use of readily available and substantially less-expensive penicillinase (PNC) for ELISA tests. Penicillinase-based ELISA was equally sensitive as ALP- and HRP-based ELISA formats for detecting *Peanut mottle virus* (Sudharsana and Reddy 1989). DAS-ELISA with urease conjugate was superior to the ALP system as the color change from yellow to violet could be more easily recognized than the change of color from colorless to yellow in the ALP system. In addition, the visual detection threshold was 0.5 ng mL^{-1} of *Tobacco mosaic virus* (TMV) with urease and 500 ng mL^{-1} with phosphatase, indicating the greater level of sensitivity of ELISA with urease (Gerber and Sarkar 1988). In another investigation, the inorganic phosphatase (PPase) from *Escherichia coli* conjugated with antibodies and tetrazolium pyrophosphate were used as substrate in ELISA for the detection of 12 viruses, including *Barley stripe mosaic virus*, *Cucumber mosaic virus*, and *Soybean mosaic virus*. The high level of sensitivity, development of the bright blue-greenish color allowing easy visual detection of positive reactions, high stability of PPase-conjugated antibodies, and negligible level of background reactions in control are the advantages of using inorganic phosphatase system for virus detection (Surguchova et al. 1998).

The use of virus-free soybean seeds is the primary strategy to reduce incidence and subsequent spread of *Soybean mosaic virus* (SMV) disease. The standard DAS-ELISA procedure was found to be effective in detecting 1 SMV-infected seed in a lot of 7000 healthy seeds. In an improved DAS-ELISA procedure, the sensitivity of detection of SMV in soybean seeds was further enhanced. This procedure was suitable to meet the tolerance limit of zero infected seed in a lot of 30,000 seeds. This standard was enforced for certification of lettuce seed lots for freedom from *Lettuce mosaic virus* (LMV) (Dinant and Lot 1992). *Lettuce mosaic virus* (LMV) causes the devastating mosaic disease. Most field isolates of LMV are seedborne in susceptible cultivars, but not in resistant cultivars carrying *mo1*¹ or *mo1*² (Dinant and Lot 1992). Ten isolates of LMV were rub-inoculated on two or three plants of cultivars carrying *mo1*¹ or *mo1*², and the intensity of disease symptoms was scored at 2–4 weeks after inoculation. For each isolate, seed transmission was evaluated using ELISA by testing at least 25 batches of 200 seeds collected from infected lettuce plants cultivar Trocadero. Isolates that had been recovered from contaminated seed batches of resistant cultivars were considered resistance-breaking strains of LMV. ELISA results showed the presence of LMV in most plants with mosaic analyzed from each of the ten countries, except Brazil where many lettuce plants with mosaic appeared to be free of *Lettuce mosaic virus*. LMV was also recovered from contaminated seed batches from different seed companies (Krause-Sakate et al. 2002).

Indirect ELISA test using monoclonal antibodies was employed for efficient detection of *Bean common mosaic virus* (BCMV) in individual bean seeds. The presence of BCMV could be detected in flour samples of bean seeds by indirect ELISA format and, based on the results, the incidence of BCMV in seedlings of germplasm accessions could be determined (Klein et al. 1992). *Peanut clump virus* was detected in 16.5% of seeds by ELISA test (Konate and Barro 1993). Double-antibody sandwich (DAS-) ELISA format was employed to detect the *Indian peanut clump virus* (IPCV) in peanut (groundnut) seeds, since IPCV was transmitted at high frequency through seeds.

Although a Hyderabad isolate of IPCV-H was present in the testae, as indicated by ELISA test, infectivity tests gave negative results. The presence of IPCV-H in the cotyledon alone was correlated with transmission to the seedlings in six peanut genotypes. Each seed, except those that failed to germinate, contained the virus in the cotyledons and was ELISA-positive; grow-out tests confirmed the virus infection. A perfect correlation was observed between symptom expression and ELISA tests on the seedlings. The majority of the peanut genotypes showed about 10% IPCV-H seed transmission. In addition, IPCV-H strain was transmitted via the seeds of finger millet (5.2%), foxtail millet (9.7%), and pearl millet (0.9%), but not in sorghum seeds. Transmission of IPCV-H in the seeds of millets assumed importance, because these millets are often grown as intercrops or in rotation with peanut; these crops may function as potential sources of inoculum which may be spread by the fungal vector *Polymyxa* sp. (Reddy et al. 1998). In a later investigation, IPCV-H strain was detected in all seeds of infected wheat plants. Although natural transmission of the virus through seeds occurred only at a frequency of 0.5–1.3%, IPCV-H could not be detected in the seeds of barley which is another natural host of the virus. DAS-ELISA format was found to be sensitive and reliable in the detection of the transmission of IPCV-H via peanut seeds (Delfosse et al. 1999).

The transmission of *Cowpea aphidborne mosaic virus* (CABMV) via peanut seeds became a serious problem as a result of the free distribution of peanut seeds as germplasm materials and commercial seed trade between several countries. The presence of CABMV was tested for in 1533 peanut seeds by direct antigen coating (DAC-) ELISA format, and 8 seeds tested positive for CABMV. Seed slices (1 mm) were used for testing. This format detected CABMV in about 1–2% seeds which seemed to be greater than the percentage of seeds which could normally be expected to produce infected plants (approximately 0.15%) (Gillaspie et al. 2001).

Fourteen cowpea accessions screened from 300 regenerated cowpea germplasm from the Genebank collections of the International Institute of Tropical Agriculture (ITTA) in Nigeria were indexed for the presence of *Cowpea aphidborne mosaic virus*, using the protein A sandwich (PAS-) ELISA format. Of the 36 accessions tested, 31 were found to be infected by CABMV. Seeds were harvested from infected mother plants at maturity and the embryos excised in vitro onto hormone-free medium composed of 4.43 g MS basal salt, 30.0 g sucrose and 8.0 g agar. The seedlings were serologically indexed to detect any seedborne virus infection, after acclimatization to screen house conditions. The seedlings obtained from excised embryos from harvested seeds were ELISA-negative (Ojuederie et al. 2009).

Double-antibody sandwich (DAS-) ELISA format was employed for the detection of *Soybean mosaic virus* (SMV) in individual embryos of both dormant mottled (50%) and nonmottled seeds of certified soybean cultivars Davis, Essex, Hutchinson, Ransom, Stonewall, and Young. SMV was not detected in individual stained seed coats of mottled or nonmottled, stained seed coats of nonmottled dormant seeds, and endosperms of either type of seed. In addition, the virus was not detected in individual stained and nonstained seed coats, epicotyls, and endosperms of mottled and nonmottled seeds at 72 h post-germination. However, the virus could be detected in individual hypocotyls at an average level of 31% in post-germinated seeds (mottled and nonmottled) of all cultivars tested. Seedlings (6–8 weeks old) from mottled and nonmottled seeds of certified soybean cultivars grown in a glasshouse were asymptomatic, while only about 50% of the seedlings showed early symptoms. However, almost half of the symptomless soybean plants were DAS-ELISA positive for the presence of SMV. Virus-free soybean

plants grown to maturity from nonmottled seeds also produced mottled seeds, indicating the need to employ a reliable technique for detection of SMV in soybean seeds to raise virus-free seed stocks (Bajawa and Pacumbaba 1996). Infection of soybean with *Soybean mosaic virus* (SMV) was found to be one of the causes of seed-coat mottling. In addition, *Phomopsis* infection and genetic control have also been reported to be responsible for seed coat mottling associated with accumulation of SMV or *Phomopsis* spp. infection was investigated. Using the standard ELISA procedure, SMV antigen was detected consistently in both mottled and nonmottled seed coats. The concentration of SMV antigen, as estimated by ELISA test in the bulk-seed coat samples, was not consistently related to the extent of seed coat mottling. Further, the level of SMV antigen accumulation detected by ELISA in individual seed coats did not differ significantly between nonmottled and blemished seed.

SMV antigen accumulation in seed coat in relation to seed coat mottling and incidence of *Phomopsis* seed infection was investigated. The fungal pathogen could be isolated from seed of all mottling categories (severe, banded, blemish, and none), with incidence of infection increasing as the degree of seed coat mottling increased. SMV was detected in both mottled and nonmottled seed coats. The relationship of SMV titer to seed coat mottling was inconsistent with cultivars (Clark and Williams) and years (1996 and 1997). The results suggested that the extent of seed coat mottling might not be related directly to SMV titer and other factors might contribute to seed coat mottling in soybean (Koning and Tekrony 2003).

The problem of seed coat mottling in soybean seeds was analyzed by studying the effects of *Soybean mosaic virus* (SMV) and *Bean pod mottle virus* (BPMV). The percentages of soybean seed in eight lines with seed coat mottling were determined at harvest after inoculating plants during the growing season with SMV and BPMV and both SMV and BPMV inside in an insect-proof cage in the field. At full green pod development stage (R6 growth stage), individual plants were sampled and tested for the presence of SMV and BPMV using DAS-ELISA format; absorbance values were recorded at 405 nm. Leaves and green pod samples from all the entries were ELISA-positive for SMV, except for samples from line 3 and Williams-*Rsv1* which were ELISA-negative. SMV caused seed coat mottling in the six susceptible lines, but not in the SMV-resistant entries in both 2000 and 2001. Seed coat mottling percentages of lines 1, 2, 4, and Williams, Williams-*Im*, and Williams-*Rpv1* were significantly higher than those of the SMV-resistant lines 3 and Williams-*Rsv1*. Mottling of seed coats from individual plants (+ or -), as determined by ELISA test, were significantly correlated ($P=0.01$). BPMV also caused seed coat mottling. BPMV and SMV inoculated on the same plant did not always result in higher percentages of mottled seeds compared with BPMV or SMV alone. As no commercial soybean cultivar with resistance to seed coat mottling is available, this problem might continue to be a serious one (Hobbs et al. 2003). A strain of *Bean common mosaic necrosis virus* (BCMNV) from Idaho, USA was identified by ELISA tests using monoclonal antibodies (MAbs), and was determined to be similar to the NL-3D strain by reaction of differential bean cultivars. All cultivars of bean that became symptomatic with NL-3K strain reacted positively with group-specific potyvirus MAb. Plants infected with NL-3D or NL-3K also reacted positively with the MAb specific to BCMNV, but not MAb specific to *Bean common mosaic virus* (BCMV), consistent with reaction to NL-3D. Bean cultivars from host groups (HGs) 6, 7, 11, and 12 did not become systemically infected with NL-3K, and BCMV was not detectable by ELISA in these hosts (Larsen et al. 2005).

Pea seeds infected by *Pea seedborne mosaic virus* (PSbMV) form the primary sources of inoculum and the secondary spread of the disease is mediated by aphid vectors. Samples of 200 or 300 seeds from each plot were combined and germinated in trays, and leaf samples from seedlings were tested by DAS-ELISA format individually or in groups of 2–10. Plots were sown with seed with actual or simulated seed transmission rates of 0.3–6.5% in 2005 or 0.1–8% in 2006 and disease spread occurred through aphids. The higher the amount of primary inoculum within plots, the greater was the rate of PSbMV disease incidence. Under conditions conducive for disease spread in 2005, sowing 6.5% infected seeds resulted in yield losses up to 25%, whereas with initial 1.1–2.2% infection, yield losses were 15–21%, indicating that use of virus-free seeds was essential to restrict the spread of the virus by aphid vectors and consequent yield losses at harvest. Seed transmission rates in harvested seed were mostly greater than those in the seed sown when climatic conditions favored early virus spread (1–17%) such as in 2005, but smaller when the climatic conditions were not favorable (0.2–2%) as in 2006. A threshold value of <0.5% seed infection was established for sowing in high-risk zones (Coutts et al. 2009).

Pea enation mosaic virus (PEMV) and *Bean leafroll virus* (BLRV) are economically important viruses infecting different legume crops. An antigen-coated plate (ACP-) ELISA format was developed for the detection of PEMV and BLRV in infected plants. The coat protein (CP) gene of each virus was bacterially expressed as a fusion protein containing an N-terminal hexahistidine tag and used as an antigen to produce antisera in rabbits. The antiserum against BLRV had a detection limit of 1:1000 dilution in leaf samples, while the detection limit for PEMV was 1:6400 dilution in leaf samples. No serological cross-reaction was observed between the antisera of these viruses with heterologous antisera. The presence of PEMV and BLRV was detected in pea, alfalfa, and vetch over a three-state area in the US Pacific Northwest over a 2 year period and virus disease incidence was mapped using ACP-ELISA tests. The usefulness of ACP-ELISA for screening germplasm for resistance to virus disease(s) was indicated by this investigation (Vemulapati et al. 2014).

Two pathogroups of *Rice yellow mottle virus* (RYMV) infecting rice occurring in Sudano-Saharan areas of West Africa have been distinguished. The extent of seed transmission of these pathogroups was assessed by employing ELISA tests and infectivity assays, using the highly susceptible rice cultivar BG90-2. In 17 of 21 rice genotypes tested, RYMV was detected in all seed parts including glumella, endosperm, and embryo at a rate of 65–100%. However, no seedlings emerging from infected seeds showed disease symptoms. Infectivity of the virus appeared to decrease throughout the process of seed formation, possibly due to inactivation of the virus as a result of seed maturation and desiccation (Konate et al. 2001).

In a further study, the presence, infectivity, and transmissibility of four isolates of RYMV in seeds of rice and six wild rice species were assessed by employing ELISA tests. RYMV was successfully detected in individual seeds of all *Oryza* species. In most cases, virus incidence in seeds was greater than 60%. However, the levels of seed infection differed significantly between virus isolates and plant species ($P < 0.001$). In contrast, the detection of RYMV in seeds of *Dactyloctenium aegyptium*, *Eragrostis ciliaris*, *E. tenella*, and *E. tremula* was unsuccessful. RYMV was infectious in freshly harvested seed extracts of all plant species tested. In contrast, most infectivity was lost in dried seeds, possibly due to virus inactivation following dehydration of the seeds during seed maturation. Inoculations of extracts from individual seed of three *Oryza* spp. resulted in positive infections of cultivar BG90-2 at both stages of seed testing (Table 7.3). No evidence for transmission of

Table 7.3 Infectivity of RYMV in extracts from seeds of rice and wild host species as determined by ELISA at two stages (Allarangaye et al. 2006).

Host plant species	Stage 1 ^a	Stage 2 ^b
<i>Oryza sativa</i>	59/120	2/120
<i>O. barthii</i>	44/120	0/120
<i>O. longistaminata</i>	59/120	4/120
<i>Eragrostis ciliaris</i>	3/20	0/20
<i>E. tenella</i>	2/20	0/20
<i>E. tremula</i>	2/20	0/20
<i>Dactyloctenium aegyptium</i>	4/20	0/20

^a Tested at harvest; ^b tested after drying the seeds for one month in the open air.

Table 7.4 Confirmation of ELISA results by vascular puncture inoculation of corn plants (Forster et al. 2001).

Source plant ^a	ELISA absorbance values ^b		Number of HPV-positive plants
	HPV	WSMV	
9706-6-1	0.969	0.142	21/167
9712-2-1	0.406	0.020	23/100
Y-4	0.015	0.048	0/100
Healthy corn	0.010	0.23	—

^a Plants grown from seeds harvested from naturally infected seeds; ^b ELISA absorbance values of HPV-infected source plants against HPV and *Wheat streak mosaic virus* (WSMV) antisera.

RYMV from seeds to seedlings was obtained, although the virus was present in some of the rice seeds even after drying. The results indicated that RYMV is unlikely to be transmitted through seeds of rice or its wild relatives (Allarangaye et al. 2006).

The incidence of *High Plains virus* (HPV) in sweet corn in Idaho, USA, where about 90% of sweet corn seed for domestic supply and significant quantities for export are produced, was of great concern for farmers. The possibility of HPV transmission via sweet corn seed was examined. Sweet corn seeds of various corn cultivars harvested during 1994–1996 from 13 locations were sown in the greenhouse. A total of 38,452 seedlings from 46,000 seeds were evaluated for HPV infection. In addition, indirect ELISA format was also applied to confirm the presence of HPV in the seedlings. Three of the seedlings exhibited disease symptoms, and were also ELISA-positive. Five additional HPV-infected corn seedlings were identified by ELISA test performed to detect *Wheat streak mosaic virus* (WSMV) in addition to HPV. Extracts from two of these plants were inoculated by the vascular puncture inoculation method. Among the 167 inoculated plants, 21 plants showing HPV symptoms were ELISA-positive only for HPV (Table 7.4). One of the ELISA-positive plants was used for successful acquisition and transmission of HPV by wheat curl mite (*Aceria tosichella*), a natural biological vector of HPV.

The results showed that *High Plains virus* might be transmitted via seeds at a very low frequency in sweet corn seeds (Forster et al. 2001).

Wheat streak mosaic virus (WSMV) transmitted by the wheat leafcurl mite (*Aceria tosichella*) was reported earlier to be transmitted via seeds of wheat. Under conditions that excluded all possibility of eriophyid mite vector activity, seed transmission of WSMV was recorded in 8 of 300 different wheat genotypes at rates of 0.5–1.5%. ELISA tests gave positive results for the presence of WSMV in wheat plants grown from infected seeds of 5 of 25 genotypes in the first experiment. In the second experiment, 3 of 18 genotypes showed positive reactions for seed transmission. RT-PCR assay results confirmed the presence of WSMV in the grouped samples of these genotypes. Sequence comparisons of four seedborne isolates showed ≥ 98.6 identity with eight Australian isolates of WSMV in GenBank, indicating a common seedborne origin of WSMV. Wheat germplasm movement as seed may result in the introduction of WSMV into other countries or areas in the same country. Seed stocks therefore have to be subjected to appropriate quarantine examination before export/import (Jones et al. 2005).

The transmission of *Tomato mosaic virus* (ToMV) and *Tobacco mosaic virus* (TMV) via tomato seeds (Broadbent 1965) and TMV in bell pepper seeds (Tosic et al. 1980) was indicated. Higher levels of seed infection and consequent yield loss were observed when the plants were infected in the early growth stages of plant development. The titer of ToMV and TMV in tomato seeds was determined by employing indirect ELISA format. The concentration of ToMV was greater than that of TMV in tomato seed. The relative concentrations of TMV in bell pepper seeds were greater than that of ToMV. The results indicated that tomato provided more favorable conditions for the replication of ToMV, while TMV replication was supported more efficiently by bell pepper (Chitra et al. 1999). *Pepino mosaic virus* (PepMV), a potexvirus originally found in pepino (*Solanum muricatum*), was able to infect tomato under natural conditions. Possible seed transmission of PepMV in tomato was examined and the experimental host range of an Italian isolate of PepMV-To was also investigated. Among the cultivated *Solanaceae*, egg plants, but not pepper or potato, were found to be susceptible to PepMV-To. The presence of PepMV-To was checked by DAS-ELISA tests in naturally infected tomato fruits. A quick field immunochromatographic system based on lateral flow with 100% correlation with DAS-ELISA was developed for the detection of PepMV in tomato samples. The virus was detected by lateral flow in individual homogenates of three dried seeds. One month after emergence of seedlings at 2–3-leaf stage, when disease symptoms were expressed, the virus could not be detected in leaf samples from 52 tomato plants. The results indicated that PepMV-To was seedborne, but not seed transmissible (or at least with a high transmission rate). DAS-ELISA and lateral flow techniques were compared for their efficiency in detecting PepMV-To using 51 additional samples. PepMV was detected in four samples of fruit or leaves by both methods. DAS-ELISA was found to be more effective in vitro, whereas lateral flow system was shown to be a reliable field detection system, providing the results within a few minutes without the use of any equipment (Salomone and Roggero 2002).

The widespread incidence of *Pepino mosaic virus* (PepMV) in many countries such as Sweden, Finland, Norway, Denmark, Slovakia, Bulgaria, Poland, Hungary, Ukraine, Chile, and Ecuador was indicated to be due to the use of infected seeds and combined routine international distribution of tomato fruits and seeds (EPPO 2005; Soler et al. 2005). DAS-ELISA format was employed to determine the rates of seed transmission of PepMV in seedlings grown from seeds obtained from symptomatic tomato fruits

of plants naturally infected with PepMV. The percentage of seeds infected with PepMV was at least 25%, as revealed by ELISA tests of grouped seeds. The seeds from symptomatic fruits were planted and the seeds were assayed by ELISA tests at cotyledon and transplant stages. Of the 168 seedlings tested, 3 seedlings were PepMV positive, corresponding to a seed-to-seedling transmission rate of 1.84%. However, none of the ELISA-positive tomato seedlings showed clear symptoms of PepMV infection. The stage of plant growth at the time of ELISA analysis affected significantly the PepMV infection rate. As PepMV is an extremely stable and persistent virus, relatively high rates of unintentional mechanical transmission might occur as in the case of *Tobacco mosaic virus* (TMV). The results suggested that PepMV introduced into greenhouses via contaminated tomato seeds/infected seedlings/plants might account for rapid spread of the *Pepino mosaic virus* in tomato, in addition to its ability to spread through leaf contact, tools, and handling by workers (Córdoba-Sellés et al. 2007). In a later investigation to determine the extent of seed transmission of *Pepino mosaic virus* (PepMV) via tomato seeds, tomato plants were artificially infected with both European and CH2 genotypes of PepMV. More than 100,000 seeds extracted from tomato fruits were indexed using bioindicator plants to confirm the presence of infective PepMV on the seeds. In addition, seeds were tested by distributing the seeds to 10 different laboratories. The young seedlings were tested by ELISA tests. Of a total of 87,000 seedlings tested, PepMV was detected in 23 seedlings, the overall transmission rate of the virus being 0.026%, with a range of seed transmission of 0.005–0.057%, depending on the seed batch tested. The results indicated, as in the earlier investigations, that PepMV could be transmitted from contaminated seed to seedlings, highlighting the risk of using seeds from PepMV-infected tomato plants which might function as potential sources of inoculum for secondary spread of the disease (Hanssen et al. 2010).

Cucumber mosaic virus (CMV) has been reported to be transmitted through seeds of some plant species to varying degrees. Seed infection rates by CMV ranged from 0.7% in pumpkin (*Cucurbita pepo*), 7.0% in bean, to 55% in wild cucumber (*Echinocystis labata*) (Doolittle and Walker 1925; Bos and Maat 1974; Sharma and Chohan 1974). *Cucumber mosaic virus* (CMV) infects spinach (*Spinacia oleracea*) crops causing significant losses. A commercial breeding line of spinach was infected by CMV and the possibility of transmission of CMV via spinach seed was examined initially by grow-out tests and then by protein A sandwich (PAS) indirect ELISA format. Approximately 50–100 seeds derived from each of 5–10 maternal plants per cross between healthy and CMV-infected male and female plants in different combinations were grown separately. These seedlings were indexed at 5-leaf stage using PAS-ELISA format to confirm CMV infection. The results showed that CMV was seed transmitted in spinach and that both the male and female parent could serve as infection sources. The incidence of CMV in the seedlings obtained by crossing infected males with healthy females was 14.5%, which was similar to the infection level observed in the parent plant seed lot (Yang et al. 1997).

Cucumber mosaic virus (CMV) epidemics in narrow-leaved lupin (*Lupinus angustifolius*) affected the production significantly in Western Australia. As the virus is known to be seed-transmitted, growing lupin by sowing seed stocks with minimal virus infection was considered as a practical approach. The seed-testing service provided an estimate of CMV infection level based on a dry seed test in which bulked subsamples of ungerminated seeds were ground to a fine powder for testing by employing DAS-ELISA format. The presence of CMV in lupin could be detected with a high level of accuracy in samples containing 1 infected seed in 100 seeds. CMV was detected only in the

embryo, but not in the seed coat of CMV-infected lupin seed. Treatment of lupin seeds with trisodium phosphate (traditionally used to disinfect the seeds contaminated with virus) did not alter the OD at 405 nm at which the virus concentration is estimated. The results indicated that the seed coat contamination with CMV was lacking in lupin seed and justified large-scale routine use of a dry seed test for determining levels of virus infection in lupin seed samples (O'Keefe et al. 2007).

Seed transmission of potyviruses facilitates introduction of these viruses into new production areas by creating primary infection sites from which the natural vectors (different aphid species) could efficiently disseminate them. Seed transmission of *Zucchini yellow mosaic virus* (ZYMV) has not been established unequivocally. The possibility of aphids acquiring ZYMV from infected fruits of watermelon and zucchini was examined because large quantities of melons were imported into France from regions where both viruses were prevalent. Imported melon fruits of the yellow canary type infected by ZYMV and *Papaya ringspot virus* (PRSV) were exposed in the fields to facilitate acquisition of the viruses from the infected fruits by aphids. Healthy melon or squash plants were used as bait plants and aphids landed and probed on the fruits. Ten melon fruits originating from Costa Rica were found to be infected by PRSV (10 fruits) and ZYMV (4 fruits) by using DAS-ELISA tests. PRSV and ZYMV isolates could be easily recovered from fruits by mechanical inoculation to melon and zucchini squash plants. Aphid transmissions were observed from infected melon fruits to melon test plants. Seeds (400) coinfecting by ZYMV and PRSV were sown. None of the 1095 plantlets developed disease symptoms or were ELISA-positive for ZYMV or PRSV. The results indicated that the infected melon and zucchini squash fruits might be a factor for spread of cucurbit potyviruses between countries or continents (Lecoq et al. 2003).

Tobacco rattle virus (TRV), a nematode-transmitted virus, infects sugar beet crops causing considerable yield losses. Protein-A-coated (PAC-) ELISA format was employed to detect TRV in racemes, seedlings grown from seeds of these plants, and seedlings from commercially available sugar beet seeds. Each raceme was tested separately with antiserum produced against TRV and also against *Broad bean wilt virus* (BBWV), which also produced yellow mosaic symptoms like TRV on leaves and raceme stipules. As the sugar beet seeds are small in size, TRV was detected in 5 day seedlings grown from seeds harvested from infected plants. TRV concentration in racemes was more than 0.3–0.5 optical units. Artificial vegetative propagation of sugar beet enhanced the transmission of TRV, reaching high levels of seed infection of 62%. Seedlings originating from seeds of 2 year old beet plant showed 20% infection by TRV. Seedlings grown from one batch of commercial seed lots were infected by TRV to an extent of 15%, while another batch had 20% infection. TRV infection of racemes was investigated in samples collected from a seed production field. TRV infection was detected in 5% of the racemes. The results suggested that the progeny of such racemes might transmit TRV under field conditions (Dikova 2005).

Indian citrus ringspot virus (ICRSV), a positive sense flexuous RNA virus, causes the Indian citrus ringspot disease, the incidence of which reached 100% in Kinnow mandarin orchards in India. The presence of ICRSV in the pollens of naturally infected flowers of Kinnow mandarins was detected. DAC-ELISA format detected the virus in seed coats, but not in the embryo and endosperm of seeds collected from fruits of ICRSV-infected Kinnow plants. Although the virus was detected in 18% of the seed coats, the grow-out test did not show the infection by ICRSV as revealed by the absence of

characteristic symptoms of the disease even after 2 years. ICRSV could be localized in the testa of seeds, but its lack of transmission to progeny was revealed by both ELISA and RT-PCR assays which did not detect the viral RNA in the seedlings (Prabha and Baranwal 2011). DAS-ELISA format was employed to detect *Apple latent spherical virus* (ALSV) in 21 apple trees surrounding two ALSV-infected trees. None of the 21 trees was found to be infected by ALSV, indicating that the virus did not spread during a period of over 25 years. ALSV was detected in 10 of 223 seed embryos (4.5%) and 10 of 227 seedlings from uninfected apple trees that were hand-pollinated with pollen from infected trees. The results showed that ALSV could be detected in only 1 of 260 seed embryo and in none of the 227 apple seedlings, using DAS-ELISA format. Seed transmission of ALSV via infected pollen was only up to 0.38%. The results showed that vertical transmission of ALSV might occur through mother plant infection to a greater level than via pollen infection (Nakamura et al. 2011).

Seed transmission of *Plum pox virus* (PPV), the incitant of Sharka disease which belongs to the genus *Potyvirus*, remained an unresolved problem concerned with development of effective disease management system. Some of the potyviruses are known to be transmitted via seeds (Ward and Shukla 1991). However, contradictory reports necessitated the reinvestigation of the possibility of transmission of PPV in seeds. Seeds collected from three plum cultivars infected with PPV-M and one apricot (*Prunus americana*) cultivar infected with PPV-Rec and *Prunus mahaleb* were tested. DAS-ELISA format was employed to detect the presence of PPV in mature seeds and seed parts, seed coats, cotyledons, radicles, and plumule of individual seed. The percentage of infected whole seeds was highest (32%) in the cultivar Tuleu timpuriu. The seed coats and cotyledons showed varying levels of PPV infection in different plum cultivars. None of the embryonic axes was ELISA-positive (Table 7.5). No planted seedlings originating from seeds of three plum cultivars infected by PPV-M strain exhibited infection by PPV-M in the embryonic axes, and the plants raised from infected trees did not exhibit disease symptoms even after one year. The results indicated that PPV-M was unlikely to be transmitted through seeds (Milusheva et al. 2008).

Kenaf (*Hibiscus cannabinus*), a potential source of cellulose, is infected by *Hibiscus latent ringspot virus* (HLRSV) belonging to *Nepovirus* group. Six commercial seed lots were tested by protein A sandwich (PAS-) ELISA format. Seed transmission of HLRSV ranging from 0 to 26% was confirmed by the PAS-ELISA format, although no symptoms

Table 7.5 Detection of *Plum pox virus*-M in whole seeds and seed parts (Milusheva et al. 2008).

Plum cultivar	Whole seeds		Seed coats		Cotyledons		Embryo	
	A	B	A	B	A	B	A	B
Stanley	26/100	26.0	40/114	35.08	10/114	8.77	0/114	0
Tuleu timpuriu	40/124	32.5	39/119	32.77	27/119	22.65	0/114	0
Valjevka	26/183	14.20	19/99	19.20	7/99	7.10	0/99	0
Modesto	33/145	24.13	30/135	22.22	7/135	5.10	0/135	0
<i>Prunus mahaleb</i>	9/81	11.1	–	–	–	–	–	–

^A Number of positive assays/number of samples tested; ^B percentages of positive detection.

of virus infection could be observed on the kenaf seeds. Of the 55 seeds dissected, 17 embryos and 7 endosperms tested positive for the virus by the immunoassay. No internal integuments contained the virus. The presence of HLRSV in the first seed wash runoff was detected by PAS-ELISA, indicating the presence of the viral antigen on seed surface. HLRSV was detected in 51% of kenaf cultivar G4 tested seed, but only 26% of the progeny derived from the same seed lot were infected, showing that seed infection of HLRSV was not necessarily correlated with the rate of infected progeny seedlings. Transmission of HLRSV through seeds of *Chenopodium amaranticolor* and *C. quinoa* was observed, but the seedlings were invariably remained asymptomatic. However, when these plants were mechanically inoculated, they exhibited systemic symptoms of yellow flecking (Rubies-Autonell and Turina 1997).

Asparagus virus 2 (AV2) is a seed-transmitted virus with worldwide distribution. The effects of AV2-infected parental sources on the development of infected seed, male, and female asparagus plants were assessed. AV2-infected or AV2-free asparagus plants were cross-pollinated and allowed to develop seed in insect-proof greenhouse. Shoots growing from the resulting seeds tested by ELISA showed that 17% of germinated seedlings were infected by AV2 when only the male parent was infected, whereas 47% of the seedlings were ELISA-positive when only the female parent was infected. When both parents were infected, infection by AV2 was detected in 95% of the seedlings. Infection of parents by AV2 resulted in reduction in successful pollination, weight, and number of seeds formed, seed germination, and seedling vigor. The high percentage of AV2-free female plants contracted the virus infection following pollination with infected pollen. The results of ELISA tests revealed that 83% of plants and 36% of shoots tested positive in the following year. On the other hand, RT-PCR assay showed that 75% of plants and 55% of shoots were infected by AV2 in the second year. The results indicated that vertical transmission of *Asparagus virus 2* via seed and horizontal transmission via pollen were of epidemiological importance (Jaspers et al. 2015).

7.1.3.2 Dot-Immunobinding Assay

Dot-immunobinding assay (DIBA), also referred as dot-ELISA (Banttari and Goodwin 1985) or enzyme-linked immunoblot assay (Wang et al. 1985), is analogous to standard ELISA procedure. Microplates used in ELISA test are replaced by nitrocellulose or nylon-based membranes on which the antigen is immobilized. The free protein-binding sites present on the nitrocellulose membrane are blocked, because of their high affinity for proteins. Based on the cost factor, nonfat dry milk (skimmed milk) powder is preferred to bovine serum albumin (BSA) and gelatin, which are more expensive. The unconjugated virus-specific antibody is allowed to react with the immobilized antigen which is probed with alkaline phosphatase (AP-), horseradish peroxidase (HRP)-labeled protein A, anti-Fc, and anti-IgG. An appropriate substrate is used for the visual detection of the colored reaction product.

Dot-immunobinding assay (DIBA) was employed for the detection of seedborne inoculum of *Barley stripe mosaic virus* (BSMV) in barley and *Bean common mosaic virus* (BCMV) in bean. DIBA was found to be effective in detecting both viruses in single seed samples and seed flours of known infection levels. The results of DIBA, immunosorbent electron microscopy, and grow-out test were highly correlated under in vitro and greenhouse conditions. Seed extracts in phosphate-buffered saline (PBS) are blotted in premarked positions on nitrocellulose membrane, followed by reaction with antibodies and enzyme conjugate and staining with nitroblue-tetrazolium substrate

solution for visualization of color development, indicating positive reaction. DIBA technique was highly suitable for routine seed health testing by certification personnel (Lange and Heide 1986).

The DIBA test was shown to be more sensitive than the ELISA technique for the detection of *Pea seedborne mosaic virus* (PSbMV). The ELISA procedure required the use of monoclonal antibodies (MAbs) to be as sensitive as the DIBA test with polyclonal antibodies (PABs) (Lange 1986). However, the DIBA test needed MAbs specific to *Peanut mottle virus* (PeMV) to provide sensitive detection as effectively as the ELISA test. The ELISA protocol was however equally efficient in detecting PeMV when either MAbs or PABs were employed (Sherwood et al. 1987).

In a later investigation, use of plain paper as the substrate instead of nitrocellulose membrane was demonstrated to be effective for the sensitive detection of several viruses such as PSbMV, BSMV, BCMV, *Pea early browning virus*, and *Squash mosaic virus* in seeds. The DIBA test therefore became still simpler without compromising the sensitivity of virus detection in seeds. The DIBA test offered several advantages over the ELISA technique that include the requirement of smaller volumes of reactants, ease of processing the samples, storing and transporting the membranes without loss of reactivity, reduction in test duration, simplicity, and cost-effectiveness (Lange et al. 1991). However, ELISA formats have been employed extensively for the detection of more seedborne viruses than any other immunoassay. *Soybean mosaic virus* (SbMV) was detected by DIBA test. Blotted membranes exhibited dark purple color following treatment with virus-specific antibodies and the substrate solution NBT/BCIP, indicating the presence of SbMV in the samples tested (Fig. 7.1; Ahangaran et al. 2009).

7.1.3.3 Petridish-Agar Dot-Immunomagnetic Assay

Petridish-agar dot-immunomagnetic assay (PADIA), requiring 5–10 times less quantities of reagents compared with DAS-ELISA test, was developed. In this test, polystyrene petridish replaces the microplates with wells. Circular areas outlined with hydrophobic cryomarker pen are marked on the inner surface of the dish. The antiserum and alkaline phosphatase conjugate are then added into the marked areas, incubated, and washed as in standard ELISA procedure. The substrate solution mixed with warm agar is added to cover the inner surface of the petridish. The sensitivity of PADIA test was shown to be the same level as the standard ELISA format (Abraham and Albrechsten 2000). As a further improvement of PADIA test, the petridish-ELISA test was developed. The inner surface of the polystyrene petridish is divided into many squares or circles, using a wax pen. Drops of reagents (50 μ L) are placed within the marked areas. All steps as in standard ELISA procedure are followed, and the absorbance values at 405 nm were recorded. Petridish-ELISA format detected *Tomato mosaic virus*, *Cowpea mosaic virus*, and *Blackeye cowpea mosaic virus* efficiently in seeds (Abdalla and Albrechsten 2001).

7.1.3.4 Fluorescent Antibody Technique

Enzyme-linked fluorescent assay (ELFA), involving the use of fluorescent antibodies, was successfully employed to detect seedborne viruses *Lettuce mosaic virus* (LMV) and *Soybean mosaic virus* (SMV) in seeds. ELFA technique could detect LMV in a sample of 500 seeds containing one infected seed. The sensitivity of ELFA procedure was 10–25 times greater than that of DAS-ELISA format (Dolares-Talens et al. 1989). Another immunoassay using fluorescent antibodies, radio-immuno assay (RIA), was employed

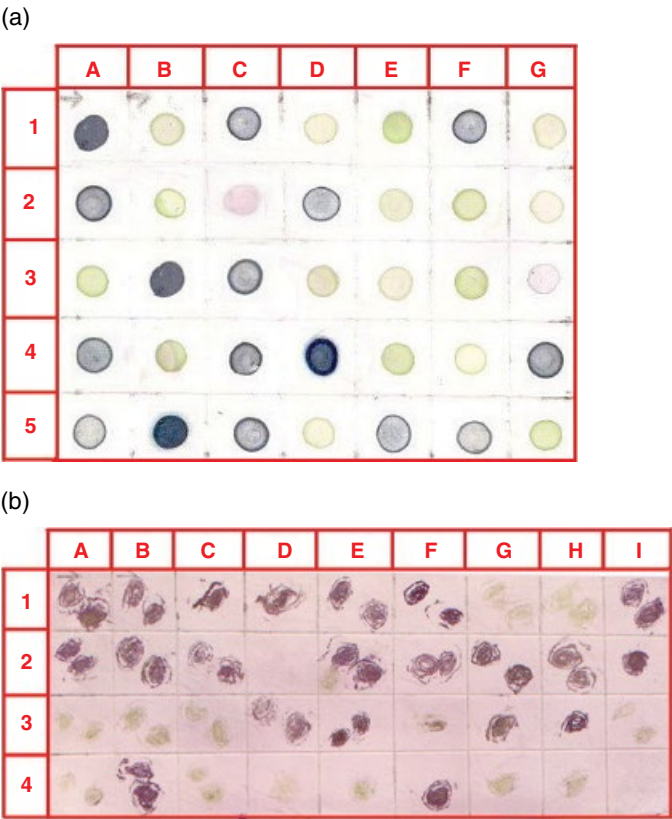


Figure 7.1 Detection of *Soybean mosaic virus* (SMV) by dot-immunobinding assay (DIBA) and tissue-print immunoassay (TPIA) using NBT/BCIP as substrate. (a) DIBA: F3: negative control; F5: positive control. (b) TPIA: A1 and B1: positive controls; H4: negative control. Development of color indicates the presence of SMV in sample extracts. Source: Ahangaran et al. (2009). Reproduced with permission of Journal of Agricultural Science and Technology, Iran.

for sensitive detection of viruses in seeds using radioactive gamma for labeling immunoglobulins. *Lettuce mosaic virus* (LMV) could be detected in a seed lot of 30,000 seeds containing only 3 infected lettuce seeds. Adequate care has to be taken for handling potentially hazardous ¹²⁵I isotopes for labeling globulins specific to the virus(es). This requirement is considered as a factor limiting wider application of RIA procedure for virus detection in seeds (Ghabrial and Sphepherd 1982; Dinant and Lot 1992).

7.1.3.5 Microscopy-Based Techniques

Microscopy-based detection techniques may be useful for confirmation of virus presence in a limited number of representative samples, but are not suitable for routine, large-scale application for detection of the viruses in large number of seed samples.

7.1.3.5.1 Immunofluorescence Microscopy

Immunofluorescence (IF) microscopy has been employed for in situ visualization of viruses in plant tissues. The mechanism of seed transmission of *Pepper mild mottle virus* (PMMoV), a tobamovirus, was studied by visualization of immunolocalized virus

in seed tissues. Two distinct patterns of virus were observed. PMMoV was present in the epidermis and parenchyma, but not in the endosperm or embryo. Alternatively, the virus was restricted to the surface of epidermis and parenchyma of seed (Genda et al. 2005). In a further study, the location of *Pepper mild mottle virus* (PMMoV) within seeds as the seeds developed on inoculated seedlings of pepper was monitored over time by detecting the virus coat protein using IF microscopy. Seedlings were inoculated with PMMoV when flower buds on the first and second branching nodes were in bloom. Fluorescence, indicating the presence of PMMoV, was first observed around immature seeds, placentas in ovaries on the fourth branching node at 20 days postanthesis (20 dpa) or 39 days postinoculation (dpi). Fluorescence of the tip of immature seeds was recorded by 34 dpa. The presence of viral antigen was observed in the embryo or endosperm but not beyond the endothelium. The results indicated that IF microscopy could be used for investigating infection cycle of PMMoV through vertical transmission (Genda et al. 2011).

7.1.3.5.2 Immunosorbent Electron Microscopy

Immunosorbent electron microscopy (ISEM) technique is suitable for visualization of viruses in the seed tissue extracts if the number of samples is limited. The electron microscopic grids are made specific for the target virus by floating the grids on the antiserum produced against the target virus. The adsorbed virus particles are observed under the electron microscope after staining with electron-dense stains. *Lettuce mosaic virus* (LMV) could be detected in the pooled seed extract containing one part of infected seed extract mixed with 99 parts of healthy seed extract. In addition, *Barley stripe mosaic virus* (BSMV), *Tobacco ringspot virus* (TRSV), and *Soybean mosaic virus* (SMV) were also detected by ISEM procedure when the disease incidence was low (Brlansky and Derrick 1979). *Alfalfa mosaic virus* (AMV) antigen was detected in situ in infected seed tissues using immunogold cytochemistry method. The presence of AMV in the cytoplasm and vacuoles of ovule integuments, microspores, mature pollen grains, and anther tapetum cells contained raft-like aggregation of virus particles (Pesic et al. 1988). Immunogold labeling procedure was shown to be effective for the detection of *Cucumber mosaic virus* (CMV) in spinach seeds. CMV virions could be visualized in the cytoplasm of ovary cells, ovule integuments and nucellus, anther, and seed coat cells as well as in the fine fibril-containing vesicles and electron-dense inclusions of amorphous aggregates in the central vacuoles of these cells in the infected seeds of spinach (Yang et al. 1997).

Fig latent virus 1 (FLV-1), a putative member of the genus *Tobravirus*, family *Flexiviridae*, infects fig trees causing mosaic disease. Symptomless seedling infection by FLV-1 was noted in the germplasm collection in southern Italy. Immunosorbent electron microscopy (ISEM) was employed to detect FLV-1 virus in leaves, hypocotyls, roots, and whole seeds of fig tree. Microscope grids were floated for 1 h at 38°C on a drop antiserum raised against FLV-1 diluted 1:50 and then placed overnight at 4°C on a drop of extract from different plant tissues. For decoration of virus particles, grids were floated on a drop of antiserum diluted 1:20 for 1 h at room temperature. FLV-1 was detected in all tissues, and roots appeared to contain the highest virus titer. All seedlings, irrespective of age and origin, were severely infected by FLV-1, indicating the identity of the virus infecting fig plants. Bundles of virus particles were seen in the ultrathin sections stained with lead citrate in the cytoplasm of mesophyll cells of symptomless seedlings. The high rate of seed transmission differentiated *Fig latent virus 1*

from other seed-transmitted plant viruses which had been reported to have lower levels of seed transmission frequency, and other viruses belonging to *Trichovirus* group, none of which is apparently transmitted through seeds. As no natural biological vector is known for FLV-1, the importance of high rates of seed transmission may not be clearly established, since fig is not propagated through seeds (Castellano et al. 2009).

7.1.4 Nucleic Acid-Based Techniques

All organisms contain nucleic acids, the storehouses of genetic information on which the characteristics of the individual organisms are based. Nucleic acid-based techniques are either based on the specificity with which nucleic acids hybridize to form double-stranded (ds) molecules, or on the similarities between nucleic acids which are identified by using restriction enzymes to cleave genomic DNA into fragments at or near defined recognition sequences. The extent of similarity of nucleotide sequences of organisms determines the relatedness of two or more closely related organisms sharing a greater level of similarity of nucleotide sequences. A highly specific/unique nucleotide sequence present in a viral nucleic acid may be useful for the detection and identification of plant viruses or its strains concerned. Generally nucleic acid-based techniques have been shown to have greater level of reliability, sensitivity, and specificity, in addition to providing the results rapidly, compared to traditional biological assays and immunoassays. As the antigenic determinants of viral coat proteins (CPs) represent only about 2–5% of viral genomes, variations in characteristics of the virus governed by a major portion of the viral genome cannot be determined by using immunoassays. Diagnostic methods based on the detection of similarities between nucleic acids of viruses and their strains have been shown to be useful for the identification of new or unusual viruses or strains. Genomes of RNA viruses have to be exposed to the action of reverse transcriptase to generate corresponding cDNA of the fragment specific to the target virus, followed by polymerase chain reaction (PCR) amplification. As the seeds contain high concentrations of inhibitors of PCR, it is essential to modify RNA extraction procedure to avoid coextraction of PCR inhibitors along with viral RNAs.

7.1.4.1 Nucleic Acid Hybridization

The target viral nucleic acid is usually fixed to a solid support. The probe (complementary nucleotide sequence) carrying a label that provides recognizable signal is allowed to anneal with immobilized target molecule. Positive reaction (hybridization) is recognized by detecting the signal from the probe. RNA probes are frequently employed, since the majority of plant viruses have RNA as their genomes. RNA:RNA hybrids have been found to be more stable than RNA:DNA or DNA:DNA hybrids. Improvement of specificity of the probes and reduction in background problems due to interference of plant sap may be accomplished by applying highly stringent hybridization conditions. Plant viral genome organization and virus–host interaction have also been investigated by employing hybridization procedures, in addition to detection and identification of plant viruses.

Viruses may be transmitted from infected plant to healthy plants by different modes; transmission via seeds has the greatest significance because of the potential of infected seeds to serve as primary sources of virus inoculum. The disease may be spread from infected seeds over short and/or long distances, as well as to several

subsequent generations. It is necessary to apply sensitive and reliable methods to detect and identify the virus(es) in the infected seed lots to facilitate elimination of primary sources of inoculum such as infected seed and propagules.

Indian peanut clump virus (IPCV), belonging to the genus *Furovirus*, is transmitted by the biological vector *Polymyxa graminis* and also via seeds which form the primary sources of inoculum. Detection of IPCV by the quarantine tests was found to be difficult because of the lack of serological cross-reactions among three IPCV serotypes. A detection procedure with sufficient broad specificity to detect all serotypes of IPCV was therefore developed using nonradioactive reporter digoxigenin (DIG-) labeled probes. Small portions of the cotyledons were excised from each seed in order to leave the embryonic axle untouched and weighed amounts were processed either by ELISA test or for the extraction of nucleic acid. The seeds were germinated and samples from the seedlings and extracts were assayed for the presence of IPCV coat protein by ELISA test. The RNA extracts were tested by dot-blot hybridization using DIG-labeled probe 4 and as buffer extracts by ELISA test. Chemiluminescent detection gave a stronger signal than chromogenic detection. A direct correlation was observed between the positive hybridization signal and the detection of IPCV by either ELISA tests or grow-out tests. No nonspecific reaction with noninfected seed samples was seen. Probe 4 was highly sensitive and able to detect IPCV in many crop plant species such as wheat and weed plant species tested. The probe did not react with samples from plants infected by other furoviruses (Wesley et al. 1996).

Soybean mosaic virus (SMV) was found to induce mottling of seed coat. Slot-blot hybridization procedure was employed to detect the presence of SMV in total RNA from SMV-resistant control seed (L78-34). On the other hand, hybridization signals of varying intensities were detected with total RNA from both mottled and nonmottled susceptible Clark and Williams seed coats. Evaluation of the band intensities revealed that the level of SMV RNA accumulation in banded seed coats was approximately twice that in nonmottled seed coats, except in one of three replications. The results indicated that plants infected with SMV produced mottled and nonmottled seed with SMV present in both types of seed coats (Koning and Tekrony 2003).

Prunus necrotic ringspot virus (PNRSV) infects several stone fruit trees including plum, peach, apricot, almond, and cherry. PNRSV is pollen- and seed-transmitted and has the potential for rapid spread in stone fruit orchards (Aparicio et al. 1999). Pollen transmission of PNRSV was indicated by earlier investigation of Digiario et al. (1992). The route of infection and pattern of distribution of PNRSV in apricot pollen were investigated using dot-blot hybridization technique. PNRSV RNA was detected in washed, infected apricot pollen grains, as well as in their washings. PNRSV was present inside and also outside of infected apricot pollen grains. On the pollen surface it was probably attached to the exine. PNRSV was detected in all floral organs, sepals, petals, and anthers and the virus invaded flower buds at an early stage of development. Viral RNA could be detected inside pollen mother cells (PMC). Uniform distribution of viral RNA in the cytoplasm of unicellular and bicellular infected pollen grains, and in the generative cell of the latter, was observed. In situ hybridization (ISH) procedure detected PNRSV in the fully mature, infected pollen grains, mainly in the apertures. Electron microscopic observations confirmed that PNRSV was localized at the apertures of infected pollen grains. PNRSV infection persisted until flower maturity, when the virus was detected in the whole flower. A PNRSV-specific signal was not detected in the nucleus of any pollen grain. PNRSV was also localized

inside pollen tube, especially in the growth zone, after pollen germination. Although PNRSV infection affected apricot pollen grain performance, the virus did not completely prevent fertilization, resulting in seed development. The infected apricot pollen tubes, once germinated, were able to reach the embryo sacs. Infected pollen could therefore play an important role in the vertical (seed) transmission of PNRSV in apricot (Amari et al. 2007).

Prunus necrotic ringspot virus (PNRSV) has been reported to be transmitted via seeds at different rates, depending on the host plant species. Seed transmission may be as high as 77–89% in cherry (Vértesy 1976; Kryczynski et al. 1992) and at a low frequency of 9% in almond (Barba et al. 1986). In order to study the route(s) of virus movement into the seed, the progress of PNRSV infection in apricot reproductive organs during their development was investigated up to seedling stage using hybridization techniques. The embryos contained the least amount of viral RNA, whereas the endosperm and testa had the maximum viral RNA concentrations as revealed by dot-blot hybridization procedure. In situ hybridization technique revealed invasion of apricot embryo in the early developmental stages. The viral RNA could be detected in the embryos in the globular development stage obtained by hand pollination of virus-free flowers with infected pollen grains. Further virus presence in seed integuments, nucellus, and testa was also revealed by in situ hybridization technique. Apricot fruits produced from hand-pollinated plants with pollen from infected apricot trees were analyzed by tissue-printing hybridization method. These fruits showed uniform distribution of PNRSV. Infection of PNRSV was detected in 10% of the seedlings grown from infected seeds, indexed by dot-blot hybridization method. Movement of PNRSV from gametes to apricot seedlings was demonstrated by different hybridization techniques employed in this investigation (Amari et al. 2009). In situ hybridization test was applied for the detection of *Apple latent spherical virus* (ALSV) in apple flower buds in infected trees. The presence of ALSV inside almost all pollen grains and in all ovary and ovule tissues, including the embryo sac and inner integument, could be detected by in situ hybridization technique (Nakamura et al. 2011).

Tomato crops are infected by viruses with RNA or DNA genome. Among them, *Tomato mosaic virus* (ToMV) and *Pepino mosaic virus* (PepMV) are seedborne; *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), and *Tomato spotted wilt virus* (TSWV) are transmitted by insect vectors; and the natural spread of *Parietaria mottle virus* (PMoV) is not clearly understood. A DIG-labeled polyprobe ribopoly-6 was designed for the simultaneous detection of these viruses by nonisotopic molecular hybridization technique. The sensitivity of the ribopoly-6 was compared with that of individual riboprobes. No significant differences in the sensitivity of the probes could be observed between the single and poly-6 hybridizations for CMV, PepMV, PMoV, and PVY. The endpoint detection limit ranged over 5 to 0.5 pg of RNA transcripts for these viruses. In the case of ToMV and TSWV, the detection limit of the ribopoly-6 was slightly lower than its corresponding individual riboprobe (5 to 0.5 pg and 50 to 5 pg, respectively). Ribopoly-6 was able to detect RNA transcripts corresponding to the CP of any of the six viruses tested, without compromising the sensitivity level in relation to the individual riboprobes, up to least 50 pg of viral RNA (Fig. 7.2). The polyprobe/tissue-printing technique was found to be a reliable alternative detection method for six viruses. In addition, the protocol reduced labor and the overall cost of testing, as well as handling and manipulation of samples to a minimum (Aparicio et al. 2009).

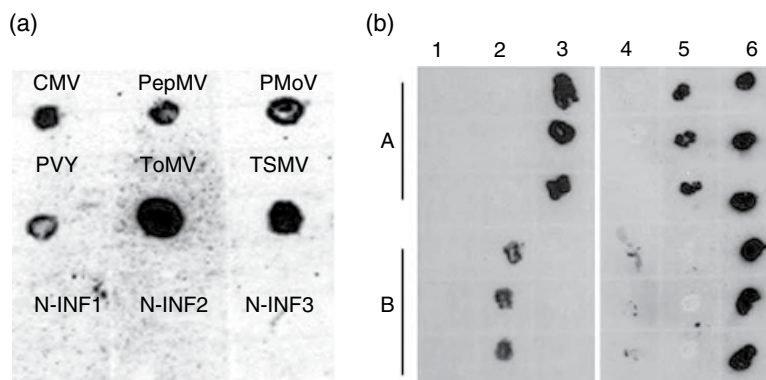


Figure 7.2 Simultaneous detection of six viruses by tissue-printing hybridization technique using nonisotopic polyprobe-6. (a) Positive reactions are indicated by development of color in samples infected by *Cucumber mosaic virus*, *Pepino mosaic virus*, *Parietaria mottle virus*, *Potato virus Y*, *Tomato mosaic virus*, and *Tomato spotted wilt virus*; lack of reaction in healthy samples N-INF1, N-INF2, and N-INF3. (b) Positive reactions are seen in field-grown plant samples when infected by any one of the viruses with which polyprobe-6 can hybridize. Source: Aparicio et al. (2009). Reproduced with permission of Springer Science, Heidelberg, Germany.

7.1.4.2 Polymerase Chain Reaction Assays

Polymerase chain reaction (PCR), in different formats and combinations, has been applied more frequently for the detection, identification, and differentiation of plant viruses because of its higher levels of sensitivity, reliability, reproducibility, and rapidity. Novel applications arising from PCR are progressively increasing as new problems are encountered demanding effective solutions. PCR is used to amplify the desired DNA fragments with known end sequences exponentially into billions of copies that can be detected efficiently. A major advantage of PCR-based assay is the possibility of detection of the pathogen-related DNA sequences present in naturally infected seeds/propagules at a concentration below the detection levels of other detection methods under natural conditions. Both ds-DNA and ss-DNA fragments can be directly amplified. Plant viruses with ds-DNA and ss-DNA are far fewer than viruses with RNA genomes.

Dahlia mosaic virus (DMV) and *Cocoa swollen shoot virus* (CSSV), DNA viruses, have been reported to be transmitted through infected seeds to seedlings, from which secondary spread of the disease occurs through their natural biological vectors. DMV, belonging to the genus *Caulimovirus*, family *Caulimoviridae*, is capable of causing serious losses in many countries where floriculture and ornamental industry have significant impact on the economy. Aphid species (16) are involved in the transmission of DMV in a nonpersistent manner. Since seed transmission of DMV was not definitely known, experiments were performed to demonstrate the seed- and pollen-borne nature of DMV. Dahlia seeds collected from different sources were tested by grow-out method in insect-proof greenhouse conditions. The seedlings were analyzed by PCR assays. Grow-out tests revealed a high rate of seed transmission of DMV. Using three primer pairs specific for DMV, the virus presence was detected in cotyledons. The virus could not be detected in seed coats. All seedlings obtained from seed germinated on filter paper tested positive for the presence of DMV with each of the three primer pairs. Likewise, DNA extracts from 4 week old leaf samples taken from plants grown in the

greenhouse also were positive for DMV by PCR assays, although plants were asymptomatic. Further, DMV could be amplified in total DNA extracts of pollen collected from flowers of infected dahlia plants. The results clearly indicated that DMV was transmitted through pollen and seeds of dahlia and the need for effective seed testing programs to eliminate the virus from infected seed samples was emphasized (Pahalawatta et al. 2007).

Cocoa swollen shoot virus (CSSV), a DNA virus belonging to the genus *Badnavirus*, poses a serious threat to cocoa production and is transmitted by coccid vectors. As the plants are propagated by seeds, it is essential to establish the seedborne nature of the virus and eliminate the infected seeds that would become the primary source of inoculum. Primers for CSSV were designed based on the conserved regions of six published sequences of the CSSV genome. An internal control targeting a nuclear cocoa microsatellite was used in a multiplex PCR with the CSSV assay. All pollen grains isolated from the anthers of infected Amelonada cocoa trees (labeled T2 and T4) tested 100% positive for the presence of CSSV by PCR/agarose gel electrophoresis (AGE) and PCR/capillary electrophoresis (CE). The component parts of cocoa seeds – testa, cotyledon and embryo – from T2 and T4 revealed varying levels of CSSV infection as shown by PCR/AGE, while PCR/CE detected CSSV in all component seed parts (100%). PCR detected CSSV in seedlings raised from seeds harvested from CSSV-infected trees. The results provided evidence for transmission of CSSV via cocoa seeds to seedlings (Quainoo et al. 2008).

Plant viruses with RNA as their genomes are predominantly found, and the RNA genomes have to be transcribed, using reverse transcription (RT), into complementary DNA (cDNA) prior to PCR amplification in the reverse-transcription (RT-) PCR format. The RT-PCR assay was shown to be effective in detecting *Pea seedborne mosaic virus* (PSbMV) in pea seeds (Kohnen et al. 1992). In a later investigation, RT-PCR assay was employed to detect and differentiate the pathotypes of PSbMV. The PSbMV pathotype P4 was isolated from seedlings of USDA pea (*Pisum sativum*) in New York. The P4-NY sequence-specific primers were constructed and compared for their detection sensitivity by RT-PCR and by pathotype-selective ELISA test. The pathotype PSbMV-P4 (0–7%) was seed-transmissible at much lower rates than P1 (0–32%) in selected pea cultivars and was not ELISA positive in reproductive tissues (pollen or embryo axes) of pea, whereas the pathotype P1 was readily detectable in these tissues. Detection of PSbMV isolates P1-DPD1 and P4-NT by RT-PCR was based on the sequence-specific priming and subsequent amplification of reverse-transcribed viral RNA. The primer pair for P1, nucleotide positions 6179–6204 and 7370–7393, respectively, facilitated amplification of a P1-specific 1200bp product. By comparison, a predicted 850bp product unique to P4 was consistently generated by P4-sequence-determined primers A and B in tissue samples containing ≥ 10 pg of P4 RNA/ μ g of tissue-extracted nucleic acid. P4-specificity was indicated by the formation of this RT-PCR product only in the presence of P4 RNA. The protocol developed in this investigation appeared to have potential for the detection of PSbMV in samples containing virus titer below the detectable levels of the ELISA test (Kohnen et al. 1995).

The comparative efficiency of the grow-out test and RT-PCR assay was assessed for the detection of *Cowpea aphidborne mosaic virus* (CAMV) in peanut seeds. The presence of CAMV was detected in a greater number of peanut seeds by RT-PCR assay than the number of seedlings observed to exhibit disease symptoms, as indicated by grow-out tests. The results of RT-PCR assay revealing the presence of viral RNA may not indicate the incidence of disease in the seeds, which is an important factor to be

determined in epidemiological investigations. DAC-ELISA format, employed as a pre-test, detected CAMV in 8 of 1553 peanut seeds, confirmed by RT-PCR assay. Another set of 57 seed samples comprising 5–24 seeds (in a total of 761 seeds) tested negative for ELISA test. In contrast, RT-PCR assay could detect CAMV in 7 of 57 seed samples. RT-PCR assay provided reproducible results in a greater number of samples when seed slices of 2–4 mm containing seed coat and cotyledon were tested. RT-PCR assay was more efficient and sensitive in detecting *Peanut stripe virus* (PStV) and *Peanut mottle virus* (PeMV) in a single seed from 100 seed composites using smaller seed slices (about 1 mm) compared to ELISA test (Gillaspie et al. 2001).

In a later investigation, RT-PCR assay and DAS-ELISA test were compared for their efficiency for the detection of *Peanut mottle virus* (PeMV) and *Peanut stripe virus* (PStV). RNA extracts were extracted from peanut seed slices or leaf disks using a Qiagen miniprep kit. The extracts of seed samples of each accession were then dispensed in a 96-well microplate with 4 rows of 12 wells. The other 4 rows of wells were used for testing another peanut accession. The RNA extracts of the pooled seed samples could be used for the detection of both PeMV and PStV in peanut seed lots by RT-PCR assay. DAS-ELISA tests could only detect the viruses in samples containing high virus titers. In contrast, RT-PCR assay was highly sensitive and the test could be applied to detect PeMV and PStV in pooled samples of peanut seed slices, some of which were ELISA-negative. The results indicated that RT-PCR assay could be employed for the consistent and reliable detection of both PeMV and PStV in peanut seedlots (Gillaspie et al. 2007).

Lettuce mosaic virus (LMV) is seedborne and naturally spread by aphids in a noncirculative manner. Most field isolates of LMV are seedborne in susceptible lettuce cultivars, but not in resistant cultivars carrying *moI*¹ or *moI*² gene. LMV isolates capable of breaking the resistance due to the genes *moI*¹ or *moI*² were also identified (Dinant and Lot 1992). The genetic diversity among LMV isolates was evaluated based on a 216 nucleotide sequence at the variable region encoding the Nib-coat protein function. LMV isolates were placed into five groups based on their ability to break resistance and seed-transmission properties. Three clusters of LMV isolates were distinguished: LMV-Yar, LMV-Greek, and LMV-RoW. In the latter cluster, two subgroups of isolates – LMV-Common and LMV-Most – accounted for a large proportion of LMV isolates analyzed. These two subgroups included the seedborne isolates, consistent with this property constituting a selective advantage and resulting in widespread distribution. LMV-Most isolates overcame the two resistance genes commonly used in lettuce, *moI*¹ and *moI*². These isolates might pose a potential threat to lettuce production. Of the two larger RoW groups, LMV-Common isolates were unable to overcome either *moI*¹ or *moI*², whereas LMV-Most isolates could combine seed transmission and the ability to overcome both *moI*¹ and *moI*² resistance. The complete sequence of an LMV-Most isolate (LMV-AF199) was determined to understand the genetic relationships among the three clusters of LMV isolates (Krause-Sakate et al. 2002).

Detection of *Soybean mosaic virus* (SMV) infecting soybean and its transmission via seeds and through the aphids are considered an important factor of its worldwide distribution, causing a marked impact on the production of soybean. The roles of selected SMV-encoded proteins in seed- and aphid-transmission were therefore investigated by constructing mutants in and chimeric recombinants between SMV 413 (efficiently aphid- and seed-transmitted) and an isolate of SMV G2 (not aphid- or seed-transmissible). The DAG amino acid sequence motif near the amino acid terminus of the coat protein (CP) was the major determinant of differences in aphid transmissibility of the two SMV isolates,

and helper component proteinase (HC-Pro) played a secondary role. Seed transmission was influenced by P1, HC-Pro, and CP. Replacement of the P1 coding region of SMV 413 with that of SMV G2 significantly enhanced seed transmissibility of SMV 413. Substitution in SMV 413 of the two amino acids that varied in the CPs of the two isolates with those from SMV G2, G-to-D in the DAG motif and Q-to-P near the carboxy terminus, significantly reduced seed transmission. The Q-to-P substitution in SMV 413 also abolished virus-induced seed coat mottling in plant introduction 68671. Association of P1, CP, and the DAG motif with seed transmission a potyvirus was reported for the first time, suggesting that HC-Pro interactions with CP may be important for multiple functions in the virus infection cycle (Jossey et al. 2013).

RT-PCR and immunocapture (IC-) RT-PCR formats were employed for the detection of *Soybean mosaic virus* (SMV) using a pair of primers designed to amplify a fragment in the coding region of the SMV coat protein (CP). A part of the sequence of coat protein was transcribed as the cDNA using reverse transcriptase. Both RT-PCR and IC-RT-PCR formats amplified the expected fragment from samples infected by SMV (Fig. 7.1; Ahangaran et al. 2009). Isolates of SMV were classified into strains based on differences in virulence. The complete genomic sequences of three SMV isolates with different levels of virulence occurring in China were determined. The sequences of 54 other SMV strains and isolates from other locations were compared with Chinese isolates. Genomic nucleotide diversity analysis and the Neutrality Test showed that the sequence of the 5' untranslated regions (5'UTR) of the virus genome-linked protein (VPg) and first protein (P1) genes had been altered by negative selection pressure. Phylogenetic analyses indicated that sequence variation in the cytoplasmic inclusion (CI) cistron of the virus was correlated closely with the level of virulence and with the source of the virus isolate. The CI gene sequence was shown to be crucial for classification and characterization of SMV strains, and may be a factor determining in the level of SMV virulence (Zhang et al. 2012a).

Transmission of *Peanut clump virus* (PCV), *White clover mosaic virus* (WCIMV), and *Carrot red leaf virus* (CaRLV) occurs through seeds of their natural host plants, peanut, white clover, and carrot, respectively. Virus-specific primers were constructed for the detection of these viruses using RT-PCR assays. The primers PCVuni5/PCVuni3 amplified a fragment of 631 bp from PCV, whereas WCIMV-CP5/WCIMV-CP3 produced an amplicon of 559 bp from WCIMV. In the case of CaRLV, the primer pair CL1-UP/CL1-DN was employed to amplify a product of 625 bp from CaRLV. Total nucleic acids were extracted from infected seeds and healthy plants. RT-PCR assays on the extracts were highly specific in amplifying the expected amplicons from the respective samples of seed extracts containing PCV, WCIMV, and CaRLV. RT-PCR assays proved to be better alternatives for the detection of seed-transmitted viruses, especially by quarantine programs, than ELISA tests which did not provide consistent results (Lee et al. 2004).

The viral genus *Potyvirus* includes 111 confirmed species and 86 tentative species, making it one of the largest genera of viruses (Fauquet et al. 2005). A novel pair of universal primers Nib2F and Nib3R was developed based on two conserved sites in the Nib region of potyvirus genomes. RNAs from 40 potyvirus isolates, representing 23 recognized species and 3 possible new species, was tested. The Nib assay detected all 40 virus isolates. The Nib2F and Nib3R primers targeted highly conserved sites that are located quite close to each other in potyvirus genomes, factors which probably contributed to the successful amplification. For most isolates, the Nib primers consistently generated a PCR product of 350 bp in size. The smaller size of PCR products amplified

by the Nib primers made cloning and sequencing easier for identification purposes. The viruses that are known to be seed-transmissible, such as *Bean common mosaic virus*, *Cowpea aphidborne mosaic virus*, *Papaya ringspot virus*, *Watermelon mosaic virus*, and *Zucchini yellow mosaic virus* were also detected in plant samples, using Nib2F and Nib3R primers. The results showed that these primers have the potential for detecting several potyviruses efficiently (Zheng et al. 2010).

Pea enation mosaic virus (PEMV), causing pea enation mosaic disease, has two obligately symbiotic viral genomes: PEMV-1, the sole member of the genus *Enamovirus* within the family *Luteovirus*; and PEMV-2, a member of the *Umbravirus* genus. A satellite RNA (RNA-3) is associated with PEMV-2 (de Zoeten and Skaf 2001). Quantitative RT-PCR was employed to estimate the relative abundance of PEMV-1 and PEMV-2 genomic RNAs in tissues, including seed coats, cotyledons, and embryo axes dissected from developing seed and systemically infected pea plants. Further, seed transmission was assessed in an extensive breeder seed multiplication grow-out and by ELISA testing of seedlings grown from seed harvested from infected pods. PEMV-1 and PEMV-2 genomes accumulated in leaves, stipules, developing whole pea, pea seed coat, and pea embryo. Both genomes accumulated in all tissues with large relative differences in mean normalized accumulation (MNA) between tissues (Fig. 7.3). The relative accumulation of PEMV-1 and PEMV-2 was c. 1240 and 13,000 times higher in leaf than in embryo tissues, respectively. Accumulation of PEMV-1 and PEMV-2 was also significantly higher in pod walls and seed coats than in cotyledons or embryo axes. The qRT-PCR analyses showed that PEMV-1 and PEMV-2 were seedborne. However, when 286 seedlings grown from seeds of infected pods were tested by ELISA at 27 days post-germination, none of the seedlings was ELISA positive. The results indicated that *Pea enation mosaic virus* could be seedborne, but the virus was not transmitted via seeds to developing seedlings (Timmerman-Vaughan et al. 2009).

Effectiveness of RT-PCR assay in detecting *Plum pox virus* (PPV) in anthers that are exchanged between countries for breeding purposes was assessed. Desiccated anthers of stone fruits plants stored at 0°C for 1–2 years were assayed by employing RT-PCR procedure. The presence of PPV was detected in the anthers, indicating that anthers might be a source of PPV and highlighting the need for monitoring international movement of *Prunus* germplasm to prevent the introduction of PPV inoculum into other countries and subsequent spread of the disease (Levy et al. 1995). RT-PCR assay was able to detect PPV in both seed coat and cotyledons. The results showed that RT-PCR assay was more sensitive, as the ELISA-negative cotyledons were found to be infected by PPV using RT-PCR procedure (Pasquini et al. 1998).

The primers based on the sequences of coat protein (CP) gene of *Plum pox virus* (PPV) amplified a 243 bp fragment in the C-terminus CP coding region. The PPV isolates infecting plum cultivars and wild apricot were identified as D strain of PPV (Spiegel et al. 2004). In a later investigation, a 243 kb fragment was amplified from 56 samples using RT-PCR format which detected PPV in peach (73%), apricot (88%), and plum (75%) trees with generic P1/P2 primer pair. Strain-specific primer results showed that 49 PPV isolates gave an amplification product with primer specific for M type, whereas seven peach cultivars gave a positive reaction with D-specific primer pair. RT-PCR format was found to be more sensitive and accurate in detecting PPV and its strains compared to DAS-ELISA test (Papayiannis et al. 2007). Immunosorbent electron microscopy (ISEM) technique was employed to detect the presence of *Fig latent mosaic virus 1* (FLV-1) in seedlings grown in the greenhouse. All seedlings, irrespective of age

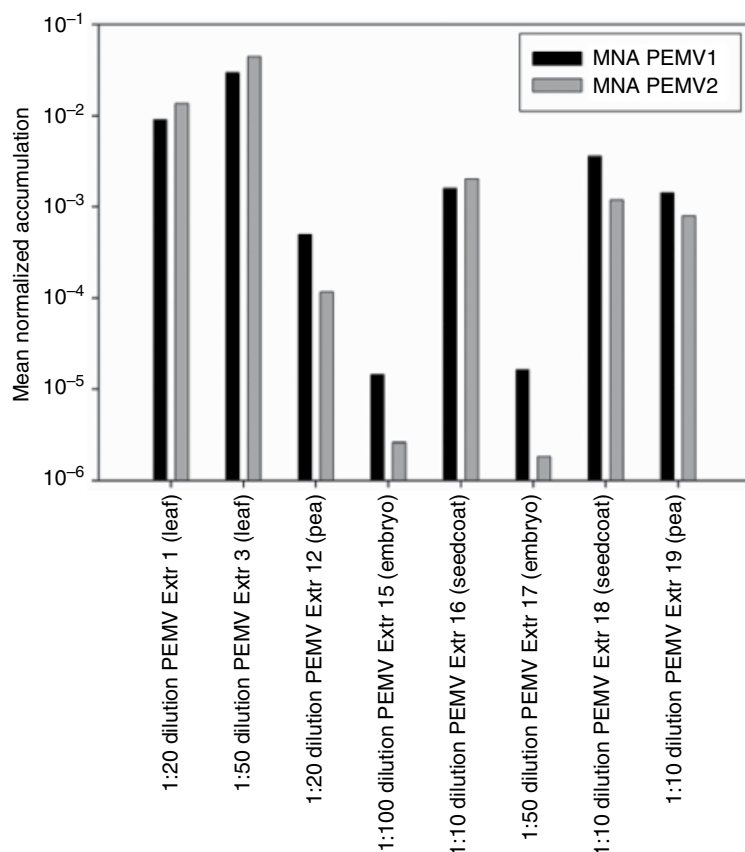


Figure 7.3 Estimation of genomic RNA concentration of two strains of *Pea enation mosaic virus* EMV1 and PEMV2, using multiplex quantitative real-time RT-PCR assay in stipule, whole pea, seed coat, and embryo tissues. Genomic RNA concentrations are expressed as mean normalized accumulation (MNA). Source: Timmerman-Vaughan et al. (2009). Reproduced with permission of the American Phytopathological Society, MN, USA).

and origin, were severely infected by FLV-1 with an incidence ranging over 73–100%. ISEM-positive seedlings were tested by RT-PCR assay using FLV-1-specific primers CPtr1f and CPtr2r, designed from the viral CP gene sequences. The expected 389 bp amplicon was generated from the extracts of infected seedlings, confirming the results of ISEM (Castellano et al. 2009).

Among the several viruses infecting pepper (chilli), *Pepper mild mottle virus* (PMMoV) (earlier known as *Tobacco mosaic virus*–pepper strain) is transmitted via seeds to seedlings. RT-PCR assay and tissue-printing immunoassay (TPIA) were used to assess the extent of seed infection by PMMoV. The effectiveness of pepper seed treatment with trisodium phosphate (Na_3PO_4) and dry sterilization of seeds at 70°C for 3 h in eliminating PMMoV from pepper seeds were determined using RT-PCR assay, using extracts of individual treated seeds. Almost all seeds (100%) were infected by PMMoV, as revealed by RT-PCR assay. Post-treatment RT-PCR analysis indicated that DNA fragments specific to PMMoV were detectable in seeds treated individually with chemical or heat. Neither treatment individually was able to eliminate the virus from all seeds tested.

On the other hand, the combined process of chemical followed by heat treatment resulted in the absence of a band specific for PMMoV in the gel, implying the elimination of the virus entirely from treated seeds. Infectivity assays performed by inoculating the extracts of seeds on the assay host *Nicotiana glutinosa* confirmed the results of RT-PCR assays. No local lesions were produced on leaves inoculated with extract of treated seed extracts, confirming the results of RT-PCR assay. The results indicated that RT-PCR assay could be reliably used not only for detecting the presence of PMMoV, but also for assessing the effectiveness of treatment for suppression of virus development (Toyoda et al. 2004).

Transmission of the potyviruses *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ringspot virus* (PRSV) through melon fruits and seeds was examined by using RT-PCR assay. Imported melon fruits of the yellow Canary type infected by ZYMV and PRSV were exposed in the fields next to healthy melon or squash bait plants. During this period, aphids were able to land on and probe the fruits. Using different fruits as sources of inoculum, 3.1–25% of the bait plants were infected by ZYMV and/or PRSV. Of these two viruses, PRSV was found to be more frequently transmitted to bait plants than ZYMV. RT-PCR assay was employed to establish the identity of the viruses present in the melon fruits. Comparison of partial sequences of the isolates from fruits and from bait plants showed a very high, if not complete, identity of the viruses, confirming that natural transmission of the virus(es) occurred from melon fruits to bait plants. The results suggested that ZYMV- and PRSV-infected melon fruits might be a means of dissemination of viruses between countries and continents (Lecoq et al. 2003). *Zucchini yellow mosaic virus* (ZYMV) transmitted through seeds of hull-less seed oil pumpkin (*Cucurbita pepo* var. *Styriaca*) at a very low rate was characterized. Three seed transmissible isolates of ZYMV occurring in Hungary were compared with the isolates from other countries. The nucleotide sequences of coat protein (CP) genes were determined and compared with the available CP sequences of ZYMV. Sequence analysis showed that the Hungarian isolates belonged to the Central European branch in the phylogenetic tree and, together with the ZYMV isolates from Austria and Slovenia, shared specific amino acids at positions 16, 17, 27, and 37 which were characteristic only to these isolates. The sequence analysis suggested that the common origin of isolates distributed in distant locations might be due to widespread seed transmission of *Zucchini yellow mosaic virus* (Tóbiás and Palkovics 2003).

Zucchini yellow mosaic virus (ZYMV), affecting several cucurbits including zucchini, cucumber, melon, watermelon, and pumpkin, has spread rapidly and achieved a global distribution within a few decades. International trading of infected fruit, seeds, and plants, vertical transmission via seed, and horizontal transmission by as many as 26 aphid species in a nonpersistent manner, have been considered as important factors responsible for the rapid and widespread occurrence of ZYMV. However, the role of seed transmission in disease spread remained unclear, necessitating experimental verification. Using RT-PCR assay, seed transmission rate of 1.6% in *Cucurbita pepo* subsp. *texana* was recorded. *C. pepo* plants from infected seeds were capable of initiating horizontal infections by ZYMV as determined by mechanical transmission and aphid vector (*Myzus persicae*). Four of the six seedlings mechanically inoculated showed disease symptoms and were tested by RT-PCR assay. Three plants derived from infected seeds were used as source plants for mechanically inoculating six healthy plants. Four plants (66.6%) were found to be infected by ZYMV using RT-PCR assay. Likewise, the aphid transmission of ZYMV from seedlings growing from infected seeds indicated a

seedling infection rate of 7.14%, confirmed by RT-PCR. In this investigation, a seed transmission rate of 1.6% for ZYMV in *C. pepo* subsp. *texana* was observed, providing evidence of vertical transmission of the virus to seedlings which could act as reservoirs of horizontal transmission by aphid vectors. This rate of seed transmission of ZYMV was considered to be theoretically high enough for infected seeds to constitute a viable route by which ZYMV epidemics may be initiated (Simmons et al. 2011).

Cucumber mosaic virus (CMV) has a wide host range, and seed transmission of CMV has been reported in some host plant species such as cucumber, pumpkin, and bean. Seed transmission of CMV in spinach (*Spinacia oleracea*) was investigated using the grow-out test, ELISA, and RT-PCR assay. In confirmation of the results of grow-out and ELISA test, RT-PCR assay was employed to amplify the expected 860 bp cDNA fragments containing the CMV coat protein (CP) gene in the reproductive tissues – embryo, endosperm, and pollen – of infected plants. The CMV CP gene cDNA was amplified to detectable levels with extracts from as few as five embryos. CMV cDNA was also amplified from nucleic acid extracts of some endosperm and pollen samples. The results of RT-PCR assay and electron microscopy revealed that CMV infected and accumulated in spinach reproductive tissues, as well as in seed tissues, and that both male and female parent could serve as infection sources of CMV for disease incidence and spread in spinach (Yang et al. 1997).

Vegetable crops are infected by several viruses of which *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV), *Tomato mosaic virus* (ToMV), *Cucumber green mottle mosaic virus* (CGMMV), and *Tomato black ring virus* (TBRV) cause significant losses in many countries. The extent of seed transmission of these viruses was assessed by employing RT-PCR assay and ELISA test on seeds of pepper (*Capsicum annuum*), tomato, melon, and lettuce obtained from farmers and companies. RT-PCR assays detected ToMV and TMV in tomato seeds and CMV, TMV, ToMV, and CMV in pepper seeds. The amplicons of expected size were generated in each virus–host combination, confirming the presence of the target virus(es) in the seeds. Likewise, the presence of CGMMV and CMV in cucumber seeds and TBRV and LMV in lettuce seeds was detected by RT-PCR assays. Comparatively, RT-PCR was more sensitive than ELISA tests since RT-PCR assay could detect the virus in some samples that were ELISA-negative (Gümüş and Paylan 2013).

A sensitive qRT-PCR assay for quantitative broad-spectrum detection of three pathotypes of *Squash mosaic virus* (SqMV) simultaneously in squash seed was developed. Converting SqMV RNA into cDNA prior to multiplexing stabilized the viral template that was then mixed with two other DNA templates (*Acidovorax avenae* subsp. *citrulli* (Aac) and *Didymella bryoniae* (DB)). To facilitate seed health testing, a generic plant nucleic acid extraction method was developed for cucurbit seed. Nucleic acid extracted from seeds yielded strong signals for each target pathogen in the multiplex qPCR format. The protocol developed in this investigation provided the opportunity of using a general nucleic acid extraction method followed by PCR to detect diverse fungal, bacterial, and viral pathogens. The procedure may therefore be used as a universal system for cucurbit seed health testing (Ling et al. 2011).

The possibility of *Cucumber green mottle mosaic virus* (CGMMV) transmission via pollen and seed was investigated. Cucumber seedlings at 3-true leaf stage were mechanically inoculated with CGMMV. At anthesis, scanning electron microscopy (SEM) and RT-PCR assay were employed to verify the presence of CGMMV. Healthy noninoculated cucumber plants were hand-pollinated with the pollen collected from the flowers of the infected plants. The rate of CGMMV transmission to the resulting fruits ranged

over 17.1–51.2%, compared with 33.3–100% for mechanically inoculated plants. RT-PCR assay was employed to detect CGMMV in seeds harvested from cucumber fruits of both treatments and tested for the presence of CGMMV. RT-PCR-positive seeds harvested from two treatments were sown separately. The seed transmission rates for the inoculated and noninoculated plants were 16.7–100% and 12.8–76.7%, respectively. The results indicated that CGMMV might be transmitted both horizontally via cucumber pollen and vertically to the next generation in cucumber. The rate transmission of CGMMV appeared to be at far higher levels compared with rates of seed transmission reported for other seed transmitted viruses (Liu et al. 2014).

Watermelon mosaic virus (WMV), belonging to the genus *Potyvirus*, family *Potyviridae*, infects soybean crops in many countries all over the world. The genetic diversity of 36 isolates of WMV from Slovakia and Iran were investigated, since analysis of genetic diversity and understanding of relatedness of viral strains is required for effective disease management and assessing the risks of emerging new diseases. RT-PCR amplification products were purified, using Wizard PCR Peps DNA Purification System (Promega Corp.), and sequenced directly from both directions by printing the sequencing reaction with the same oligonucleotides as used for PCR in order to obtain a master (dominant) sequences of isolate concerned. Sequence analysis targeting two opposite genomic regions (P1 and Nib-CP) was carried out. Phylogenetic analysis using partial sequences of the PI genes showed that Slovak WMV isolates had greater genetic diversity, representing two groups (group 1 and group 2), whereas all Iranian isolates belonged to a single group 2 with relatively low divergence. The results indicated that at least two independent introductions of WMV might have occurred and further spread of the virus via aphid vectors could be possible (Glasa et al. 2011).

Artichoke yellow ringspot virus (AYRSV) belongs to the genus *Nepovirus*, members of which are transmitted through seeds of their respective susceptible plant hosts. Transmission of AYRSV through seeds of broad bean and vetch (*Vicia sativa*) has been reported (Avgelis et al. 1992; Terzakis et al. 2002). Onion crops infected by AYRSV suffered considerable qualitative and quantitative losses. Biological and RT-PCR assays were applied to determine the transmission of AYRSV via onion seeds. All symptomatic onion plants grown from infected seeds were tested by RT-PCR assay using virus-specific primers that amplified a unique 530 bp fragment from the extracts of onion plants. A relatively high percentage of seedlings (20%) showed AYRSV infection as revealed by RT-PCR assay. Further, the results of RT-PCR assay showed that transmission of AYRSV occurred through pollen and seeds, which might help AYRSV in its survival and perpetuation in the absence of plant hosts and nematode vectors (Maliogka et al. 2006). Seed transmissibility in papaya of *Papaya meleira virus* (PMeV) occurring in Brazil and Mexico was investigated. Three types of seed materials – whole dry seed, seed coat, and embryo-endosperm from the papaya cultivar Maradol infected by PMeV – were tested using RT-PCR assay. Seed transmission rate of c. 81% was observed, indicating the importance of seed infection by PMeV in the incidence and spread of the virus in these countries (Tapia-Tussell et al. 2015).

7.1.4.3 Combination of PCR Assay with Other Diagnostic Methods

A combination of immunocapture (IC) and reverse-transcription polymerase chain reaction (IC-RT-PCR) procedure was developed to enhance the sensitivity and specificity of detection of *Peanut stripe virus* (PStV) and *Peanut mottle virus* (PeMV) in peanut seed lots. A small slice was removed from each seed distal to the radicle of a random

100-seed sample. The slices from seeds were extracted in buffer, followed by centrifugation. A portion of the supernatant was incubated in a tube that had been coated with antiserum specific to either PStV or PeMV. The virus was captured by the respective antiserum and the tubes were washed. RT-PCR mix (with primers designed from conserved sequences within the capsid region of each virus) was placed in the same tube and the amplification of DNA fragments specific to each virus was accomplished. The amplified band of 611 bp representing PStV and 339 bp product representing PeMV were observed after electrophoresis in 1.5% agarose gels in TE buffer and stained with ethidium bromide. The threshold of detection by IC-RT-PCR was one peanut seed infected by either virus in 100 seeds. The IC-RT-PCR assay had greater level of sensitivity than ELISA test used earlier and this protocol showed the potential for testing a large number of seed lots of peanut germplasm entries (Gillaspie et al. 2000).

Soybean mosaic virus (SMV), belonging to the family *Potyviridae*, exists in the form of many strains. Precise identification and differentiation of SMV strains will be useful for developing SMV-resistant soybean cultivars. A RT-PCR/RFLP combination was employed for efficient detection and identification of SMV strains. A primer pair amplifying a 1385 bp fragment of the cylindrical inclusion (CI) coding region was used for RT-PCR and RFLP. Profiles of the amplified products were subjected to restriction digestion with *RsaI*, *EcoRI*, or *AccI* restriction endonucleases. The RT-PCR/RFLP patterns of eight SMV isolates collected from local soybean cultivars were compared with the five reference strains. Based on the banding patterns, 13 SMV (5 strains and 8 isolates) could be grouped into 6 or 8 categories when restriction-digested with *EcoRI* or *RsaI*, respectively. RT-PCR/RFLP differentiated SMV strains that could not be distinguished by the screening procedure using differential soybean cultivars. RT-PCR/RFLP was highly sensitive and therefore only required tiny amounts of viral RNA. The results of RT-PCR/RFLP correlated well with strain identification by symptom phenotypes produced on differential cultivars inoculated with strains and isolates. The seedborne SMV isolates from local soybean cultivars could be differentiated by RT-PCR/RFLP analysis. The strains inducing necrosis were in low concentrations and the sensitive RT-PCR/RFLP procedure could also detect and differentiate these strains. As there was 99% nucleotide sequence homology in the amplified 1385 bp region between two SMV strains, RT-PCR/RFLP technique was also able to differentiate them (Kim et al. 2004). Seed samples of cowpea (136) collected from different regions of India were indexed for the presence of seedborne viruses by employing grow-out test, differential host response assay, electron microscopy, ELISA, and immunocapture (IC-) RT-PCR assays. Infection of 43 cowpea seed lots by *Bean common mosaic virus* (BCMV-BCIM) was detected. The identity of three of these isolates as BCMV-BCIM was established conclusively by sequencing the nucleotides in the 3' region of the viral genome. Seed transmission of BCM-BCIM varied over the range 0.67–13.49% (Udaya Shankar et al. 2009).

Plum pox virus (PPV) causing “sharka” disease infects plum, apricot, peach, and sour and sweet cherries. PPV isolates were classified into four groups, namely D, M, EA, and CD, based on serological and biological properties which may have a bearing on the ability of the isolates to be transmitted via seeds. Extent of seed transmission of PPV isolates – PPV-D (more common in apricot and plum) and PPV-M (common in peach) – was investigated by applying ELISA and IC-RT-PCR assay. Isolates were characterized by restriction analysis of amplicons with *RsaI* restriction enzyme and analysis of coat protein migration on 15% SDS-polyacrylamide gel followed by Western blotting.

The presence of PPV was checked on fully ripe seeds, germinating seeds, and seedlings. One half of the apricot seed stock was analyzed by ELISA and IC-RT-PCR assays, separating cotyledons, also containing the embryo, from seed coats. IC-RT-PCR assay detected the virus up to 100%, whereas ELISA was unable to detect the virus in the same extract. PPV was mainly localized in seed coats (69% of PPV-D and 53.2% of PPV-M) in infected seeds tested. The virus was present in the cotyledons at lower percentages (34.8% of PPV-D and 10.0% of PPV-M) in infected seeds. Cotyledons, plumule, and radicle tested separately by IC-RT-PCR assay showed different levels of PPV infection. When tested by IC-RT-PCR assay, seedlings of apricot (over 3 years) and peach (over 6 months) did not show the presence of the virus or any disease symptoms. The results showed that seeds did not have any role in PPV-M and PPV-D infection (Pasquini et al. 2000).

The possibility of transmission of *Apple latent spherical virus* (ALSV) via apple seeds was examined using reverse-transcription (RT-) quantitative PCR (qRT-PCR) assay. The cotyledons of seeds and true leaves of seedlings emerging from the seeds of infected apple trees were tested for the presence of ALSV. The amplification curves of ALSV RNAs from cotyledons were classified into three patterns: fast (F); slow (S); and no amplification (N/A). Viral RNA was present at high titer (F pattern) in cotyledons from approximately 1% of seeds, a proportion broadly consistent with earlier reports on ALSV seed transmission rates. The majority of seeds (94.4%) had minute concentrations of virus-related RNA (S pattern). Low concentrations of virus-related RNA were also detected by siRNA analysis using next-generation sequences. However, no viral RNA could be detected in the true leaves following seed germination. The results indicated that viral RNA present in the seed possibly failed to replicate. No ALSV RNA was detected (N/A pattern) in the remaining 4.6% of seeds. For all practical purposes, almost all apple seedlings (c. 99%) from ALSV-infected apple trees might be considered as virus-free (Kishigami et al. 2014).

7.2 Detection of Viruses in Propagules

Plants are propagated to increase the plant population in the field through true seeds produced after sexual reproduction or through propagules such as tubers, bulbs, corms, suckers, cuttings, setts, or budwood for grafting onto rootstocks. Transmission of viruses via seeds to seedlings generally occurs at low percentages. On the other hand, the viruses carried by propagules are transmitted more frequently and at higher percentages, and they may exhibit visible symptoms of virus infection or remain asymptomatic. Selection of virus-free planting materials is the basic requirement for growing healthy crops that can provide better yields and for preventing buildup of virus inoculum by planting infected materials repeatedly. This is a crucial factor in virus disease epidemiology; once diseased plants are once established in commercial orchards, there is no effective method of curing the infected plants. Under such a situation, the most effective method of containing the secondary spread of the disease is the removal of infected plants as other options are ineffective or impractical or uneconomical.

The principal approach for managing virus and virus-like diseases transmitted through propagules is the production of nuclear stocks free of diseases by efficient certification/clean stock programs, by employing sensitive, reliable, and rapid techniques for detecting and identifying the viruses present in the plant materials, especially

when they are asymptomatic. It may then be possible to ensure the supply of virus-free planting materials to the growers. Application of inappropriate/less-sensitive methods may result in the escape of infected planting materials. Adequate care therefore has to be taken in applying the correct technique and selecting proper sampling of planting materials. Use of multiple detection methods may be required for detection of viruses in perennial fruit crops in which the viruses may have long incubation periods extending to several weeks. The mother plants from which the planting materials are obtained have to be tested at the proper time/season, when the viruses are actively multiplying. Detection techniques have been developed based on the biological, immunological, and nucleic acid properties of the target virus(es) (Narayanasamy 2011).

7.2.1 Bioindexing Methods

The presence of viruses in mother plants or propagules is visually examined by personnel with sufficient experience. The infected plants with early symptoms and propagules with doubtful symptoms are eliminated. Plants which were infected late in the season may not show any recognizable symptoms. Asymptomatic plants and propagules are indexed by taking the inoculum from them and inoculating on assay or indicator plants which express characteristic symptoms (either local lesions or systemic symptoms) rapidly within a few days. Several plant species such as *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus*, *Nicotiana benthamiana*, *N. clevelandii*, and *N. occidentalis* react with chlorotic or necrotic local lesions to several viruses.

In the case of viruses infecting stone fruit trees, propagules are indexed by graft-inoculating *Prunus* spp. and varieties that respond to virus infection rapidly. *Prunus persica* cultivar GF305 (peach) was used as the bioindicator plant for detecting *Plum pox virus* (PPV). Characteristic symptoms of PPV infection was expressed on leaves of indicator plants (Desvignes et al. 1999; Gentit 2006). *Strawberry crinkle virus* (SCV) was detected in mother plants by inoculating *Nicotiana occidentalis* which developed countable local lesions, the number of which represented virus titer in the infected strawberry sample (Klerks et al. 2004). Mild strains of *Strawberry mild yellow edge virus* (SMYEV) induced delayed or no symptoms. *Fragaria vesca* “EMC” already infected with the latent A strain of *Strawberry crinkle virus* (SCV) developed symptoms of infection following inoculation with very mild strains of SMYEV. The latent A strain infection therefore increased the sensitivity of the indicator plants that produced epinasty of newly emerging leaves together with chlorotic flecks. *Strawberry mottle virus* (SMoV) and *Strawberry vein banding virus* (SVBV) could be detected by performing leaflet grafting onto *F. vesca* and *F. virginiana* clones (Martin and Tzanetakis 2006).

Tobacco rattle virus (TRV), belonging to the genus *Tobravirus*, has nematodes as the biological vectors. The virus could be transmitted by sap inoculation onto *Nicotiana clevelandii* and *Petunia* hybrid, and they were used as bait plants by planting them in the soil naturally infested with the nematode vector *Paratrichodorus minor*. The bait plants were placed in growth chambers at 20°C and 30°C for two days. Virus-like symptoms were observed in the bait plants. Infection of bait plants by TRV was confirmed by employing antigen-trapped indirect ELISA format (Pérez et al. 2000).

The ability of viruses to infect one or more plant species is one of the biological properties studied, and the host range of the virus/strains may be useful in identifying the virus concerned. *Potato virus Y* (PVY), the type species of the genus *Potyvirus*, causes serious diseases in a large number of plant species including potato, tobacco,

and tomato worldwide. PVY isolates represent three major strain groups: ordinary (PVYO); tobacco vein necrosis (PVYN); and aphid nontransmissible stiple streak (PVYC). Many recombinant and nonrecombinant forms of PVY occur in different countries around the world. PVY was inoculated using infective sap and aphid on 802 and 3712 seedlings, respectively, representing 21 weed species. Infection of *Erodium cicutarium*, *Geranium pusillum*, *Lactuca serriola*, and *Lamium purpureum* by PVY was detected by ELISA tests, although the plants remained asymptomatic. The presence of PVY in selected plants of the four species was confirmed by back-inoculations to *Nicotiana tabacum* (tobacco). PVY infection of *L. serriola* in Central Europe was observed for the first time (Kaliciak and Syller 2009).

Biological indexing of budwood sources was the foundation for the first certification program commenced in California (Hiltabrand 1957). Indexing was performed by grafting tissue to sensitive indicator plants. Young seedlings of sweet orange (*Citrus sinensis*) were used for testing, and they were placed in a temperature-controlled glasshouse after graft-inoculation. Observations were made for the development of characteristic symptoms of *Citrus psorosis virus* (CPsV) which included a shock reaction with leaf shedding and die-back of the first flush and/or chlorotic leaf flecking and spotting on successive flushes. As this procedure required a long time and glasshouse facility, immunoassays replaced the biological indexing procedure for the detection of CPsV (Martin et al. 2002). The usefulness of biological indexing for graft-transmissible viruses infecting citrus in the presence of additional pathogens was evaluated. Mexican lime (ML) was used as the primary indexing host for both *Citrus tristeza virus* (CTV) and *Citrus vein enation virus* (CVEV), whereas sweet orange (SWO) and Dweet tangor (DwT) were used as indicator plants for *Citrus psorosis virus* (CPsV). Multiple infections did not preclude symptom expression or reduce the diagnostic efficacy of the primary indexing hosts for CTV, CPsV, and *Citrus tatter leaf virus* (Apple stem-grooving virus). Symptoms of CVEV and the diagnostic efficacy of Mexican lime were suppressed by the T-30 group CTV isolates, but not by other CTV isolates tested. An application of alternate bioassay hosts for indexing was investigated. Dweet tangor and Citrus excels detected CVEV in single infections, whereas in sweet orange CVEV was detected only when CPsV was present. CTV was readily detected using alternate indicator plant Citru excels, whereas only shock-reaction-inducing CPsV isolates could be effectively indexed by Mexican Lime (Vidalakis et al. 2004).

7.2.2 Immunoassays

Polyclonal and monoclonal antisera have been prepared against sap transmissible viruses. On the other hand, purification of non-sap-transmissible viruses was found to be difficult because of the low virus titer and uneven distribution in the source plants. Several immunoassays have been tested for their sensitivity, reliability, and reproducibility. Double-antibody sandwich (DAS-) enzyme-linked immunosorbent assay (ELISA) and indirect DAS-ELISA formats have been employed with different degrees of success in detecting the viruses in asexual propagules. It was estimated that about 11×10^6 ELISA tests were carried out in the Netherlands annually on propagative materials, including about 5×10^6 tests for indexing seed potatoes and about 6×10^6 tests for indexing ornamental plant materials. This high volume of tests demonstrates the suitability and cost-effectiveness of ELISA tests, which could be performed with ease even by less trained personnel (Clark and Adams 1977; Huttinga 1996).

Significant advancements have been made in the production of antibodies specific for plant viruses. A novel method of preparing antisera was developed for viruses for which purified preparations or viral proteins were not readily available. The coat protein (CP) gene of the virus is cloned and expressed in *Escherichia coli* cells and the polypeptide(s) produced in bacterial cells were used as immunogens for production of polyclonal antibodies (PABs) in the rabbits. The N-terminal P1 proteinase of *Potato virus Y* (PVY), associated with the cytoplasmic inclusion bodies induced by PVY, was expressed in *E. coli*. The antiserum raised against the expressed protein detected the P1 protein associated with the cytoplasmic inclusion bodies, characteristic of PVY infections (Arbatova et al. 1998). Isolates of *Citrus tristeza virus* (CTV) (often referred to as *Citrus tristeza virus* complex) differed in their serological properties. They were broadly assigned to four groups based on their pathology in different citrus cultivars. The most important groups are those that caused incompatibility between sour orange rootstock and susceptible scion cultivars (decline-inducing isolates (D-I)) and those cause stem-pitting (SP-isolates) of scion cultivars in decline-tolerant scion-rootstock combinations. CTV isolates that did not induce decline or stem-pitting were considered as mild isolates. Some isolates cause severe seedling yellows (SY) on sour orange, lemon, and grapefruit. SY reaction is commonly used as a diagnostic symptom for severe CTV isolates. Epitopes for a panel of 30 monoclonal antibodies (MAbs) specific for CTV were mapped on CTV coat protein (CP) expressed in *E. coli* cells. Expression constructs that generated different portions of CTV CP were screened against MAbs by Western blotting and ELISA test. All MAbs could be placed into five groups (I to V), four of which have continuous sequential epitopes. The linear, continuous epitope for MCA-13 (aa 118–128) which distinguished Florida quick decline-inducing (D-I) CTV isolates from mild isolates, was expressed in *E. coli* cells as a peptide fused to a maltose-binding protein (MBP). This fusion protein was purified and used as an antigen to generate a rabbit PAB antiserum. This aa 118–128-specific polyclonal antiserum had the same serological properties as MAb MCA-13 and reacted with Florida quick D-I CTV isolates, but not with mild isolates (Nikolaeva et al. 1996).

Another significant development is the production of phage-displayed recombinant antibodies, specific to target virus M13 phage (with ss-DNA as the genome) that infects strains of *Escherichia coli* carrying the *f'* episome (plasmid). The infected bacterial cells act like a factory producing M13 phage components continuously without undergoing lysis. The phage components include phage DNA gene 8 coat proteins, gene 3 attachment proteins, and other proteins that may be linked or fused to these proteins. The gene 3 proteins (3–5 copies/phage particle) located on the tip of the phage facilitate the attachment of phage particles to receptors on the *Escherichia coli* cells. The receptor sites are present in the hair-like projections (pili) expressed on the surface of *E. coli* strains carrying *f'* episome. For the production of M13 phage-displayed recombinant antibodies, the DNA from antibody-producing β -lymphocytes or hybridomas is genetically linked to the phage gene 3 DNA. The proteins encoded by the antibody DNA and gene 3 DNA are coexpressed or fused to one another, resulting in the production of an antibody–gene-3 fusion protein. When the phage carrying the gene fusion infects *E. coli*, expression of an antibody molecule occurs on its tip. Any antibody DNA linked to phage DNA and any antibody protein fused to phage proteins are assembled and secreted in a manner similar to that of the phage proteins. The recombinant antibodies are equally specific as MAbs and can be used for the rapid identification and differentiation of plant viruses (McCafferty et al. 1990).

The fragment of the IgG (antibody) molecule in the C-terminal end is designated Fc fragment. Different antibodies (IgM, IgA, IgD, and IgE) exhibit a unique antigen-binding site, as they retain a common three-dimensional structure since the framework of the Fv region is not affected by the hypervariable residues present in loops that extend into the solution. The constant region located in the C-terminus of the antibody molecule defines the class of antibody such as IgG, IgM, IgA, IgD, and IgE. The IgG molecule has a Y shape and the variable regions (V_H and V_L) are located at the extremity of two arms and have one variable and constant region. Generally, IgG and IgM are formed in response to antigenic stimulation. Most of the monoclonal antibodies generated may therefore be of IgG or IgM isotype. Plant viruses may induce predominantly IgG which is resistant to mercaptoethanol. The Fc fragment of IgG has high affinity for protein A molecule (MW 42,000) isolated from cell walls of the bacterium *Staphylococcus aureus*. A single-chain Fv antibody fragment (scFv) may be prepared from a synthetic phage antibody after several rounds of selection against a purified preparation of antigen (viruses). A scFv fragment may be engineered to recognize a specific epitope of antigenic protein and expressed in plants by employing an appropriate vector such as an epichromosomal expression vector derived from *Potato virus X* (PVX). The gene encoding the secretory scFv is then used to transform the plant. The engineered scFvs may become inexpensive tools for the detection of antigen. These "plantibodies" may find wide applications, including detection of human viruses. A bispecific scFv product in transgenic tobacco plants was able to recognize certain neotopes (new reactive surfaces) and cryptotopes (hidden reactive surfaces) of *Tobacco mosaic virus* coat protein (TMV CP) (Fischer et al. 1999). Functional expression of a scFv both in *E. coli* and plant cytoplasm was observed (Tavladoraki et al. 1999). A genetic immunization system by engineering scFv fragments capable of recognizing epitopes on plant virus capsids (coat proteins) was developed. A scFv fragment was engineered to recognize an epitope of the glycoprotein G1 conserved among large number of tospoviruses (type member *Tomato spotted wilt virus*). The gene encoding the secretory scFv was used to transform *Nicotiana benthamiana* plants following a procedure similar to that employed for TMV-CP (Franconi et al. 1999).

A recombinant single-chain variable fragment (scFv) antibodies with specificity to *Citrus tristeza virus* (CTV) strain, which causes the most severe disease in citrus accounting for heavy losses worldwide, were produced from the hybridoma cell lines 3DF1 and 3CA5. These scFvs were genetically fused with dimerization domains and alkaline phosphatase, respectively. Diagnostic reagents were produced by expressing these fusion proteins in bacterial cultures (*E. coli*). The engineered antibodies were successfully used for detection in plants by DAS-ELISA and tissue-print (TP-) ELISA. The fully recombinant ELISA tests showed that the recombinant proteins, as diagnostic reagents, had similar efficiency in detecting CTV in plant tissues as their parental MCAs, 3DF1, and 3CA5, indicating that the recombinant antibodies might be used for routine detection of CTV (Terrada et al. 2000).

Citrus psorosis virus (CPsV) causes citrus psorosis disease which is widespread and economically important, affecting citrus production. The virus transmission by mechanical inoculation to herbaceous plants was erratic and the virus titer was low in infected plants. The difficulty in purifying the virus made the preparation of antisera against CPsV laborious. In order to prepare effective antisera against CPsV, the CP gene of an Italian isolate of CPsV with almost identical amino acid sequence similarity to a Florida CPsV was cloned and expressed as fusion protein with maltose-binding protein

(MBP) in *E. coli* cells. Polyclonal antiserum to the fusion protein (As-PsRc1) and IgGs purified from it were used successfully in Western blotting and ELISA formats for the detection of antigen in total protein extracts, crude sap from infected *Chenopodium quinoa*, or citrus plants. The AsPsRc1 antiserum was specific and was able to detect CPsV in infected plants without the need for a pre-absorption step to remove host plant proteins (antigens). The availability of an antiserum against recombinant virus CP represents an additional and/or alternative tool for manufacturing standardized kits for the serological detection of CPsV. The use of recombinant coat proteins for antiserum production eliminates the need to maintain and purify viruses, and allows the process of antiserum production to be standardized (Loconsole et al. 2006).

The effectiveness of polyclonal antibodies to recombinant p25 *Citrus tristeza virus* (CTV) coat protein (rCP) was assessed for use as both trapping and the intermediate antibody in indirect DAS-ELISA test. The p25 CP gene of three CTV isolates was amplified by reverse-transcription (RT-) PCR procedure and further cloned and expressed in *Escherichia coli* cells. The rCP was injected into rabbits and goats for antibody production. Western blotting assays with rCP CTV-specific antibodies reacted positively with the homologous and heterologous rCP of the three CTV isolates, and with corresponding native coat protein present in crude sap extracts of CTV-infected citrus tissue, but not with extracts from healthy trees. The rCP antibodies from goat and rabbit reacted as both plate trapping and intermediate antibodies in DAS-ELISA, discriminating healthy and CTV-infected citrus plants; OD405 absorbance values ranged over 0.151–2.415 for CTV-infected samples and <0.100 for healthy tissues. Commercially available antibodies developed to recombinant antigens against CTV were employed as functional antibodies for trapping and intermediate antibodies for reacting specifically with *Citrus tristeza virus* isolates in DAS-ELISA tests. After fractionation with PAGE and dialysis against phosphate-buffered saline, the antibodies were used as immunogen (Iracheta-Cárdenas et al. 2008).

Single-chain fragment variable (scFv) antibody (scFvLR3) directed against the CP of *Grapevine leafroll-associated virus 3* (GLRaV-3), a major viral pathogen infecting grapevine, was expressed in *E. coli* and used to develop a diagnostic ELISA Kit. The antibody was fused to the light chain constant domain of human immunoglobulin to create the bivalent CL-LR3 which was purified from the periplasmic fraction, giving a yield of c. 5 mg L⁻¹. The bivalent antibody could be effectively used as coating and detection reagents, providing a distinct improvement in the sensitivity over original monomeric scFvLR3. The sensitivity of the reagent against recombinant GLRaV-3 CP was 0.1 ng. The sensitivity, specificity, and durability of the reagent was similar to a commercial kit. The CL-LR3 showed a weak cross-reaction in immune electron microscopy assays on GLRaV-1 and GLRaV-7, but not with the phylogenetically more distant GLRaV-2. A fully recombinant kit was developed with the inclusion of a recombinant GLRaV-3 CP expressed in bacteria to avoid problems associated with virus propagation and purification. The protocol was found to be a rapid, simple, sensitive, and reliable tool for detection of *Grapevine leafroll associated virus-3*. Freedom from GLRaV-3 of grapevine planting materials in the nursery might be ensured by the DAS-ELISA test, using the antibody scFvLR3. This antibody was fused to the light chain constant domain of human immunoglobulin to create the bivalent reagent C_L-LR3. In immunosorbent electron microscopy (ISEM) assay, C_L-LR3 strongly reacted with GLRaV-3 but weakly with GLRaV-1 and decorated GLRaV-7. Strong binding of C_L-LR3 with GLRaV-3 in ISEM assay corroborated the results of ELISA tests (Fig. 7.4). The protocol developed in

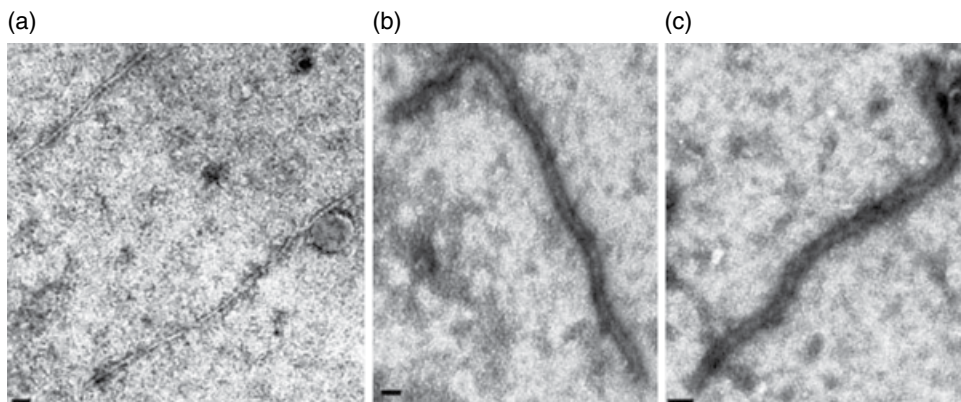


Figure 7.4 Detection of grapevine leafroll viruses by immunosorbent electron microscopy (ISEM) using the bivalent reagent C_L-LR3. The bivalent antibody could trap (a) GLRaV-1 or decorate (b) GLRaV-7 and (c) GLRaV-3; bar = 100 nm. Source: Cogotzi et al. (2009). Reproduced with permission of Springer Science, Heidelberg, Germany.

this investigation maintained the robustness and sensitivity of the conventional DAS-ELISA, with the advantage of the use of fully synthetic and highly standardized reagents that could be made available in unlimited quantities (Cogotzi et al. 2009).

7.2.2.1 Enzyme-Linked Immunosorbent Assays

Double-antibody sandwich (DAS-) enzyme-linked immunosorbent assay (ELISA) and its variants have been demonstrated to be effective in detecting several viruses transmitted through propagules to the subsequent generations of crops. Viruses infecting fruit and vegetable crops have been detected by ELISA tests, facilitating the elimination of infected plants/planting materials, resulting in the reduced disease incidence level and consequent prevention of crop losses. Plant viruses detected in mother plants or propagules using ELISA formats are presented in Table 7.6.

DAS-ELISA format was effective in detecting *Citrus tristeza virus* (Bar-Joseph et al. 1979), *Plum pox virus* (Voller et al. 1976), and *Strawberry latent ringspot virus* (Kristek and Polak 1990). Polyclonal antisera raised against the respective viruses were used for the detection of the viruses in plant extracts. Indexing of fruit trees and propagules was performed using ELISA tests. *Apple mosaic virus* (Clarke et al. 1976), *Plum pox virus* (Adams 1978), and *Prunus necrotic ringspot virus* (Barbara et al. 1978) were detected using ELISA tests with polyclonal antibodies (PABs). Screening of plum germplasm entries for the presence of PPV was taken up using ELISA tests. Flowers, leaves, and bark of clones were tested and the presence of *Plum pox virus* (PPV), *Apple mosaic virus* (ApMV), and *Prunus necrotic ringspot virus* (PNRSV) was confirmed rapidly, facilitating the selection of varieties resistant to the target virus(es) (Karešová and Paprštejn 1998). Transgenic plum trees (*Prunus domestica*) expressing CP gene of PPV were inoculated by viruliferous aphids or chip budding. The inoculated plants were tested by DAS-ELISA to determine infection by PPV. Among the five transgenic clones tested, clone C-5 was found to be resistant to PPV (Ravelonandro et al. 1997).

Bacterially expressed virus coat protein (CP) fragments were used for preparing antibodies specific for the virus. The antibodies were employed for the detection of *Citrus tristeza virus* (CTV) in ELISA tests (Nikolaeva et al. 1996). The fully recombinant

Table 7.6 Viruses detected in propagules of crops using immunoassays.

Immunoassay/ crop plant	Viruses detected	References
ELISA formats: (a) Fruit crops		
Apple/Pear/Plum	<i>Apple mosaic virus</i>	Matic et al. (2008)
	<i>Apple stem pitting virus</i>	Jelkman and Keim-Konrad (1997)
	<i>Pear vein yellowing virus</i>	Jelkman and Keim-Konrad (1997)
	<i>Plum pox virus</i>	Adams (1978); Spiegel et al. (2004); Michelutti et al. (2005)
	<i>Prune dwarf virus</i>	Clarke et al. (1976)
	<i>Prunus necrotic ringspot virus</i>	Barbara et al. (1978); Michelutti et al. (2005); Matic et al. (2008)
Cherry	<i>Little cherry virus 1</i>	Matic et al. (2009)
Citrus	<i>Citrus psorosis virus</i>	D'Onghia et al. (1998); Kaparia-Isaria et al. (2007)
	<i>Citrus tristeza virus</i>	Bar-Joseph et al. (1979); Nikolaeva et al. (1996); Terrada et al. (2000); Martin et al. (2002); Loconsole et al. (2006)
	<i>Citrus fastidiosa</i>	Bar-Joseph et al. (1979); Nikolaeva et al. (1996); Terrada et al. (2000); Martin et al. (2002); Loconsole et al. (2006)
Grapevine	<i>Grapevine Bulgarian latent virus</i>	Tomažič et al. (2008)
	<i>Grapevine fan leaf virus</i>	Kamenova et al. (2007); Tomažič et al. (2008)
	<i>Grapevine fleck virus</i>	Kamenova et al. (2007); Tomažič et al. (2008)
	<i>Grapevine virus B</i>	Kamenova et al. (2007); Tomažič et al. (2008)
	<i>Grapevine leafroll-associated virus-1 and -5</i>	Monis (2000); Xu et al. (2006); Tomažič et al. (2008)
ELISA formats: (b) Vegetable crops		
Onion	<i>Onion yellow dwarf virus</i>	Mahmoud et al. (2007); Conci et al. (2010)
	<i>Leek yellow stripe virus</i>	Mahmoud et al. (2007); Conci et al. (2010)
Potato	<i>Potato leafroll virus</i>	Salim Khan et al. (2003)
	<i>Potato mop-top virus</i>	Sokmen et al. (1998)
	<i>Potato virus A, M, S, X, and Y</i>	De Bokx (1987); Varveri (2000); Salim Khan et al. (2003)
	<i>Tomato spotted wilt virus</i>	van Schadewijk (1996)
Sweet potato	<i>Sweet potato chlorotic fleck virus</i>	Jones and Dwyer (2007)

Table 7.6 (Continued)

Immunoassay/ crop plant	Viruses detected	References
Yam (<i>Dioscorea</i> sp.)	<i>Sweet potato feathery mottle virus</i>	Jones and Dwyer (2007)
	<i>Sweet potato virus 2</i>	Jones and Dwyer (2007)
	<i>Dioscorea bacilliform virus</i>	Odu et al. (1999)
ELISA formats: (c) Ornamental plants		
Iris	<i>Iris mottle virus</i>	van der Vlugt et al. (1993)
Lily	<i>Cucumber mosaic virus</i>	Derks et al. (1997)
	<i>Lily mottle virus</i>	Derks et al. (1997)
	<i>Lily symptomless virus</i>	Derks et al. (1997)
	<i>Lily virus X</i>	Derks et al. (1997)
Tissue-blot immuno assay		
Citrus	<i>Citrus psorosis virus</i>	Martin et al. (2002); Loconsole et al. (2006)
	<i>Citrus tristeza virus</i>	Cambra et al. (1994); Terrada et al. (2000); Lin et al. (2006)
Garlic	<i>Garlic latent virus</i>	Tsuneyoshi and Sumi (1996)
	<i>Garlic mosaic virus</i>	Tsuneyoshi and Sumi (1996)
	<i>Garlic virus A, B, C, and D</i>	Tsuneyoshi and Sumi (1996)
Prunus	<i>Apple chlorotic leaf spot virus</i>	Cambra et al. (1994)
	<i>Plum pox virus</i>	Cambra et al. (1994)
	<i>Prune dwarf virus</i>	Cambra et al. (1994)
	<i>Prunus necrotic ring spot virus</i>	Cambra et al. (1994)
Immunoblot assay		
Cherry	<i>Cherry mottle leaf virus</i>	James and Howell (1998); Gispert et al. (1998)
	<i>Little cherry virus 1</i>	Matic et al. (2009)
Citrus	<i>Citrus tristeza virus</i>	Nikolaeva et al. (1996); Alibach-Marti et al. (2000)
Grapevine	<i>Grapevine leafroll-associated virus</i>	Monis and Bestwick (1997); Monis (2000)
Dot-immunobinding assay		
Cherry	<i>Cherry mottle leaf virus</i>	James and Mukerji (1996)
Grapevine	<i>Grapevine rupestris stem pitting-virus</i>	Minafra et al. (2000)

(Continued)

Table 7.6 (Continued)

Immunoassay/ crop plant	Viruses detected	References
Yam	<i>Yam mosaic virus</i>	Njukeng et al. (2005)
Immunosorbent electron microscopy		
Banana	<i>Banana streak virus</i>	Thottappilly et al. (1998); Harper et al. (2002)
Citrus	<i>Citrus psorosis virus</i>	Martin et al. (2004); Loconsole et al. (2006)
Cymbidium (orchids)	<i>Cymbidium mosaic virus</i>	Hsu et al. (1992); Vejaratpimol et al. (1999)
Garlic	<i>Carnation latent virus</i>	Maeso et al. (1997)
	<i>Garlic virus A</i>	Helguera et al. (1997)
	<i>Onion yellow dwarf virus</i>	Maeso et al. (1997)
Sugarcane	<i>Sugarcane bacilliform virus</i>	Viswanathan and Premachandran (1998)
In situ immunoassay		
Citrus	<i>Citrus tristeza virus</i>	Lin et al. (2000)

antibodies used in the ELISA tests were shown to be as specific and sensitive as the conventional ELISA tests performed with parental MAbs, indicating the usefulness of recombinant antibodies for routine detection of CTV in woody plants such as citrus (Terrada et al. 2000). The sanitary status of all clonal Genebank collection of stone fruit trees was evaluated using ELISA tests. A total of 645 trees comprising 197 peach and nectarines, 183 sweet and sour cherries, 106 plum, 106 apricot, and 53 other cherries were tested by ELISA tests for the presence of *Plum pox virus* (PPV), *Prunus necrotic ringspot virus* (PNRSV), and *Prune dwarf virus* (PDV). PPV infection was not detected in any of the trees tested. PNRSV and PDV were frequently detected in single and mixed infections. The overall average of virus infection rate was determined to be 20.3%. PNRSV was predominantly found, accounting for 74% of the infections in all trees tested, with the highest rate in apricot (90.9%). PDV infection was observed at higher rates in sweet and sour cherries (64%) and plum (60%) (Michelutti et al. 2005). The presence of ilarviruses in stone fruit trees was monitored throughout the year (2005) using ELISA tests. All samples were tested by DAS-ELISA with a commercial kit. All PNRSV-infected trees gave positive reactions to DAS-ELISA during November or January. During other months, the highest number of positives was obtained between February and May with nine, eight or nine out of eleven PNRSV-infected trees. All trees infected by PDV tested positive during March-April-May. Detection by ELISA was high in January and February, ranging over 78–89%. In the case of ApMV, detection of the virus in all trees was only accomplished in November. In other months, detection rates varied over 0–60%. The results indicated that the virus titer might vary with different months of the year; samples should therefore be acquired at the appropriate time using the correct plant tissues (Matic et al. 2008).

Plum pox virus (PPV) occurs in the form of four strains or serotypes, differing in their pathogenicity, host range, aphid transmission efficiency, and geographical distribution. An unusual isolate of PPV, considered to originate in Eastern Europe, could not be classified as a member of any of the strains recognized already. Determination of the identity of any new strain is necessary to develop an effective management system for the strain of the virus concerned. Detection of PPV in apricots from wild and cultivated plum in Kazakhstan was attempted using ELISA tests. All plum samples from 11 trees, one sample from 11 apricot trees, and the positive control reacted positively when tested by triple-antigen sandwich (TAS-) ELISA format using PPV-specific universal MAb 5B as the secondary antibody. All healthy controls tested negative for PPV in TAS-ELISA test. Based on the TAS-ELISA tests, the variation in the isolates of PPV in Kazakhstan did lead to any conclusion (Spiegel et al. 2004). *Prunus necrotic ringspot virus* (PNRSV) was detected using DAS-ELISA format in woody host *Prunus mahaleb*. The sensitivity of the ELISA test was determined using serially diluted extracts of infected and healthy leaf samples of *P. mahaleb*, up to 1/180 dilution of extract (Sipahioğlu 2005). ELISA formats were employed to detect viruses in propagation plant materials, rootstocks, and seedlings of fruit tree cultivars. PNRSV was detected in plum rootstock sample, whereas *Prune dwarf virus* (PDV) was detected in cherry seedlings. *Plum pox virus* (PPV) was detected in peach and cherry rootstocks and seedlings. Mixed infection by PDV + PPV was observed in cherry seedling sample. None of the viruses tested was detected in apple and pear rootstock and seedling samples. Overall, 6.13% of propagative plant materials scheduled to be imported into Turkey showed infection by one or two of the viruses investigated (Özdemir and Kaya 2008).

ELISA tests were employed to detect *Citrus psorosis virus* (CPsV) in citrus plants from which propagative materials had to be prepared. ELISA tests could detect CPsV in a greater number of infected citrus accessions in which the bioindexing method did not detect CPsV infection, indicating the suitability of ELISA tests for eliminating infected citrus plants because of its greater sensitivity, rapidity, and reliability (D'Ongnia et al. 1998). CPsV could be detected in citrus plant tissue extracts by using polyclonal and monoclonal antibodies (PABs and MABs) in ELISA tests. Two formats, DAS-ELISA and TAS-ELISA, were applied for the detection of *Citrus tristeza virus* (CTV) in young shoots. All samples reacting with A322 in DAS-ELISA also reacted with MAb 13C5 in TAS-ELISA, but two samples reacting with 13C5 were not recognized by MAb2A3. For each MAb, CPsV detection by TAS-ELISA and another immunoassay, direct tissue-blot immunoassay (DTBIA), were always coincident. Detection of CPsV by DAS-ELISA and TAS-ELISA in previously untested field trees correlated well with psorosis diagnostics based on biological indexing, specifically with the results of cross-protection tests (Martin et al. 2002).

The use of grafting materials from infected citrus trees for nursery propagation is the principal method for the dissemination of virus and virus-like diseases, since they are graft-transmissible. ELISA tests were found to be efficient in detecting *Citrus tristeza virus* (CTV) in sweet orange (*Citrus sinensis*) cultivar Musambi source plants. Microbudding technique was employed for early propagation of virus-free plants. Thermotherapy combined with indexing procedure based on DAS-ELISA test reduced the time required for the development of virus-free Musambi nuclear stocks. When results were needed rapidly and suitable indicator plants were not available, the ELISA test was found to be highly useful for the production of virus-free citrus plants (Abbas et al. 2006). For the selection of elite mother plants of citrus for producing clean stocks,

a survey was taken up in Cyprus. All mother plants were tested by biological indexing and ELISA test. Plants free of *Citrus tristeza virus* (CTV) and *Citrus variegation virus* (CVV) were selected for propagation. However, some mother plants were found to be infected by *Citrus psorosis virus* (CPsV). A standard procedure of shoot-tip grafting technique was applied for eliminating CPsV in selected citrus accessions. Micro-grafted plants regrafted on sour orange seedlings in vivo and successfully established were transferred to the glasshouse. These plants were tested 6–9 months later using ELISA tests. Virus-free plants were used as the primary source material for the basic citrus plantation (Kaparia-Isaia et al. 2007).

Grapevine leafroll is a graft-transmissible disease caused by nine serologically distinct viruses (Gugerli 2003). Monoclonal antibodies (MAbs) are monospecific and function as better immunologic reagents for sensitive detection and precise identification of components of complex diseases. Grapevine LR102 was used as the viral source infected with a number of grapevine leafroll-associated viruses. An optimized ELISA using the LR102 PAb and 15F1-AP conjugates allowed the specific detection of GLRaV-5 and GLRaV-8 present in LR100 and LR102 virus sources. Incubation of coating antiserum samples and conjugates at 4°C overnight were the best conditions for detection of viral antigens. No reaction occurred with crude extracts of tissues infected with GLRaV-1, -2, -3, -4, or -6, indicating the specificity of antibodies.

Monoclonal antibodies reactive to the previously uncharacterized *Grapevine leafroll-associated virus* (GLRaV) coat protein were developed. A novel 37 kDa protein was found to be associated with grapevine leafroll disease in mixed virus infection. The polypeptide was separated from the 38 kDa polypeptide associated with GLRaV-1 using tricine sodium dodecylsulfate-phenylamide gel electrophoresis (TSDS-PAGE) system. Concentrated p37 preparations were used to immunize the mice for the production of MAb which reacted with the 37 kDa polypeptide, but not with the 38 kDa polypeptide associated with GLRaV-1 using Western blot assay. The MAbs displayed reactivities to either the 37 kDa polypeptide alone, the 37 kDa polypeptide and 36 kDa polypeptide associated with GLRaV-4 and -5, or the 36 kDa polypeptide associated with GLRaV-5 and the novel 37 kDa polypeptide. The 36 kDa polypeptide associated with GLRaV-4 and the 37 kDa polypeptide associated with the novel GLRaV-8 therefore share at least one peptide with p37 protein. The protocol developed for ELISA using the 15F1 MAb and LR102 PAb allowed detection of GLRaV-5 and -8 in crude plant extracts. The use of MAbs increased the specificity and sensitivity of detection by ELISA and Western blots by reducing the background due to the presence of host-specific antibodies in polyclonal antiserum (Monis 2000). Detection of GLRaV-3 in flowers or fruits of symptomatic and asymptomatic grapevines using DAS-ELISA was generally erratic throughout the growing season. However, ELISA test was effective for detecting GLRaV-3 in petioles of symptomatic and asymptomatic vines, with nearly all tested samples being positive from the developing berries stage onward. The bark scrapings of canes from vines or dormant cuttings were found to be the best tissues for reliable detection of GLRaV-3 by ELISA tests (Ling et al. 2001).

Grapevine leafroll-associated virus 2 (GLRaV-2), one of the viruses associated with leafroll disease of grapevine, is also implicated in disorders known as graft incompatibility, young vine decline, and rootstock stem lesion. The antisera were raised to recombinant virus proteins for the detection of GLRaV-2 infection in diseased grapevines. The genes encoding RNA-dependent RNA polymerase (RdRp) and coat protein (CP) of Chinese isolate of GLRaV-2-CF were cloned in the vector pET-28a (+) and sequenced.

Comparisons of these sequences with those of other GLRaV-2 isolates available from databases revealed homology levels with GLRaV-2 PN and GLRaV-2 Sem higher than 98% at the nucleotide and amino acid levels. The cloned RdRp and CP were successfully expressed in *Escherichia coli* BL-21 and the resulting recombinant proteins were used to raise antisera in rabbits. Both antisera were tested for the presence of GLRaV-2 in grapevine samples by protein-A sandwich (PAS-) ELISA and indirect dot-blot immunobinding assay (IDBIA). The antiserum to recombinant CP could be used effectively to detect GLRaV-2 by either method. The results were comparable to those obtained from DAS-ELISA tests using a commercial kit. The recombinant RdRp-based antiserum provided somewhat different results. The efficiency of the antisera varied depending on the grapevine accessions. Suitable dilutions of antiserum to RdRp for use in PAS-ELISA were 1:500 and 1:250 for trapping and revealing, respectively, whereas those for the antiserum to CP were 1:1000 and 1:500, indicating the greater sensitivity of antiserum to CP. The results showed that antiserum to CP had greater potential for detecting GLRaV-2 in plant tissues, whereas antiserum to RdRp might be more useful for studying virus–host relationships at the cellular level (Xu et al. 2006).

The infection of grapevines by over 70 agents (viruses, viroids, and phytoplasmas) was recognized by the International Council for the Study of Virus and Virus-like Diseases of Grapevine (ICVG) in 2003. Plant growth yield and fruit quality are significantly reduced by virus diseases which spread through infected propagules (buds, cuttings, rooted cuttings from infected canes) over long distances. Secondary spread of the diseases may be due to activities of the natural biological vectors present in those locations. Effective control of the disease(s) therefore depends on clean stock programs. In Bulgaria, the propagative grapevine materials were checked by AgroBio Institute, Sofia, by employing DAS-ELISA tests. In accordance with the European Legislation and the National System for Production of Certified Vine Growing Material operating in Bulgaria, 1295 samples from grapevine varieties and rootstocks were checked for their virus status during 2004–2006. A total of 30.5% of plant materials tested were ELISA-positive for one (86.08%) or more (13.92%) viruses. *Grapevine fleck virus* (23.3%), *Grapevine fanleaf virus* (5.05%), and *Grapevine leafroll-associated virus-1* and *-3* (3.34% and 2.78%, respectively) were more frequently detected. *Grapevine Bulgarian latent virus* (0.37%) was also detected occasionally. The results clearly underscore the need for constant monitoring of incidence of virus diseases in planting materials, which might remain asymptomatic, to avoid appreciable losses induced by viruses in the subsequent generations of grapevines (Kamenova et al. 2007). An ELISA protocol was optimized for the detection of *Grapevine leafroll-associated virus 2* (GLRaV-2) and *Grapevine virus B* (GVB) in grapevine propagative planting materials, using extraction buffer in BIORIBA Kit. Both viruses could be detected in the planting materials and the suitability of the protocol for virus detection for certification program in Slovenia was suggested by this investigation. The results were confirmed by Western blotting and electron microscopy coupled with decoration procedure (Tomažič et al. 2008).

Little cherry virus 1 (LChV-1), an unassigned species of the family *Closteroviridae*, causes the little cherry disease (LChD). Different immunization strategies for producing antibodies against viral coat protein (CP) of LChV-1 were applied. Antisera were produced using either purified virus preparations, bacterially expressed proteins, or a DNA vector that expressed the cloned CP gene in vivo. Effective induction of specific antibodies to LChV-1 CP was obtained using DNA intramuscular immunization followed by a single boost with the recombinant protein. The entire coat protein sequence

was cloned in a mammalian expression vector and, after being coated by an amphiphilic nontoxic reagent, was delivered into rabbit. A protein boost increased the specific immune response against the virus protein. Purified antibodies from the DNA-CP boost IgG preparation detected LChV-1 in plants grown in vitro infected by ITMAR and ITSPA isolates. They were effective in capturing LChV-1 from infected leaves at IgG dilutions of 1:500 ($2\mu\text{g mL}^{-1}$) and at a conjugated antibody dilution of 1:250. Infected sample extracts had 29 times higher OD values on average than in-vitro-grown healthy cherries. Further, the antibodies detected LChV-1 in field-grown trees at the same dilution used for plants grown in vitro. Of the 50 stone fruit trees LChV-1 was detected in 20 trees by ELISA, although infection by LChV-1 was indicated by RT-PCR in greater number of trees, revealing the lower level of sensitivity of ELISA tests. Although ELISA failed to detect the virus in almond (isolate ITPER) and peach (isolate ITMAY), it detected LChV-1 in cherry (75%) and plum (39%). The DNA-CP boost antiserum provides a promising option for the serological detection of LChV-1, since the antiserum to the virus was not available commercially (Matic et al. 2009).

Indexing of propagules has to be preceded by application of some treatments to break dormancy or activate dormant tissues of the propagules. Viruses begin to replicate and reach higher concentrations, enabling successful detection of viruses in the planting materials after breaking the dormancy. Detection of *Potato virus Y* (PVY) and *Potato virus A* (PVA) by ELISA test was possible only after breaking dormancy of potato tubers (Vetten et al. 1983; De Bokx 1987). Storing potato tubers at 20°C for three weeks facilitated detection of *Potato mop top virus* (PMTV) by ELISA (Sokmen et al. 1988). Storage of dahlia tubers seemed to have some influence on detection of *Tomato spotted wilt virus* (TSWV). Dahlia tubers stored for a minimum period of 4 weeks followed by taking mixed samples of three roots per tuber were suitable for reliable detection of TSWV by ELISA test, since the virus distribution in tubers was uneven (van Schadewijk 1996). Removal of small pieces of bulb tissue, followed by storing at 17°C or 20°C for 3 weeks, was required for detection of *Iris severe mosaic virus* by ELISA test (van der Vlugt et al. 1993). For the *Lily mottle virus* (LMOV), the scales of lily had to be stored at 20°C for 2–3 weeks under fluorescent light (12–16 h/day) for the detection of LMOV using ELISA test. In contrast, *Lily symptomless virus* (LSV), *Cucumber mosaic virus* (CMV), and *Lily X virus* detection by ELISA did not require any pretreatment (Derks et al. 1997). The *Dioscorea bacilliform virus* (DaBV) infecting yam was detected rapidly and identified precisely by employing ELISA tests. In addition, by using ELISA tests it was possible to establish the serological relatedness of DaBV with *Sugarcane bacilliform virus* and *Banana streak virus* (Phillips et al. 1999). A new strain of *Potato virus Y* infecting *Dioscorea alata* was identified by applying ELISA tests (Odu et al. 1999).

Potato is infected by probably the largest number of viruses, dispersed by the infected potato tubers to different locations (Salazar 1996; Brunt 2001). *Potato virus Y* (PVY), the type species of the genus *Potyvirus* (family Potyviridae), occurs worldwide, infecting a wide range of crops including potato. A prerequisite for producing virus-free seed potato tubers is the availability of accurate and rapid methods for detection of low levels of virions and viral genome molecules. An antiserum against PVY was produced against a local strain in Greece. DAS-ELISA format was applied for the detection of PVY. The dilution endpoint of DAS-ELISA tested was determined to be 10 ng mL^{-1} of virus with a mean absorbance value at 405 nm of 0.118, whereas the absorbance values of healthy control was 0.034. The sensitivity of detection of PVY was improved further by employing the one-step immunocapture (IC-) RT-PCR assay (Varveri 2000).

In-vitro-generated plantlets of six potato cultivars – Cardinal, Diamant, Dhera, Mutta, Cilena, and Sieglinde – were mechanically inoculated with seven potato viruses, namely A, Y, V, M, S, X, and *Potato leafroll virus* (PLRV), and the inoculated plants were monitored for 4–6 weeks. DAS-ELISA tests were performed using the antisera specific to each virus to be detected. Plantlets inoculated with viruses were infected, except PLRV which was not mechanically transmissible. *Potato virus S* did not induce recognizable symptoms in all six potato cultivars inoculated with PVS. However, DAS-ELISA test detected the virus in all infected plantlets (12/12) with strong positive reaction with the antiserum. In the case of other potato viruses, visible symptoms were produced and detection of the respective virus by ELISA tests could be achieved effectively. The limit of detection of most viruses in ELISA tests ranged between 1 and 10 ng mL⁻¹ using the chromogenic substrate p-nitrophenyl phosphate (NPP). The extracts of noninoculated plants did not respond positively with the antisera in the ELISA tests (Salim Khan et al. 2003).

Potato seed-tuber samples were analyzed using DAS-ELISA commercial kits for detection of *Potato leafroll virus* (PLRV), *Potato virus X* (PVX), and *Tomato spotted wilt virus* (TSWV), whereas TAS-ELISA format was applied using broad-spectrum monoclonal antibodies for the detection of *Potato virus Y* (PVY). Infection by PVY in the ware potato and noncertified potato seed tubers in all areas were up to 100% of suspected plants or tubers. PLRV was widespread (30–38%) in the upland crops and in noncertified seed tubers (29%). A small number of field plants were also infected by TSWV. More than 50% of potato seed lots produced in two locations in Greece were rejected in 2005 and 2006 during their final stage of certification due to heavy PVY infection (up to 60%). Both PVY and PLRV were also detected in several important lots of the “basic” and “certified” categories of seed tuber. In basic seeds, 25 of 96 lots and 33 of 88 lots showed PVY infection, and PLRV was detected in 12 and 13 lots in 2005 and 2006, respectively. PVX was detected occasionally in 9 and 7 basic seed lots in 2005 and 2006, respectively. The certified seed tuber lots also showed significantly higher levels of infection by PVY (86/302 and 145/336 lots), while PLRV infections were at lower levels compared to PVY. The presence of PVY and PLRV in certified seed tubers indicated the need for applying more sensitive detection methods to restrict the spread of these viruses through seed tubers (Chatzivassiliou et al. 2008).

Tomato spotted wilt virus (TSWV) has the potential to cause significant losses in potato production. Five popular potato cultivars were found to be susceptible to two tobacco isolates of TSWV. The rate of TSWV transmission from infected foliage to tubers and from infected tubers to developing progeny potato plants was affected by cultivars, while virus transmission efficiency was independent of TSWV isolates tested. ELISA test was applied to detect TSWV in different potato tissues used for sample preparation. Virus presence was revealed in all samples of leaves and tubers by ELISA tests. However, composite sampling from six different tuber parts was found to be the most reliable for assessing the extent of infection by TSWV in potato plants (Bulajić et al. 2014). In the ELISA tests, the key step is the incubation of microtitre plates for 3–4 h with IgG and a conjugate in an incubator at a temperature of 37°C. The effectiveness of mechanically shaking the plates using a shaker to induce a vibrating movement, instead of incubation of plates in the incubator, was assessed. Replacing the stages of incubation of plates with IgG and conjugate at 37°C with mechanical shaking allowed the duration of the whole process of DAS-ELISA format to be reduced to less than 3–4 h without any significant impact on the quality of detection of potato viruses. The process

of shaking the microtitre plates for 90 min was found to be equally efficient as the 3 h long incubation for the detection of *Potato virus Y* (PVY), *Potato virus M* (PVM), and *Potato leafroll virus* (PLRV). The reaction background value for healthy sap as control was very low in all combinations, irrespective of the time of reading after the substrate was placed. In the case of PLRV, a decrease in the sensitivity of the shaking method was observed compared to incubation in the incubator (Wróbel 2014).

The performance of the conventional ELISA format was compared with two PCR-based methods for the detection of *Potato virus Y* (PVY) in potato seed tubers. The seed tubers were permitted to have 2% infection by PVY as per rules of certification for seed potatoes in Idaho, USA. The PCR-based methods (Nes Multi and IPC Multi 2), as a group, were found to be superior to ELISA test. However, the PVY detection performance of one the PCR-based methods (New Multi) was not statistically different from the performance of ELISA tests. The results suggested that application of PCR methods in the potato certification programs would result in unjustifiable costs for seed potato producers, unless other economic benefits from using PCR-based methods are realized. In addition, the type of a sample material affected the PVY detection outcome. PVY might be detected more readily in leaves rather than in potato tubers (Bolotova et al. 2009). For the detection of *Iris yellow spot virus* (IYSV) infecting onion crops, the antiserum produced in rabbits using bacterially expressed recombinant viral (N) protein was employed as the immunogen. The specificity and sensitivity of the antiserum in detecting a wide range of IYSV isolates were determined by employing an indirect ELISA format. The antiserum was specific by reacting with the extracts from IYSV-infected onion plants, but not with extracts of healthy plants. IYSV could be detected in up to 1:1000 dilution of IYSV-infected onion tissue extracts of samples collected from several states in the USA. No cross-reactivity with other tospoviruses, *Tomato spotted wilt virus* and *Impatiens necrotic spot virus* (INSV), was observed, indicating the specificity of the antibodies generated against IYSV. The indirect ELISA format provided the advantage of using the commercially available secondary conjugated antibody. IYSV was found to be the predominant tospovirus infecting onion crops in the USA (Pappu et al. 2008). Calla lily plants are infected by four potyviruses, *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TuMV), *Konjac mosaic virus* (KoMV), and *Zantedeschia mild mosaic virus* (ZaMMV) in Taiwan. The sensitivity and specificity of detection of these potyviruses by indirect (I)-ELISA format and multiplex RT-PCR assay were compared. The antisera for the viruses were prepared using the recombinant capsid proteins of respective viruses as immunogens. The sensitivity of detection of the viruses was 25–265 times lower than those of multiplex RT-PCR, depending on the virus concerned. KoMV was found to be the dominant virus in the field-grown plants (Hu et al. 2010).

Onion yellow dwarf virus (OYDV) belonging to the genus *Potyvirus* (family Potyviridae) is one of the major viruses infecting garlic crops. *Chenopodium amaranticolor* reacted with local lesions and was used for biological purification of OYDV for further analysis. Polyclonal antiserum was produced by injecting the rabbits with purified virus preparations. Repeat-indirect ELISA format was applied for the detection of OYDV in infected garlic plants. Green leaves and clove storage leaves were tested by DAS-ELISA format to assess the variations in the virus concentrations. OYDV concentration was low in the apical leaf and then increased up to about the sixth leaf below the apex. Virus concentration was higher in older leaves compared with apical leaves. OYDV was successfully detected in cloves of garlic. The DAS-ELISA format was found

to be sensitive enough to detect OYDV in clove storage leaf than in the leaf initials of the dormant plantlets. This test could be used to screen during the summer season, before the bulb was separated into planting material (Mahmoud et al. 2007). Under natural conditions, garlic plants are infected simultaneously by two or more number of viruses. Virus-free garlic plants obtained by meristem culture were rapidly infected when planted in the field. The pattern of virus movement and fluctuations in virus titer in leaves and cloves of garlic plants after infection, were investigated. DAS-ELISA format was employed for the detection of *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV) in different plant parts and cloves of garlic. Significant differences in virus concentrations were observed in different leaves, but the distribution of the viruses was variable. Hence, no one type or position of leaf was preferable for detecting virus presence. Sampling any leaf at the end of the crop cycle (about 200 days after planting) appeared to be best time, since the older plants had higher concentrations (several fold) than the younger plants. Analysis of virus distribution in bulbs revealed that the virus concentration was higher in early-inoculated than in the late-inoculated plants. All cloves in 81% of bulbs were either ELISA-positive or -negative. Individual cloves were either infected or remained uninfected in only 6% of the bulbs. Other bulbs (13%) gave negative results coexisting with an uncertain status as revealed by ELISA tests (Conci et al. 2010).

7.2.2.2 Tissue-Blot Immunoassay

In tissue-blot immunoassay (TBIA) the viral antigens are transferred from the freshly cut plant tissue surface to nitrocellulose membranes. The cut surface is pressed gently and a tissue imprint is made on the membrane. The blots are probed with antibodies specific to the target virus labeled with enzymes. In the indirect method, the antigens are allowed to react first with the primary antibodies specific to the virus and then with enzyme-labeled secondary antibodies. Using both direct and indirect methods, several viruses infecting fruit trees, namely *Apple chlorotic leaf spot virus*, *Prunus necrotic ringspot virus*, *Prune dwarf virus*, *Plum pox virus*, and *Citrus tristeza virus*, were detected reliably and consistently in infected mother plants. This method is simple, sensitive, and rapid, providing the results in 3 h. The imprinted membranes could be stored for long periods for reference and transported from the field to the laboratory (Cambra et al. 1994). The comparative effectiveness of ELISA and tissue-blot immunoassay (TBIA) was assessed for the detection of *Lily symptomless virus* (LSV) in lily bulb scales. TBIA detected LSV in a greater number of samples than ELISA test. LSV was detected in some samples that were ELISA-negative, indicating a greater level of sensitivity of TBIA procedure (Gera et al. 1995). Six viruses infecting garlic, namely *Garlic mosaic virus* (GMV, *Potyvirus*), *Garlic latent virus* (GLV, *Carlavirus*), and four *Garlic virus*-type viruses (Gar V-A, Gar V-B, Gar V-C, and Gar V-D), were detected in infected plants and identified by using direct tissue-blot immunoassay (DTBIA) and RT-PCR assay. The respective viral coat protein (CP) genes were expressed as fusion proteins in *Escherichia coli* and the products were used as immunogens for producing antibodies capable of reacting with respective virus particles. The antisera specifically recognized each of three types of rod-shaped virus particles as revealed by immunoelectron microscopy (ISEM) and ELISA test. DTBIA was found to be a convenient and practical detection technique for garlic viruses. The viruses GMV, GLV, and Gar V-A to -D were readily detected in infected tissue blots on nitrocellulose membranes. In contrast, no signal appeared on blots from virus-free garlic samples produced by shoot-tip

cultures with antisera to different viruses. The results were in agreement with those of RT-PCR assay. The sensitivity of DTBIA was comparable to that of ELISA tests, indicating the practical applicability of DTBIA under field conditions. DTBIA was found to be useful for surveying the incidence of garlic viruses. DTBIA was also able to detect Gar V-C in plants also infected by Gar V-A or -B or -D (Tsuneyoshi and Sumi 1996).

A direct tissue-blotting assay (DTBA) was applied to track the movement of *Potato leafroll virus* (PLRV) from newly infected foliage to the tubers. Russet Burbank potatoes at different maturities were inoculated using viruliferous aphids. Stems and tubers were periodically tested for the presence of PLRV with DTBA after inoculation. Indexed tubers were tested by the grow-out test and ELISA in the greenhouse during the winter to confirm the results of immunoassays performed in the previous summer. DTBA was found to be effective in detecting PLRV in foliage of plants grown from infected tubers, but less accurate in detecting primary infection of tubers by this virus (Whitworth et al. 2000).

The comparative efficacy of direct tissue-blot immunoassay (DTBIA), DAS-ELISA, and triple-antibody sandwich (TAS-) ELISA formats were assessed for the detection of *Citrus psorosis virus* (CPsV) in samples from various citrus varieties grown in the glasshouse and in the field. In young leaves and shoots CPsV was readily detected by all three procedures, whereas detection of CPsV by DTBIA in old leaves was less consistent. CPsV was detected with similar sensitivity using nitrocellulose or nylon membrane. The reliability of DTBIA in detecting CPsV in different citrus varieties, plants of citrus cultivars Salustiana, Valencia Late, and Washington Navel which were inoculated by grafting, was assessed. Tissue prints from all inoculated plants positively reacted with the antibody mix, regardless of the citrus cultivars and CPsV isolate combination, whereas no reaction was seen in tissue prints from uninoculated controls. Results of detection of CPsV by DTBIA, DAS-ELISA, and TAS-ELISA correlated perfectly with psorosis diagnosis based on biological indexing, specifically with the capacity of those sources to cross-protect against challenge inoculation with psorosis B. The DTBIA test provides the advantage of being simpler, cheaper, and faster than ELISA formats and can offer the same degree of specificity and sensitivity, if young tissue in early spring through mid-autumn season is used for detection of CPsV in citrus cultivars (Martin et al. 2002). In a later investigation, transversely cut tender shoots or whole flowers and rolled old and young leaf blades of citrus plants were tested by DTBIA test for the detection of CPsV. The virus could be detected readily in tissue prints from flowers, tender shoots, and young leaves collected in spring, but not from hardened leaves (Loconsole et al. 2006).

Tissue-print enzyme-linked immunosorbent assay (=direct tissue-blot immunoassay or (DTBIA)) was performed using a mixture of recombinant antibodies 3DFiscFV-AP/S and 3CA5scFV-AP/s for detecting *Citrus tristeza virus* (CTV) in sweet orange and Mexican lime (*C. aurantium*). Tissue-print ELISA along with DAS-ELISA format was employed successfully for routine detection in different laboratories of previously characterized CTV isolates, as well as field infected trees. The excellent performance of the recombinant antibodies obtained may obviate the need for conventional monoclonal antibodies using hybridoma cells (Terrada et al. 2000). An improved direct tissue-blot immunoassay (DTBIA) procedure was developed for the detection of *Citrus tristeza virus* (CTV) in citrus plants within 1 h. Prints of fresh young stems of citrus plants that were infected or uninfected with CTV were made by gently and evenly pressing the fresh-cut surface of the stems onto nitrocellulose membranes.

The CTV-specific polyclonal antibody 1212 (PCA 1212) and monoclonal antibodies 17G11 (MAb17G11) and MCA13 (MAbMCA13) were employed for detecting CTV in infected citrus samples. All samples from greenhouse plants that were infected with CTV decline-inducing isolate T-30 were positive to CTV-specific PCA1212, MAb17G11, and MAbMCA13, whereas samples from greenhouse-grown plants infected with nondecline-inducing isolate T-30 were positive to PCA1212 and MAb17G11, but not to MAbMCA13. Uninfected samples were negative to all antibodies. The sensitivity of the improved DTBIA procedure with prereaction solution of $1.0\text{ }\mu\text{g mL}^{-1}$ CTV-specific antibodies, 1/1000 dilution of labeled secondary antibodies, and a 20 min incubation was equal to that of the standard DTBIA procedure. The improved DTBIA (I-DTBIA) procedure could detect CTV within 1 h with CTV-specific PAb and MAbs and commercially available secondary antibodies. The sensitivity of I-DTBIA was similar to that of standard DTBIA, ELISA, and PCR assay. However, the I-DTBIA was more expensive than standard DTBIA and ELISA, since the concentration of labeled secondary antibodies required for I-DTBIA was greater than the other two immunoassays. The increased chemical cost might be offset by reduced cost of labor and rapidity of obtaining the results from this diagnostic test, if the results were needed early (Lin et al. 2006; Section 7.5.1).

A nondestructive tissue-blot immunoassay (TBIA) procedure was developed for the detection of *Tomato spotted wilt virus* (TSWV) in *Ranunculus asiaticus* tubers and other ornamental plants. *R. asiaticus* is grown for tuber production, potted plants, and cut flowers. The sensitivity of TBIA in detecting TSWV in tubers was comparable to ELISA tests and the results of both tests were in agreement for 96.5%. The size of the tubers in infected plants was affected by TSWV infection, small tubers showing 44% infection and larger tubers having only 19% infection. Among the tubers that tested positive for TSWV infection, 48% of those that germinated produced foliage in which TSWV was detected. The remaining 52% of the infected tubers that germinated developed into plants that were asymptomatic and in which TSWV was not detectable after germination. Only 4% of tubers that tested negative produced infected plants after germination. The ease of sample preparation and processing was the principal advantage of TBIA over ELISA test. Further, assays may be performed in disposable bottles without any special laboratory facilities. Samples may be blotted in one location and sent for testing in laboratory located elsewhere or for batch processing over time, when a large number of samples has to be indexed. TBIA therefore has the potential for the efficient indexing of planting materials and may become an integral part of TSWV management programs (Whitfield et al. 2003; Section 7.5.2).

As alternatives to ELISA formats for monitoring potato virus disease incidence, suitable for seed potato certification systems, multiplex tissue-print immunoassay (TPIA) and dot-blot immunoassay (DBIA) were evaluated for the detection of *Potato leafroll virus* (PLRV), *Potato virus S* (PVS), *Potato virus X* (PVX), and *Potato virus Y* (PVY) in the extracts of green and/or tuber tissues. In TPIA, the specific precipitation patterns in infected tissues of leaf petioles or stem cross-sections observed with each virus allowed identification of the specific virus or mixed infections in a single multiplex assay. For detection of PVY in green tissues, DBTA was found to be over 50 times more sensitive than ELISA test. TPIA and ELISA were effective for the detection of PVY and PVS in the tuber stem end or from eyes in seed tubers without prior germination. PVS was evenly distributed in potato tuber tissue, while PVY was localized in the vascular tissue beneath the epidermis with irregular distribution along the periphery of the tuber. In countries

that lack facilities for tests based on tuber germination, PVS and PVY may be monitored using TPIA for the detection of these viruses in tuber tissues as an alternative to ELISA tests (Samsatly et al. 2014).

7.2.2.3 Immunoblot Assay

Immunoblot assay involves the transfer of electrophoresed viral proteins from the gel matrix onto a membrane and making them accessible for subsequent analysis by specific immunoprobos. The virus capsid protein or nonstructural protein present in the infected plants can be detected. Polyclonal and monoclonal antibodies generated against the target virus have been employed as immunoprobos. The coat protein (CP) and movement protein (MP) of *Grapevine virus A* (GVA) were detected by immunoblot analysis in rugose wood disease-affected grapevines. The MP was detected earlier than CP, so detection of MP might be a basis of early detection of GVA in grapevine plants (Rubinson et al. 1997). The association of flexuous filamentous rods with an average length of 888 nm with peach mosaic disease and its serological relationships with *Cherry mottle leaf virus* could be established by Western blot analysis (James and Howell 1998; Gispert et al. 1998). The proteins extracted from sweet orange bark tissue infected with *Citrus tristeza virus* (CTV) were subjected to electrophoresis in 8–20% gradient PAGE in a discontinuous Tris-glycine SDS system. The separated proteins were transferred onto nitrocellulose membranes followed by probing with different MAb and PABs generated with recombinant proteins expressed in *Escherichia coli*. Both MABs and PABs readily reacted with severe isolate T36 and distinguished it from mild isolates (T30 and T55) of *Citrus tristeza virus* (Fig. 7.5) (Nikolaeva et al. 1996). The native p25 coat protein (CP) of CTV was purified to allow comparison of five isolates of CTV by immunological analysis of peptide maps using both MABs and PABs. The CTV-CP peptides were electroblotted to polyvinylidene difluoride (PVDF) membrane. Specific MABs

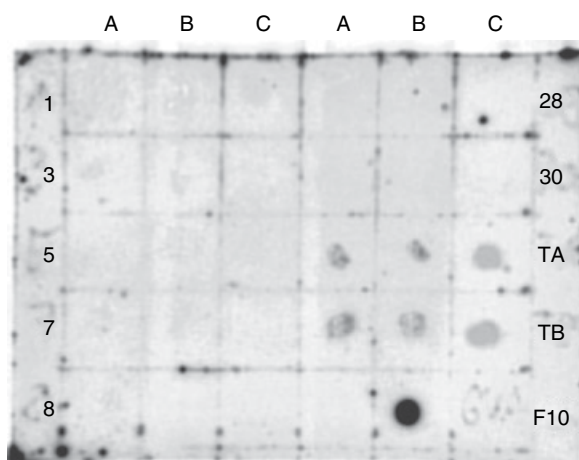


Figure 7.5 Detection of *Grapevine virus A* (GVA) in tissue prints and extracts of petiole tissues in *Vitis* spp. and hybrids. Tissue imprints: Columns A and B; Dot blots: Column C. Samples: 1: 110 Richter; 3: Kober 5BB; 5: *Vitis rupestris*; 7: *V. riparia* 8- LN 33 (interspecific hybrids of different *Vitis* spp.); 28: Interspecific hybrid Prim (Palatina); 30: *V. vinifera* cultivar Guzal Kara; TA: positive grapevine cultivar Traminer, lower leaves; TB: positive grapevine cultivar Traminer, upper leaves; F10: plasmid. Source: Komínek et al. (2008). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

were allowed to react with immobilized peptides. The migration distance of bands was measured and the color intensity was estimated using a scale of 0–4 (0 for no color and 4 for a dark blue color). The serological analysis of peptides was useful to obtain an accurate comparison of CTV isolates. It was superior to other techniques available for differentiating isolates of *Citrus tristeza virus*, since the indirect DAS-ELISA test was unable to distinguish some of the CTV isolates (Alibach-Marti et al. 2000).

Immunoblot analysis may be effectively employed to differentiate the components of mixed infections of two viruses. Infection by serologically related and unrelated *Grapevine leafroll-associated viruses* (GLRaVs) and *Grapevine bark-associated virus* (GBaV) resulted in the development of complex symptoms, differing from those caused by these viruses individually. By applying immunoblot assay, the components of the complex disease of grapevine were identified (Monis and Bestwick 1997). A previously uncharacterized *Grapevine leafroll-associated virus* (GLRaV) coat protein (37 kDa) was found associated with grapevine leafroll disease in a mixed infection. The polypeptide was separated from the 38 kDa polypeptide associated with GLRaV-1, using tricine sodium dodecylsulfate-PAGE system. Concentrated p37 preparations were used to immunize mice for the production of MAbs. The MAbs reacted positively with 37 kDa polypeptide associated with GLRaV-1 using Western blot assay. The mixed antibody Western blot assay allowed sensitive detection of GLRaV-1, -2, -3, -4, -5, -8, and *Grapevine rootstock scion incompatibility-associated virus*. The MAbs used in the mixed antibody Western blot assay reduced the monospecific background as compared to the PAb reactive to GLRaV-2 employed earlier. The new virus in the mixed infection was identified as *Grapevine leafroll-associated virus-8* with a 37 kDa capsid protein (Monis 2000).

Bacterially expressed proteins (coat protein (CP) and RNA-dependent RNA polymerase (RdRp)) were used to produce specific antisera against CP and RdRp of *Grapevine leafroll-associated virus 2* (GLRaV-2). In Western blots, antisera against recombinant RdRp diluted to 1:5000 or recombinant CP diluted to 1:10,000 reacted strongly with homologous proteins expressed in *Escherichia coli*. The antisera recognized the corresponding recombinant proteins in Western blots. CP antiserum detected infection by GLRaV-2 in all samples, whereas the antiserum to RdRp detected the virus in most samples obtained from petioles of infected grapevine plants (Xu et al. 2006). The isolate *Grapevine leafroll-associated virus-Pr* infecting the Greek grapevine cultivar Prevezamko was investigated to determine its molecular identity for detection in grapevine plants. A virus-specific antibody was raised against the recombinant CP protein of GLRaV-Pr by immunizing the New Zealand rabbit. A band of c. 37 kDa, recognized by the antiserum in Western blotting, was consistently detected in all preparations from GLRaV-Pr-infected plants. This band was not detectable in extracts from healthy grapevines or other tissues of plants singly infected with related GLRaV-4, -5, -6, or -9 viruses. Western blot analysis revealed that the pattern expressed from the CP coding ORF (ORF5) had an electrophoretic mobility indistinguishable from the GLRaV-Pr CP expressed from infected tissues. This virus was proposed as a new virus species within the genus *Ampelovirus* (Maliogka et al. 2009).

Citrus yellow vein clearing virus (CYVCV) causes yellow vein clearing disease in lemon trees in many countries. The CYVCV isolate Y was used for deep sequencing analysis of the virus-induced small RNA fractions and for mechanical and graft-inoculation of herbaceous and citrus indicator plants. A polyclonal antiserum was developed from CYVCV-Y1 purified from *Phaseolus vulgaris* and used in Western blot

assays to characterize the coat protein of CYVCV-Y1 and determine its serological relationship with related viruses. The CYVCV genome organization and size resembled that of flexiviruses. The search for sequence homologies revealed that *Indian citrus ringspot virus* (ICRSV) (*Mandarivirus*, genus; *Alphaflexiviridae*, family) was found to be the most closely related virus. However, CYVCV had an overall nucleotide sequence identity of c. 74% with ICRSV. Although these two viruses had similar genome organization, virus particles, and herbaceous host range, CYVCV caused different symptoms in citrus. Further, it was serologically distinct from ICRSV. Primer pairs designed based on the sequences of CYVCV were used to detect the virus by conventional and quantitative RT-PCR assay on yellow vein clearing symptomatic field trees, as well as graft- and mechanically inoculated host plants. The results showed that CYVCV was the causal agent of yellow vein clearing disease and represented a new species in the genus *Mandarivirus* (Loconsole et al. 2012).

Western blot procedure was employed for the detection of *Little cherry virus 1* (LChV-1), causing little cherry disease (LChD). Different immunization strategies were adopted for producing antibodies against the viral coat protein of LChV-1. Purified virus preparations, bacterially expressed protein, or a DNA vector that expressed the cloned CP gene in vivo were used. Effective induction of specific antibodies to LChV-1 CP was obtained using DNA intramuscular immunization followed by a single boost with the recombinant protein. A protein boost increased the specific immune response against the virus protein. When the DNA-CP boost IgG preparation was tested for detection of LChV-1 in infected sour cherry plants grown in vitro, it clearly recognized the 46 kDa CP protein from extracts of leaves from plants infected by ITMAR isolate. A 46 kDa protein band was also observed in extracts of plants infected by ITSPA isolate, but this protein was not detected in healthy little cherry plants (Matic et al. 2009).

Cryptic viruses are seedborne ds-RNA viruses which coexist with host plants lifelong without inducing any visible symptoms. The long-term effects of the presence of *Carnation cryptic virus* (CarCV) in Hungarian *Dianthus* germplasm collection were investigated. Different species (18) of in vitro propagated *Dianthus* and two members of *D. caryophyllus* (var. *Club* and *Silene vulgaris*) maintained in tissue cultures for 18 years were examined. The ds-RNA pattern was visualized by immunoblotting using ds-RNA-specific monoclonal antibodies. Genomic ds-RNAs of CarCV were detected in *D. caryophyllus*. In four additional species, *D. superbus*, *D. giganteus*, *D. gratianopolitanus*, and *Silene vulgaris*, several ds-RNA species in the same size range as the genomic ds-RNA of CarCV were detected. In addition, three other cryptic viruses, *Beet cryptic viruses* BCV-1, -2, and -3 were also found to persist in tissue-culture-derived plants under in vitro conditions. The results indicated that cryptic viruses were so well adapted to their hosts that they could persist for 7 years of in vitro culturing, despite a dramatic change in the environment that might affect their survival under environmental conditions such as exposure to thermotherapy. The presence of cryptic viruses might be highly valuable for confirming that plants are virus-free (Szego et al. 2005).

7.2.2.4 Dot-Immunobinding Assay

Dot-immunobinding assay (DIBA) is similar to the ELISA test and is performed on nitrocellulose membrane instead of the microtitre plates used for the ELISA method. The DIBA test employing MAbs or PAbs specific to *Cherry mottle leaf virus* (CMLV) was shown to be more efficient than TAS-ELISA format or Western blot assay in detecting CMLV. Young leaves and flowers of cherry were found to be the most suitable

tissues for detecting CMLV (James and Mukerji 1996). The DIBA test was applied to detect *Lily symptomless virus* (LSV), *Tulip breaking virus-lily* (TBV-L), and *Cucumber mosaic virus* (CMV) efficiently in the scale segments of *Lilium* sp. (Nümi et al. 1999). *Grapevine rupestris stem-pitting-associated virus* (GRSPaV) could be detected in leaf petioles and cortical scrapings from dormant canes of grapevine plants by employing antiserum generated against bacterially expressed coat protein of GRSPaV. DIBA was effective in detecting the virus during large-scale field survey, while ELISA tests were not effective in providing reliable results for the detection of GRSPaV in grapevine (Minafra et al. 2000). Dot-immunobinding assay effectively detected *Yam mosaic virus* (YMV) in stems, petioles, leaves, and tubers of yam (*Dioscorea rotundata*). Younger leaves were more suitable for detection of YMV than other yam tissues tested. DIBA was as sensitive as TAS-ELISA format (Njukeng et al. 2005).

Nitrocellulose membrane (NCM-) ELISA format was employed for the detection of viruses infecting sweet potato. *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato mild mottle virus* (SPMMV), and *Sweet potato chlorotic fleck virus* (SPCFV) were detected. SPFMV was predominantly present, accounting for 67% and 27% in symptomatic and asymptomatic plants, respectively. The presence of other viruses was detected in varying percentages of sweet potato plants, with SPCSV being the next frequently detected virus in symptomatic (64%) and asymptomatic (13%) sweet potato plants. Occurrence of these two viruses – SPFMV and SPCSV – was found in all 15 districts of Kenya surveyed. Dual infection of sweet potato plants with SPFMV and SPCSV was the most common multiple infections, detected in 52% and 12% of the symptomatic and asymptomatic plants, respectively (Nyaboga et al. 2008). Propagules such as potato tubers accumulate viruses through several generations if they are not indexed and the infected tubers are not eliminated effectively. In order to detect and identify the viruses present in potato tubers, an optimized dot-blot immunoassay was developed using viral proteins bound to polyvinylidene fluoride (PVDF) membrane. The presence of virus protein was detected with alkaline-phosphatase-labeled antibodies and a chemiluminescent reagent. For the detection of multiple viruses, antibody array was spotted onto the PVDF membrane and used for the detection of *Potato virus Y*, *Potato leafroll virus*, and *Hosta virus X* (Palma et al. 2013).

7.2.2.5 In Situ Immunoassay

Detection of *Citrus tristeza virus* (CTV) in various plant tissues such as stems, petioles, and leaf veins of young new shoots or mature shoots of citrus plants was accomplished by using both polyclonal antibody PCA1212 and monoclonal antibodies MCA13 or 17G11. In situ immunoassay (ISIA) was useful for visualization of the presence of the virus in the plant tissues directly. The plant tissues were fixed with 70% ethanol and incubated with the PAb or MAbs. Bound antibodies were labeled with enzyme-conjugated species-specific secondary antibodies and exposed to a substrate mixture containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate. Development of a purple color, as indicated by observations under a light microscope, confirmed the presence of CTV antigen in the phloem tissues of infected citrus plants. The health status of citrus plants was revealed by the absence of the purple color signal. ISIA test detected all isolates, both severe and mild using PCA1212 and the broad spectrum MAb17G11, but strain-selective MAb13 only reacted with severe isolates. The location of CTV antigens could be monitored directly and accurately by ISIA test in both fresh tissues and samples stored in plastic bags at 4°C or frozen for 4 weeks. ISIA

procedure provided sensitive detection of CTV to the level of DTBIA test. ISIA was found to be a simple, rapid, specific, and practical procedure for CTV detection and identification in field samples (Lin et al. 2000).

7.2.2.6 Immunosorbent Electron Microscopy

Immunosorbent electron microscopy (ISEM) technique is useful for spot-checking the presence of viruses in infected tissues and for monitoring the movement and distribution of viruses in different tissues of infected plants. The electron microscope grids are coated with polyvinyl formaldehyde (Formvar) strengthened with a layer of evaporated carbon. The crude antiserum raised against the target virus or fractionated α -globulins may be used to sensitize the coated grids. The grids coated with antibodies are treated with virus suspension or plant tissue extract at appropriate dilution and incubated at room temperature. The virus particles adsorbed to the grids are stained with 2% uranyl acetate and observed under the electron microscope for the presence of virus particles. Several viruses have been detected rapidly in purified virus preparations or in plant extracts. The sensitivity and effectiveness of ISEM may be improved to some level by coating the grids with protein A obtained from *Staphylococcus aureus* which exhibits specific affinity to the Fc portion of the IgG molecule, facilitating the detection of viruses present in low concentrations (Gough and Shukla 1980).

The presence of *Onion yellow dwarf virus* (OYDV) and *Carnation latent virus* (CarLV) infecting garlic was efficiently detected by ISEM in tissue-culture-derived garlic plants, demonstrating the usefulness of this technique for production of virus-free garlic stocks (Maeso et al. 1997). In another investigation, the effectiveness of ISEM for detecting *Garlic virus A* by employing the antiserum against the virus coat protein was revealed (Helguera et al. 1997). Infection of several sugarcane clones by *Sugarcane bacilliform virus* could be visualized by employing ISEM procedure (Viswanathan and Premachandran 1998). ISEM technique was found to be efficient in detecting *Cymbidium mosaic virus* (CyMV) isolates and was more sensitive than ELISA test. The detection of CyMV by ISEM was shown to be twice as sensitive than ELISA in crude sap of infected orchid leaves (Hsu et al. 1992). In a later investigation, ISEM was shown to be useful for detecting CyMV in 74.43% of orchid plants suspected to be infected, whereas biological indexing and electron microscopic observations could only detect CyMV in 51.43% and 42.86% of orchid plants tested, respectively (Vejaratpimol et al. 1999). By applying ISEM, infection by a Nigerian isolate of *Banana streak virus* (BSV) was detected in asymptomatic banana plants more efficiently than triple-antigen sandwich (TAS-) ELISA format, as reflected by the number of banana plants that were positive to ISEM observations (Thottapilly et al. 1998). In a later investigation, ISEM technique effectively detected BSV in various plant samples collected from different locations in Uganda (Harper et al. 2002). ISEM procedure was shown to be efficient in detecting *Citrus psorosis virus* (CPsV) in cross-protected plants, revealing the extent of effectiveness of the mild strains in protecting the citrus plants against CPsV (Martin et al. 2004). *Chenopodium quinoa* (the bioindicator) plants infected by CPsV were used for the detection of the virus by ISEM. Electron microscope grids sensitized by floating them on drops of crude antiserum generated against CPsV were floated on extracts of plants infected by the virus at 4°C overnight. The grids with adsorbed virus particles, followed by staining with 2% uranyl acetate, were observed under the electron microscope. The CPsV particles trapped from crude plant extracts of infected leaves by the virus-specific antibodies could be visualized (Loconsole et al. 2006).

Leek yellow stripe virus (LYSV) infecting garlic and leek was detected by immunosorbent electron microscopy decoration procedure (ISEM-D). An antiserum to LYSV-L-Arg strain was produced and the titer of the antiserum was estimated to be 1:256,000 by PTA-ELISA format. Leek plants infected with LYSV-L-Arg were negative to other viruses infecting garlic. When this isolate was tested by decoration with different LYSV and OYDV antisera, all virus particles were uniformly decorated with different antiserum dilutions. When LYSV-L-Arg and OYDV were compared by decoration with different LYSV and OYDV antisera, cross-reactions were detected (Lunello et al. 2002). Onion crops were previously only known to be infected by OYDV in Greece, but another isometric virus with similarities to nepoviruses was isolated from infected onion plants. After partial characterization, the virus was identified as *Artichoke yellow ringspot virus* (AYRSV). ISEM and decoration techniques were applied for the detection and assessment of serological relationship between AYRSV and other viruses belonging to the genus *Nepovirus*. AYRSV was mechanically transmitted to *Nicotiana benthamiana*, the indicator plant for the virus. The crude sap extracts of *N. benthamiana* plants infected with the onion isolate of AYRSV did not react with the antisera produced against any of the 18 nepoviruses tested by ISEM and decoration procedures. Sequence analysis showed that the onion isolate of AYRSV was the causative agent of the disease affecting onion crops (Maliogka et al. 2006).

The comparative efficacy of ISEM and triple-antigen sandwich (TAS-) ELISA techniques was assessed for the detection of a Nigerian isolate of *Banana streak virus* (BSV). ISEM was found to be more efficient and sensitive by detecting BSV in greater number of infected banana plants than the TAS-ELISA format (Thottapilly et al. 1998). The usefulness of ISEM procedure was revealed by the tests conducted on various plant samples collected from different locations in Uganda, where BSV was responsible for causing serious losses to banana production (Harper et al. 2002). ISEM technique provides certain advantages such as direct visualization of viruses in infected plants tissue extracts, and a sensitivity comparable to and even better than ELISA test in the case of some virus infections. The possibility of using unfractionated crude antiserum or antigen samples (5–10 µL), noninterference by antibodies to host proteins and establishing relationship between viruses, in addition to its use for spot-checking randomly selected samples to confirm the presence of viruses, have been indicated (Narayanasamy 2011).

7.2.3 Nucleic Acid-Based Techniques

Plant viruses have either RNA (the majority) or DNA as their genomes. Infectivity and type of symptoms induced are determined by specific sequences (genes) of the viral nucleic acids. As the nucleic acids remain stable in their respective host plants, nucleic acid-based techniques provide more reliable and sensitive detection of viruses in seeds, plants, and propagules, even when they remain asymptomatic, because of uneven distribution or low virus titers. Generally nucleic acid-based techniques are more sensitive and specific, giving the results rapidly, than compared to immunoassays. Various kinds of assays such as nucleic acid hybridization, polymerase chain reaction (PCR), reverse-transcription (RT-) PCR, real-time PCR, and DNA array technology have been reported to be effective to different degrees for the detection, identification, quantification, differentiation, and establishment of relationships between viruses/strains of the same virus.

7.2.3.1 Nucleic Acid Hybridization

The target nucleic acid is usually fixed to a solid support in hybridization procedures employed for the detection of viruses. The probe (complementary nucleotide sequence) carrying a label that provides the recognizable signal is allowed to anneal with immobilized target molecules. Hybridization is recognized by detecting signal from the probe. As the majority of plant viruses have RNA as their genome, RNA probes are commonly employed. RNA:RNA hybrids have been shown to be more stable than RNA:DNA or DNA:DNA hybrids. DNA probes labeled with radioactive (^{32}P) probes are hazardous to human beings. The cDNA copies of viral RNA can be produced by employing a retrovirus reverse transcriptase and labeling with a radioactive or nonradioactive reporter. The probes are useful for the detection and identification of the virus and its strains.

Dot-blot hybridization method provides qualitative detection, indicating whether or not a plant is infected. Dot-blotting does not differentiate the number and size of hybridized molecules, since hybridization signal is the sum of all sequences recognized by the probe. However, this procedure can provide results rapidly by specific nucleic acid sequences in samples containing crude plant extract/purified virus preparations. Dot-blot hybridization has therefore been employed for routine and large-scale testing required for certification and quarantine programs.

The presence of viruses in propagules has been detected less frequently using hybridization techniques. The dot-blot hybridization technique provided sensitive detection of *Banana bunchy top virus* (BBTV) when BBTV-specific clones and radioactive or nonradioactive probes were used. This procedure was as sensitive as the ELISA test in detecting the virus in infected plants (Xie and Hu 1995). By using a nonradioactive digoxigenin (DIG-) labeled cRNA probe of approximately 2100bp, *Potato leafroll virus* (PLRV) was readily detected in dormant potato tuber tissues. No cross-reaction with *Potato virus X* (PVX) or *Potato virus Y* (PVY) was observed in the reaction with DIG-labeled cRNA probe, indicating the specificity of hybridization procedure. The assay was 2000-fold more sensitive than the ELISA test in detecting PLRV in dormant potato tuber tissues (Loebenstein et al. 1997). Nonradioactive DIG-labeled sense and antisense cRNA probes were employed for the detection of *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* (ORSV) in crude leaf extracts or total RNA from infected leaves of orchids. As the probes remained stable for more than a year, it was possible to perform the dot-blot hybridization tests for sensitive and reliable detection of CyMV (Hu and Wong 1998). Nonradioactive tissue-blot hybridization procedure was employed for the detection of *Tobacco rattle virus* (TRV), causing corky ringspot disease of potato tubers. Necrotic potato tuber tissue was extracted with cork borer and blotted on nylon membrane for 15 s. The cRNA probes specific for the 16kDa of the Florida isolate of TRV were prepared from the gel-purified DNA fragment amplified from the cloned PCR product. The 463bp fragment was labeled with nonradioactive biotin and employed for hybridization with the immobilized targets in the nylon membrane. A positive signal was detected when the membranes with four samples of each of the potato cultivars were tested. Red Lasoda and Siebago and three samples of cultivar Atlantic with typical symptoms of TRV infection were tested. No signal was detectable in membranes containing healthy tissue samples (Pérez et al. 2000).

Several viruses infect potato crops and are transmitted through seed tubers which are likely to become sources of primary inoculum for these viruses. Natural biological vectors are involved in the secondary spread of the diseases from the plants growing from the infected tubers and the virus diseases may be introduced to new geographical

locations where they may be less important. Potato plantlets initiated into tissue culture have therefore been tested for several viruses prior to propagation for seed potato production. Commercially available ELISA kits were found to be unsuitable for indexing the plantlets for the detection of different viruses belonging to the genera *Potyvirus*, *Carlavirus*, *Potexvirus*, *Luteovirus*, *Promovirus*, *Tobravirus*, *Tospovirus*, *Alfamovirus*, and *Tymovirus*. An optimized nonradioactive nucleic acid-subtractive hybridization (NASH) assay was applied for its simultaneous detection of carlavirus *Potato virus S* (PVS), potexvirus *Potato virus X* (PVX), and *Potato virus Y* (PVY) in potato tissue culture plantlets. This procedure was adaptable for detection of these viruses since it required a single extraction from a small portion of a tissue culture plantlet. Further, this assay could save time, labor, and the cost of testing for the simultaneous detection of multiple virus infections.

The sensitivity of NASH protocol was determined by hybridization of PVS-, PVX-, and PVY-specific probes with membranes spotted with a dilution series of tissue cultured plantlet extracts. The viruses could be detected individually with specific probes at a dilution of 1:40 of plant extracts. It was found that the probe which was amplified from plantlets infected with PVY^O could also detect other strains of PVY in plantlets infected with PVY^N, PVY^{NTN}, and PVY^{N:O}. Furthermore, when a cocktail of different probes was used, the multiplex hybridization test was effective in detecting PVS, PVX, and PVY in a single hybridization assay (Janczur et al. 2006). Plant virus transcripts may be localized by a simple, rapid, and inexpensive hybridization procedure. This procedure is based on fluorescent in situ hybridization (FISH) using short DNA oligonucleotides complementary to an RNA segment representing a virus transcript in infected plant. The DNA probe contained a fluorescent molecule at its 5' and 3' ends. This technique was applied for the detection of *Potato leafroll virus* (PLRV) in potato plants. The viral transcripts were localized in the phloem sieve elements of infected potato plants, producing specific fluorescence. No signal was observed in the healthy negative control plants. This protocol was found to be simple, rapid, and inexpensive, and had the potential to provide reliable and convincing background-free results (Ghanim et al. 2009).

Citrus tristeza virus (CTV) infecting *Citrus* spp. exists in the form of several strains differing in their biological properties, particularly in symptoms produced in various *Citrus* species or cultivars. These strains have to be detected rapidly and identified precisely to plan an efficient and effective system for containing the incidence and spread of the disease. A hybridization procedure for DNA or cRNA probes was developed to provide an easy and powerful method for detecting differences in any genomic region of CTV. A nonisotopic hybridization technique was developed to detect and differentiate isolates of CTV, using digoxigenin (DIG-) labeled cDNA probes and different kinds of target RNA. Hybridization of DIG-labeled probes with purified ds-RNA or concentrated total RNA extracts spotted on nylon membranes allowed detection of CTV nucleic acid equivalent to as little as 0.1–1.0 ng of infected tissues when the reaction was developed with a chemiluminescent substrate (LPPD or CSPD, Boehringer Mannheim) following manufacturer's recommendations, or chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT). The sensitivity was similar or slightly better than that obtained by hybridization with a ³²P-labeled probe. CTV was also detected by hybridization of DIG-labeled probes with tissue prints from freshly cut young citrus shoots. A signal localized in the phloem area of citrus plant tissue was observed in prints from CTV-infected plants, but not in healthy plant tissues. Chemiluminescent detection provided a stronger signal in nylon than in polyvinylidene

difluoride (PVDF) membranes, whereas the performance in PVDF membrane was better when chromogenic substrate was used. Hybridization of tissue prints with DIG-labeled probes under stringent condition (60°C and 50% formamide) could differentiate CTV isolates in citrus grown in the greenhouse or in the field. The DIG-labeled probes were found to be specific and sensitive for the detection of CTV and effective for efficient differentiation of CTV isolates. The sensitivity of the hybridization assay was at the highest level when nylon membranes and chemiluminescent substrates were used. The protocol developed in this investigation allowed detection of ds-RNA obtained from 0.1 g of tissues, yielding a 10-fold greater sensitivity than chromogenic detection. Hybridization tests with DIG-labeled probes showed similar sensitivity as the ^{32}P -labeled probes, providing safe, sensitive, and faster detection of CTV isolates. Further, the probes could be stored without loss of activity and used in any laboratory without safety concerns.

By employing hybridization of tissue prints with DIG-labeled probes, the need to obtain purified ds-RNA or total RNA could be avoided (Narv  ez et al. 2000; Section 7.5.3). The tissue-print hybridization method was employed for the detection of *Citrus psorosis virus* (CPSV) in citrus trees during a field survey. The detection of CPSV always coincided with diagnosis of psorosis by cross-protection, but not with the presence of symptoms considered to be characteristic of CPSV infection in field trees or in graft-inoculated indicator plants. The tissue-print hybridization procedure was considered to be the most suitable technique among ELISA, RT-PCR, and ISEM methods tested, because of advantages such as the absence of sample processing and handling risks for quarantine personnel. Further, samples stored for 2–3 years gave similar results as those observed from fresh or desiccated citrus tissues (Martin et al. 2004).

Prunus necrotic ringspot virus (PNRSV), *Prune dwarf virus* (PDV), and *Apple mosaic virus* (ApMV) belonging to the genus *Ilaravirus* (family *Bromoviridae*) have a worldwide distribution and cause significant losses. PNRSV and PDV are seed- and pollen-borne and may be spread by pollinating insects (Hamilton et al. 1984; Kelley and Cameron 1986; Aparicio et al. 1999; Amari et al. 2007). However, ApMV is known to be transmitted only through hazelnut seed (Cameron and Thompson 1985). PNRSV, PDV, and ApMV are spread through infected propagules and nursery productions. The use of rapid, sensitive, and reliable methods for the detection and identification of these viruses in planting materials is therefore required to prevent their spread.

Tissue-printing (TP) molecular hybridization and the ELISA test were compared for their efficiency in detecting PNRSV, PDV, and ApMV in different hosts known to be infected with one or mixture of viruses. Trees of almond (7), apricot (2), cherry (6), peach (3), and plum (1) were tested once a month in 2005 to determine the influence of cropping seasons on detection limits of TP and ELISA procedures. Both detection techniques could detect all the three viruses, but optimal detection occurred in different months. All PNRSV-infected trees gave positive reaction in January and March–April using TP, but the ELISA test was more successful in November and January. PDV was detected in all trees by both tests in March–April. Only in November were all trees positive for the presence of ApMV by both tests, and it was more difficult to detect ApMV compared with the other two viruses, possibly due to low virus titer and uneven distribution. The highest rates of detection for PNRSV, PDV, and ApMV were observed in the winter in the buds of dormant cuttings, regardless of methods applied. Although PNRSV, PDV, and ApMV are closely related phylogenetically and have similar coat protein sequence identity, they did not respond in the same manner to TP and ELISA tests.

The differential behavior might be due to the fact that TP analyzed a small portion of the host tissue (only printed area), whereas ELISA interacted with a larger amount of plant tissue (0.5 g). Further, TP targeted viral RNA and ELISA reacted with coat protein, whose stability and concentrations were likely to be influenced by the season. Overall, for ApMV the tissue-printing (TP) procedure was more successful; ELISA was more suitable for the detection of PNRSV and PDV. However, TP assay had the advantage of simplicity with no manipulation of samples, can be adapted to large-scale surveys, and has the potential to simultaneously detect all three viruses belonging to the genus *Ilarvirus* (Matic et al. 2008).

Grapevine virus A (GVA) is the type member of the genus *Vitivirus* and has positive sense ss-RNA as the genome. A specific dot-blot hybridization assay was developed using an in vitro transcribed nonradioactive RNA probe for the routine detection of GVA. Of the 83 samples tested, 7 grapevines were found to be positive for GVA using the riboprobe. Analysis of comparative efficacy of the riboprobe, DAS-ELISA, and RT-PCR assays showed that RT-PCR failed to detect GVA in two samples (EL1 and VostA). GVA could be detected in EL1 only by using riboprobe. Sample VostA was positive for GVA using riboprobe and ELISA, but not with RT-PCR assay. It is possible that the greater reliability and sensitivity of the riboprobe could be due to the use of probe for longer than the relatively short oligonucleotide PCR primers, and the ability of riboprobes to overcome minor variations in the primer region of viral sequence. When the plant extracts were tested by dot-blot hybridization, positive results were obtained only with infected plant extracts, independent of leaf positions (both upper and lower leaves). Dot-blot hybridization assay was found to be preferable because of the possibility of using plant extracts, instead of the complicated procedure of isolating RNA from plant, reducing overall cost of the procedure considerably. Tissue prints from infected tissues also provided positive results for the presence of *Grapevine virus A*. The RNA probe was employed for detecting GVA in plant extracts and tissue prints. The presence of GVA both in upper and lower leaves was detected, independent of plant morphology. It was possible to employ the tissue-printing method for large-scale detection of GVA under field conditions (Fig. 7. 5). The possibility of using plant extracts for GVA detection, without the need for RNA isolation, could reduce the overall cost of testing (Komínek et al. 2008).

7.2.3.2 Polymerase Chain Reaction Techniques

Viral pathogen-related DNA sequences present in naturally infected host plants may be increased to levels that can be detected by employing polymerase chain reaction (PCR) amplification. Both ds-DNA and ss-DNA molecules can be directly amplified by PCR assay. Plant viruses with DNA as the genome may be detected directly by using specific primers by PCR assays. On the other hand, the majority of plant viruses have RNAs as their genomes. They have to be subjected to the reverse-transcription step to generate complementary DNA (cDNA) prior to PCR amplification, and the assay is referred to as reverse-transcription (RT-) PCR procedure.

Banana bunchy top virus (BBTV), belonging to the genus *Nanavirus*, infects banana production seriously in several countries. The infected suckers form the primary sources of inoculum. The presence of BBTV in different plant parts was detected by PCR assay (Hafner et al. 1995). In another investigation, a PCR procedure was applied for the detection of BBTV in banana plants and the assay was 100 times more sensitive than dot-blot hybridization and ELISA tests (Xie and Hu 1995). Three pairs of primers

constructed based on the nucleotide sequences of DNA component 1 of BBTV containing viral putative replicase gene were employed for the detection of the virus. These primer pairs amplified DNA fragments of 436, 446, and 447 bp from extracts of banana leaves infected by BBTV in tissue-culture-derived banana and viruliferous aphids (Shamloul et al. 1999). In another investigation, the primer pair designed from the putative replicase region of viral component 1 was employed. BBTV was detected in field-grown banana plants and also in viruliferous aphids. The primer pair amplified a 1 kb fragment of the viral DNA (Manickam 2000). The presence of BBTV was detected in three Egyptian banana cultivars by PCR assay. Two specific primers were designed, based on the nucleotide sequence of BBTV-Eg DNA2-2 (component 2). A fragment with a size of about 1056 bp was amplified from the DNA extracts obtained from BBTV-infected banana plants, but not from the extracts from healthy banana plants used as the negative control. All infected plants (100%) of three cultivars reacted positively by amplifying virus-specific amplicons. The BBTV could be detected in the extracts of asymptomatic plants of three cultivars, indicating varying degrees of BBTV infection (29–38%). Among the three banana cultivars, cultivar Baradica showed a higher level of BBTV infection. BBTV was also detected in tissue-culture-derived symptomless plantlets. The protocol developed in this investigation had the potential for testing the BBTV infection in banana germplasms (Soweha 2005). Detection of BBTV could be achieved by preparing total DNA extracts from infected leaves of banana plants and using the primers designed based on the coat protein (CP) gene sequences of BBTV. A major DNA fragment of the expected size of 50 bp was amplified from the extracts of leaves from infected banana plants. In contrast, no amplification occurred in extracts from healthy banana plants, indicating the specificity of the primer employed in the PCR assay (El-DougDoug et al. 2006).

Banana tissues contain high contents of polyphenols, polysaccharides, and tannins that inhibit PCR amplification of target DNA fragments from microbial pathogens. In order to overcome this problem, a simple extraction protocol (SEP) was developed for extracting *Banana bunchy top virus* (BBTV) from banana leaves and aphids for use in PCR assay. Sodium sulfite and polyvinylpyrrolidone were included in the extraction buffer to minimize the inhibitory effects of polyphenols and polysaccharides that may be coextracted with viral nucleic acid. The level of detection efficiency of the PCR assay using the templates obtained with SEP procedure was comparable to that of CTAB method. BBTV DNA could be detected at $0.1 \text{ pg } \mu\text{L}^{-1}$ concentration. The virus could be detected in freeze-dried and CaCl_2 -dried leaf samples from BBTV-infected plants. The SEP protocol showed potential for wide application due to its simplicity, rapidity, and cost-effectiveness (Selvarajan et al. 2015).

Pineapple bacilliform virus (PBV) was detected by employing specific primers designed from PBV sequences which yielded a 403 bp product after amplification. The positive detection of the PBV-specific amplicon only in the extracts of infected pineapple plants, but not in the extracts of plants infected by *Sugarcane bacilliform virus* or *Banana streak virus*, revealed the specificity of the primers and the identity of PBV (Thompson et al. 1996). The genomic identity of *Citrus mosaic virus* was established as the DNA genome for the virus infecting citrus as indicated by PCR amplification by degenerate oligonucleotide primers based on the conserved *Badnavirus* genomic sequences (Ahlawat et al. 1996). A good-quality DNA has to be obtained from virus-infected plants to achieve reliable detection of the virus causing various kinds of diseases. The efficiency of extraction of DNA and quality and stability of the viral

genome could be improved by the addition of sodium sulfite as in the case of *Citrus yellow mosaic virus* (CYMV), a nonenveloped bacilliform virus infecting sweet oranges in India. Further, the viral DNA extracted with sodium sulfite improved the stability of virus particles at various temperatures, compared with those extracted using commercial kit DNeasy Kit (Qiagen) (Baranwal et al. 2003).

A polymerase chain reaction (PCR) assay was developed for the detection of geminiviruses in a variety of sweet potato cultivars. A reliable and simple method of extracting high-quality DNA from infected plants was developed for large-scale sample preparation. Degenerate and virus-specific primers were employed to amplify the PCR products of the expected sizes which were not amplified from healthy sweet potato plants. The protocol developed in this investigation successfully detected 20 different geminivirus species or isolates in both in vitro plantlets and greenhouse-grown sweet potato plants. The extraction method using CTAB buffer and a semiautomatic homogenizer yielded suitable-quality DNA for PCR assay from both in vitro plantlets and three *Ipomoea* indicator hosts and greenhouse-grown plants. PCR assays using primers PW285-1/PW285-2, SPG1/SPG2, and SPG3/SPG4 yielded amplicons of size 515, 912, and 1148 bp for two uncharacterized sweet potato geminivirus isolates and *Ipomoea leafcurl virus* (ILCV). Very weak or no amplification occurred in DNA extracts of other non-sweet-potato geminiviruses by the primers PW285-1/PW285-2 and SPG3/SPG4. However, DNA fragments of different sizes were amplified from seven non-sweet-potato geminiviruses by PCR using degenerate primers SPG1/SPG2. All PCR amplicons except that of *Beet curly top virus* (BCTV) were smaller than those for sweet potato geminiviruses. When in vitro plantlets were tested, the virus was detected in a greater number of plants (40 of the 62 plantlets) using primers SPG1/SPG2 as compared with primers PW285-1/PW285-2 (29 of the 62 plantlets; Fig. 7.6). Consequently, the SPG1/SPG2 primers together with the host primers (MDH-H969 and MDH-C1163) were selected as the best primers for PCR-based detection of geminiviruses in sweet potato.

The use of host primers in the PCR allowed for the simultaneous detection of host DNA and target viruses under the same PCR conditions. The results of grafting assay for 16 plants correlated well with those of PCR using primers SPG1/SPG2. Several in

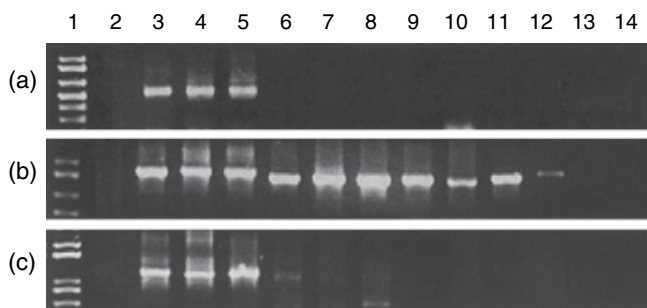


Figure 7.6 Detection of sweet potato viruses by PCR assays in sweet potato using three different primer pairs: (a) PW285-1/PW285-24; (b) SPG1/SPG2; or (c) SPG3/SPG4. Lane 3: an uncharacterized Jamaican isolate infecting sweet potato; lane 4: an uncharacterized Puerto Rican isolate infecting sweet potato; lane 5: *Ipomoea leafcurl virus*; lane 6: *Tomato yellow leafcurl virus*; lane 7: *Tomato mottle virus*; lane 8: *Bean golden mosaic virus*; lane 9: *Cabbage leafcurl virus*; lane 10: *Squash leafcurl virus*; lane 11: *Cotton leaf crumple virus*; lane 12: *Beet curly top virus*; lane 13: a potyvirus infecting sweet potato (negative control); lane 14: water control. Source: Li et al. (2004). Reproduced with permission of the American Phytopathological Society, MN, USA.

Table 7.7 Comparative efficiency of the polymerase chain reaction (PCR) and grafting for detection of geminiviruses in sweet potato plants (Li et al. 2004).

Plantlet number	First PCR ^a	Second PCR ^b	Symptoms on <i>I. setosa</i> ^c
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	–	+	+
6	–	+	+
7	–	+	+
8	–	–	–
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	+	+	+
14	–	–	–
15	+	+	+
16	–	–	–

^a Primer pair PW285-1/PW285-2 employed in the PCR assay; ^b primer pair SPG2/SPG2 used in the PCR assay; ^c tested plants grown in a greenhouse and graf-inoculated onto indicator host *Ipomoea setosa*.

vitro clones that were negative by PCR and grafting assays were propagated and released as virus-free material to growers. PCR assays using the three primer pairs detected nine uncharacterized isolates of the geminiviruses in sweet potato from Asia and America. However, the most effective detection was achieved with degenerate primers SPG1/SPG2, which detected a Taiwan isolate of *Sweet potato leafcurl virus* (SPLCV-Taiwan) in a sample diluted to 10⁻⁹. Viral identities of these amplicons from SPLCV-Taiwan were confirmed by sequencing. The degenerate primers had a broader spectrum of detection than virus-specific primers. The PCR assay was found to be effective and reliable for virus detection as grafting (Table 7.7; Li et al. 2004).

PCR primers with the desired degree of selectivity may be synthesized, in comparison with serological reagents, at a much lower cost than that required for the production of MAbs or PAbs. In addition, the availability of nucleotide sequence data increasingly with time, has made the use of PCR-based methods more attractive for large-scale applications. In the case of plant viruses with RNA genomes, the RT-PCR approach has proven to be more effective than the ELISA format, particularly for the detection of viruses in asymptomatic plants/planting materials which may have low virus titer and uneven distribution in the infected tissues. The principal limiting factor in the application of PCR-based assays in routine virus disease diagnosis is the preparation of good-quality viral nucleic acid free of PCR inhibitors which may be coextracted along with plant nucleic acids, especially in the case of woody plants such as grapevine and fruit tree varieties belonging to the genera *Malus*, *Prunus*, and *Pyrus*. An efficient procedure

was developed for the extraction of high-quality RNA from woody plants without use of phenolics, organic solvents, or alcohol precipitation. The protocol uses the commercially available spin-column matrices, and restricts the inhibitory effects of plant polysaccharides and polyphenolic compounds commonly observed on subsequent PCR amplification when conventional extraction methods are applied to woody plant species. By using this extraction procedure, several viruses – *Apple stem-grooving virus* (ASGV), *Apple stem-pitting virus* (ASPV), *Prunus necrotic ringspot virus* (PNRSV), *Plum pox virus* (PPV), *Grapevine fan leaf virus* (GFLV), *Arabidopsis mosaic virus* (ArMV), and *Grapevine leafroll-associated virus 3* (GLRaV-3) – could be detected. The procedure was equally effective for the extraction of viral RNA from either budwood, leaves, or flower blossoms, as determined by the equivalent RT-PCR detection of ASGV and PNRSV from these plant tissues. The detection of viral RNA in samples of total plant RNA extracted by the procedure developed in this investigation was as sensitive as the immunocapture (IC-) RT-PCR method that requires virus-specific antiserum and longer time for *Plum pox virus* (PPV) detection (MacKenzie et al. 1997).

The reverse-transcription (RT-) PCR assay has been demonstrated to be effective and reliable for the detection and differentiation of viruses infecting potatoes and other vegetatively propagated crops. RT-PCR assay was more reliable and sensitive in detecting *Potato leafroll virus* (PLRV) in dormant tubers from field-grown plants and in vitro propagated microtubers when compared to ELISA test. Further, ELISA was able to detect PLRV only in microtubers, whereas RT-PCR could detect the virus in both types of tubers (Spiegel and Martin 1993). For the detection of PLRV, a common electric drill was employed for sampling a large number of dormant tubers by simultaneously removing and macerating tuber eye samples. This method of sampling was found to be rapid, simple, and inexpensive. The dormant tubers from plants with primary infection were detected by both RT-PCR and ELISA tests for the presence of PLRV (Souza-Dias et al. 1999). In another investigation, the relative incidence of PLRV in different locations was assessed during field inspection by employing RT-PCR assay. Higher levels of potato leafroll disease were detected by RT-PCR assay than by visual inspection. Infection levels of sprouts from potatoes planted in the greenhouse were determined by ELISA test and RT-PCR assay. The infection of sprouts up to 85% was revealed by both ELISA and RT-PCR procedures (Russo et al. 1999).

An efficient method of extraction of total RNA from virus-infected tissues of woody plants was developed involving desiccation of plant tissues or exposure to higher temperatures to overcome the inhibitory effects of polyphenols present in plants. Infected leaves from different host plant species were dried at 65°C for 2 days and conserved at 4°C under air-proof conditions. The treated leaves yielded good-quality RNAs of *Apricot latent virus* (ApLV), *Plum bark necrosis stem-pitting-associated virus* (PBNSPaV), *Prunus necrotic ringspot virus* (PNRSV), and *Potato virus Y* (PVY). The procedure was also effective in extracting the RNA of *Apple scar skin viroid* (ASSVd). The viruses ApLV, PNRSV, and PVY and the viroid ASSVd could be easily detected by RT-PCR assay, while nested RT-PCR format was efficient in detecting PBNSPaV from infected leaves processed by drying the plant tissues at 65°C. No significant difference could be observed between ethidium-bromide-stained profiles of air-dried and fresh leaves from infected plants. This simple and inexpensive method of treating the virus-infected plant tissues might be useful for successful detection of plant viruses in plants containing high concentrations of inhibitor of PCR assays (Sipahioğlu and Ocak 2006).

Reverse-transcription (RT-) PCR assay has been demonstrated to be useful for the detection of a wide range of viruses infecting various herbaceous plant species and in different types of host tissues, including asexually propagated planting materials. In addition, it is also possible to detect viruses in crude plant extracts by employing RT-PCR assay, avoiding the need for purification of viruses. *Prunus necrotic ringspot virus* (PNRSV) was detected in dormant peach trees by employing a modified RT-PCR procedure. This procedure was effective for indexing budwood materials in post-entry quarantine (PEQ) programs and also for ensuring the virus-free nature of the planting materials in certification programs (Spiegel et al. 1996). In a later investigation, another modified RT-PCR assay was employed for the detection of PNRSV in dormant peach and almond trees. Two different pairs of primers yielding short (70 bp) and long (200 bp) products were employed. Amplification of short PCR product was effective for the detection of PNRSV in plant tissues with low virus titer as in dormant trees, while the long PCR product was amplified in tissues with high virus titer (Rosner et al. 1997).

RT-PCR and ELISA tests were employed for determining their comparative efficacy in detecting *Prunus necrotic ringspot virus* (PNRSV). Total RNA extracts were made from serially diluted fresh leaf tissue, root, bark, and 1 year old green bark tissue, using the silica-based method. Purified total RNA extracts were used as template for cDNA synthesis by reverse transcription. The amplicon (616 bp) produced following PCR amplification was resolved by gel electrophoresis and visualized by ethidium bromide staining. RT-PCR assay was more sensitive in detecting local PNRSV isolate present in Turkey. Among the different plant tissues tested, leaves of infected *Prunus mahaleb* were found to be the most suitable tissues for detecting PNRSV. The detection limit for PNRSV using leaf tissue extract was 1:2560, suggesting that PNRSV was well distributed in actively growing plant tissues and more concentrated in young leaves (Sipahioğlu 2005).

The RT-PCR assay has been applied for the detection of plant viruses more frequently than any other diagnostic methods as the number of viruses for which nucleotide sequence data available increases rapidly. *Citrus psorosis virus* (CPsV), causing one of the oldest-known graft-transmissible diseases of citrus, was investigated to assess the extent of infection of citrus by six psorosis-like isolates in Spain. Detection of CPsV was achieved by using RNA extracts from citrus leaves and the primers CPV1 and CPV2, based on the RNA3 sequence of the CPsV-4 isolate from Florida. The presence of all six isolates in leaf extracts was detected by the RT-PCR assay and the results were similar to that of biological indexing and electron microscopy (Martin et al. 2002). RT-PCR assay was found to be more sensitive than ELISA test in detecting *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV). These two viruses could be readily detected at any time throughout the growing season (Mekuria et al. 2003). Crop losses due to infection by *Plum pox virus* (PPV) increased enormously, necessitating the development of a rapid, reliable, and sensitive diagnostic method for its detection to facilitate eradication of infected plants. ELISA test was not sensitive enough to provide reliable and consistent results. A real-time fluorescent RT-PCR format was found to be more sensitive than ELISA or conventional PCR method. The results of the real-time fluorescent RT-PCR assay were highly reproducible and could be obtained rapidly. PPV could be detected in the propagative plant materials by real-time assay which could be employed for detecting PPV in multiple hosts and various tissues of test plants (Schneider et al. 2004).

The incidence and distribution of *Grapevine leafroll-associated virus 7* (GLRaV-7) in China were investigated. Samples (213) from 92 grapevine cultivars from 13 Chinese

provinces and regions were tested using RT-PCR format and sequencing. GLRaV-7 was detected in 40.4% of the samples, indicating the wide distribution of GLRaV-7 in major grapevine-growing areas in China. More than 50 grapevine cultivars, including popular cultivars Manicure Finger (100%), Cabernet Sauvignon (83.3%), Merlot (60%), Fujimineri (60%), and Red Globe (50%), were found to be infected by GLRaV-7. The genetic variability of GLRaV-7 isolates was determined based on partial nucleotide sequences (nucleotides 945-1329 and 97-598 of ORF) of heat shock protein 70 homologue (HSP70h) and 61 kDa protein (*p61*) (nucleotides 1073-1572 of the ORF) genes. The overall mean values of nucleotide diversity were low (ranging from 0.009 to 0.066) and phylogenetic analysis based on *p61* showed that the GLRaV-7 isolates segregated into three phylogenetic clusters. A new primer pair developed in this investigation based on the *p61* gene, and its associated downstream intergenic region detected more GLRaV-7 Chinese isolates compared to the primers reported in earlier studies, indicating the greater sensitivity of the RT-PCR assay in detecting GLRaV-7 isolates in grapevine in China (Lyu et al. 2014).

Apple stem-pitting virus (ASPV) is widespread in commercial apple cultivars which remain asymptomatic. Infection by ASPV could be recognized only when they were grafted on sensitive rootstocks. *Cherry rasp leaf virus* (CRLV) and *Cherry necrotic rusty mottle virus* (CNRMV) have a considerable negative impact on cherry production. RT-PCR protocols were developed to detect these viruses rapidly and reliably. A total of 14 primer sets were designed and tested for the specific detection of each virus. Two of the 14 virus-specific primer sets for each virus were found to specifically detect each virus. Total RNAs extracted from healthy and from ASPV-, CRLV-, and CNRMV-infected plant tissues were used to synthesize cDNA using oligo-nt primer, and then amplified by virus-specific primers for each virus. Each primer specifically amplified DNA fragments of: 578 bp and 306 bp products for ASPV (prASCP-C and prASCP-N primers, respectively); 370 bp and 257 bp products of CNRMV (prCN4 and prCN6-NEG1 primers, respectively); and 697 bp and 429 bp products of CRLV (prCR4 and prCR5-JQ3D3 primers) by RT-PCR assays. DNA sequencing of amplified DNA fragments confirmed the nature of each amplified DNA. Application of RT-PCR assay for the detection of RNA viruses provided many advantages including high sensitivity, specificity and high sample throughput. In addition, ASPV, CRLV, and CNRMV could be detected in infected leaves, stem, and bud tissues of infected plants (Park and Kim 2004).

Potato virus Y (PVY), the type member of the genus *Potyvirus*, has a worldwide distribution, infecting several crops in addition to potato. Infected tubers form the sources of inoculum and, when introduced into the field, form several foci of infection from which secondary spread occurs through the activities of different species of aphids (*Myzus persicae* is the most common and efficient vector in different geographical locations). Spread of the disease may occur from the plants growing from infected seed tubers or inoculum may be transported from outside the field by the viruliferous aphids. The PCR-microplate hybridization technique was employed for the detection of *Potato virus Y* (PVY) in potato plants. A cDNA fragment from coat protein (CP) region of PVY RNA genome was amplified by RT-PCR protocol, followed by detection of the amplicons employing digoxigenin (DIG-) labeled cDNA probe. The detection limit of the assay was 10 fg of PVY genomic RNA and the sensitivity of detection was enhanced by 10,000 times that of ELISA test (Hataya et al. 1994).

The detection of immobilized amplified product in a one-phase system (DIAPOPS) was effective for the detection of four strains of *Potato virus Y* (PVY). Specific detection

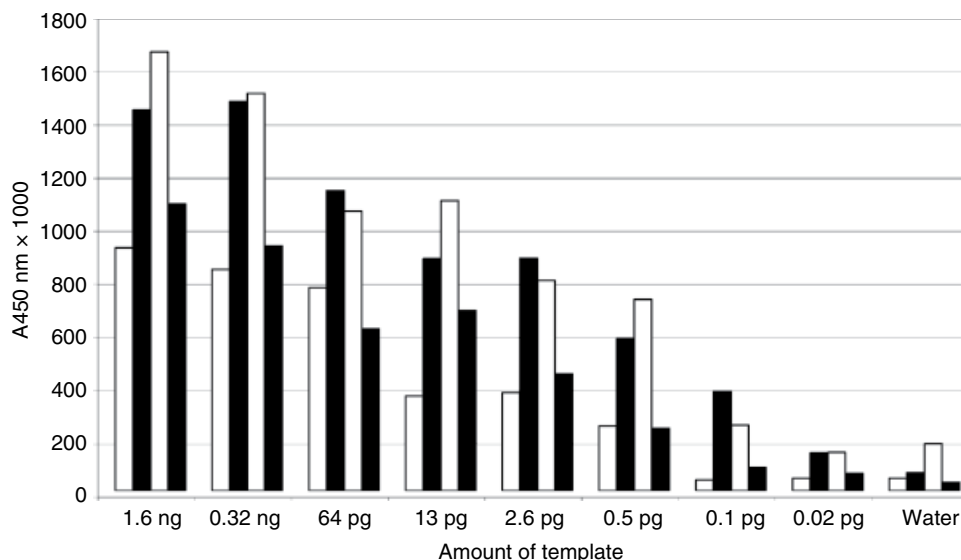


Figure 7.7 Detection of purified *Potato virus Y* added directly at different concentrations to RT reaction mixture by RT-DIAPOPS procedure. Four columns represent each dilution of PVY in four independent experiments. Source: Nicolaisen et al. (2001). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

of PVY strains, O, N, NTN, and C, was achieved by using primers designed for RT-PCR assay. *Potato virus A* (PVA) and *Potato virus V* (PVV) were not detected, indicating the specificity of DIAPOPS technique. However, ELISA test detected PVY strains in some samples which were negative to DIAPOPS (less than 1/10 of ELISA samples) (Nielson et al. 1998). An RT step was included prior to DIAPOPS assay for the detection of *Potato virus Y* (PVY). NucleolinkTM Strips (NUN A/S, Røkilde, Denmark) were covalently coated with an oligonucleotide which served as one of two primers in the following PCR amplification. During PCR, the amplifications were covalently attached to the microwell surface. The amplicons served as targets for a biotin-labeled probe which could be subsequently detected in an ELISA reader, after the addition of a suitable streptavidin-enzyme/substrate combination. The use of a solid phase eliminated transfer steps, resulting in reduction of contamination risk (Fig. 7.7) (Nicolaisen et al. 2001).

The reverse-transcription (RT-) PCR assay has been demonstrated to be effective and reliable for the detection and differentiation of viruses infecting potatoes and other vegetatively propagated crops. RT-PCR assay was more reliable and sensitive in detecting *Potato leafroll virus* (PLRV) in dormant tubers from field-grown plants and in-vitro-propagated microtubers when compared to ELISA test. Further, ELISA was able to detect PLRV only in microtubers, whereas RT-PCR could detect the virus in both types of tubers (Spiegel and Martin 1993). For the detection of PLRV, a common electric drill was employed for sampling a large number of dormant tubers by simultaneously removing and macerating tuber eye samples. This method of sampling was found to be rapid, simple, and inexpensive. The dormant tubers from plants with primary infection were detected by both RT-PCR and ELISA tests for the presence of PLRV (Souza-Dias et al. 1999).

In another investigation, the relative incidence of PLRV at different locations was assessed during field inspection by employing RT-PCR assay. Higher levels of potato

leafroll disease were detected by RT-PCR assay than by visual inspection. Infection levels of sprouts from potatoes planted in the greenhouse were determined by ELISA test and RT-PCR assay. Infection of sprouts up to 85% was revealed by both ELISA and RT-PCR procedures (Russo et al. 1999). The current-season spread of PVY in 11 potato fields planted with 6 different potato cultivars in New Brunswick, Canada was investigated. In each field, 100 plants were selected and the spread of the disease was monitored by employing ELISA and real-time RT-PCR assays. The average PVY incidence in fields increased from 0.6% in 2009 and 2% in 2010 in the leaves to 20.3% in 2009 and 21.9% in 2010 in the tubers at harvest. Real-time RT-PCR assay was more efficient at detecting PVY from leaves than ELISA tests. Further, RT-PCR assay detected PVY in a greater number of samples from growing tubers compared to leaf samples obtained from the same plant at the same sampling time. PVY incidence assessed from the growing tubers showed a significant positive correlation with PVY incidence of tubers at harvest (Fageria et al. 2013).

Certified potato seed tuber lots were evaluated in Washington (960) and Oregon (268) for the presence of *Potato virus Y* and its strains, representing 40 potato cultivars from 212 seed potato growers in 11 states and 5 Canadian provinces. The samples were assayed using ELISA tests. The ELISA-positives with MAbIF5 were considered to be PVY^{N/NTN}. Samples positive for MAb2 were considered to be PVY^O or PVY^{N:O}. When a composite sample tested positive for PVY^O or PVY^N, plants within that 10-composite sample were individually retested with appropriate antiserum to determine the number of infected plant(s). Plants confirmed as PVY^O positive were further tested by RT-PCR in 2002 and 2003 to differentiate between PVY^O and PVY^{N:O}. A coupled, one-tube duplex RT-PCR (cRT-PCR) was performed with primer pair Anti/S3 which amplified a product of 745 bp from PVY^N, PVY^{N:O}, and PVY^{NTN} isolates. The primer pair Anti/S7 generated a product of 281 bp for PVY^O isolates. The isolates amplifying the 745 bp product in cRT-PCR and detectable with MAb2 were classified as PVY^{N:O} as they were O serotype. The results showed that PVY^O occurred in 16 cultivars originating from 12 states/provinces and 70 seed growers in Washington, and this strain was detected in 15 cultivars originating from 13 states/provinces and 52 growers in Oregon. The incidence of PVY^O fluctuated over three years (2001–2003), whereas the incidence of PVY^N remained relatively constant. In contrast, the incidence of PVY^{N:O} approximately doubled in the number of states/provinces and seed growers where the presence of this strain was detected. The increased incidence of PVY^{N:O} in potato seed lots was possibly due to the difficulty in differentiating this strain from PVY^O. Certification agencies typically used only serological assays to differentiate PVY^O from PVY^N, whereas PVY^{N:O} might only be easily recognizable by employing both immunoassays and PCR-based assays. The results showed that techniques effective for differentiating PVY strains have to be employed by potato seed certification agencies (Crosslin et al. 2006).

The development of diagnostic techniques for potato seed tuber certification resulted in considerable improvements in sensitivity, specificity, reliability, and rapidity of the ELISA and PCR-based assays. However, studies to analyze the differences in virus detection performance of these techniques, using a statistical approach and relatively large datasets, have been scarce. The maximum allowable infection of *Potato virus Y* (PVY) in seed potatoes was 2% as per the rules of certification of seed potatoes in Idaho, USA. The choice of a diagnostic method for PVY might affect the outcome of the test and consequently the economic benefits received by seed potato growers. PVY detection performance of conventional ELISA- and RT-PCR methods was subjected to statistical analysis.

The PCR-based assays were considered to be superior to ELISA test. Of the two RT-PCR-based methods, the performance of IPC Multi was statistically better than the performance of ELISA and New Multi format. PVY detection performance of New Multi was not statistically different from that of ELISA test. Further, a positive outcome was considered to be less likely when potato tubers were tested and more likely when potato leaves were tested. The results suggested that selection of efficient diagnostic procedure and appropriate plant tissues were important in the outcome of the tests (Bolotova et al. 2009).

Alfalfa mosaic virus (AMV), the type member of the genus *Alfamovirus* (family, *Bromoviridae*) has a wide host range which includes potato, alfalfa, and pea. AMV infection in potato fields in several provinces in Canada was observed. The similarities and differences between eight AMV potato isolates were investigated using RT-PCR assay and sequence analysis of their coat protein (CP) gene. The sequence and phylogenetic analysis showed that eight AMV potato isolates clustered into one strain group, whereas slight differences among other Canadian AMV isolates were revealed. To detect the AMV isolates, a pair of primers (AMV-F and AMV-R) specific to the AMV CP gene was designed based on the nucleotide sequence alignment of known AMV strains. RT-PCR assay using the primer pair was specific and sensitive in detecting AMV in potato leaf and tuber samples. AMV RNAs were easily detected in composite samples of 400–800 potato leaves or 200–400 tubers. RT-PCR amplicon of 351 bp was generated from all infected leaves and tubers of potato. Restriction analysis of PCR amplicons with the enzyme *SacI* was a simple method for confirmation of PCR tests, yielding two fragments of the expected sizes of 150 and 201 bp. The results showed that composite leaf or tuber samples could be used in RT-PCR assays to reduce the time and cost of testing required for screening tubers on a large scale (Xu and Nie 2006).

Tobacco rattle virus (TRV), the type member of the genus *Tobravirus*, has bipartite RNA genome which differentiates into two classes of isolates: M-type (multiplying type) composed of larger particles (containing RNA-1); and NM-type (nonmultiplying), containing a shorter particle (containing RNA-2) that encodes for coat protein (CP) and lacks RNA-1 and coat protein. The M-type isolates causes infection in plants and was found to be common in nature, although they lack a coat protein (Harrison and Robinson 1982). RT-PCR assay detected TRV in extracts of the core of tissues of potato tubers containing total RNAs. Primer A, complimentary to residues 6555–6575, and primer B, identical to residues 6113–6132 of TRV-RSYM RNA-1, were employed for amplification after reverse-transcriptase action on viral RNA. The amplification product of 463bp fragment was cloned, sequenced, and compared to sequences of TRV isolates from Europe and Canada. Nucleotide sequence homology varied from 90% to 94% between Florida isolate and other isolates. RT-PCR was sensitive enough to detect TRV RNA from 1 µg of total nucleic acids from potato tubers. Since the protocol developed in this investigation amplified a segment from RNA-1, it was able to detect TRV in tubers infected by TRV-NM type and M-type in Florida with greater sensitivity and accuracy than ELISA tests (Pérez et al. 2000).

The viral genus *Tobravirus* includes three species, namely *Tobacco rattle virus* (TRV), *Pepper ringspot virus* (PepRSV), and *Pea early browning virus* (PEBV), and they infect a wide range of economically important crops such as potato, pepper, tomato, and beans. Detection of tobnaviruses is complicated by the bipartite genome, since only one of the RNAs can be detected in a single nonmultiplexed PCR format. Using the primers designed to the 16kb pathogenicity protein (RNA-1), TRV was detected in both

systemically infected plants and local necrotic zones. However, ELISA tests did not detect tobnavirus particles in local necrotic zones, suggesting that the local necrotic zones contained the unencapsidated M variant containing only RNA-1 of TRV (Weidemann 1995).

In order to detect all known members of the genus *Tobravirus*, a generic PCR assay was developed using two forward and three reverse PCR primers with little or no degeneracy. These primers amplified a fragment of the 194 kb RNA polymerase gene in the RNA-1 section of the bipartite *Tobravirus* genome from plant tissue infected with TRV, PepRSV, and PEBV. All six primer combinations produced a band of the expected size when used with cDNA derived from plant tissue infected with each of these three viruses, following reverse transcription using either a poly-T or specific primer. The sensitivity of the PCR assay, using serial dilutions of cDNA for the three viruses, was drastically reduced using R2 primer. The undiluted cDNA was amplified from four small samples, and at 1/10 and 1/100 dilutions the viruses were only detected in two samples, indicating the lower level of sensitivity of the RT-PCR assay (Jones et al. 2007).

Sweet potato chlorotic fleck virus (SPCFV), belonging to the genus *Carlavirus* (family *Flexiviridae*), was detected in mixed infection with *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato virus 2* (SPV2) in plant samples obtained from the Australian Capital Territory. Virus titers are often low in sweet potato plants and direct virus detection from the plant tissues extracts is unreliable; each plant was therefore top-grafted with scions of *Ipomoea setosa* (a universal indicator host for sweet potato viruses). Leaves of the grafted *I. setosa* scions were tested for virus presence by DAS-ELISA/RT-PCR assay. Total RNA was extracted by grinding the leaf tissues with liquid nitrogen. Primer pairs PMB-25a/PMB-26 amplified a 696 bp fragment from the 5' end of the CP gene; primer pair PMB-27/PMB-28 amplified a 620 bp region from the 3' end of the CP gene. For SPCFV detection, a portion of the CP gene was amplified by RT-PCR, using either a universal primer pair that amplified all strains or primer pairs that differentiated between RC and other strains. The presence of SPCFV, SPV2, and SPFMV-D was detected by ELISA and RT-PCR assay and their identity was confirmed by sequencing of viral CP gene. Using this protocol, the presence of SPCFV and SPFMV-D was detected and identified for the first time in Australia (Jones and Dwyer 2007).

The incidence of *Potato mop-top virus* (PMTV) in commercially produced potato cultivar Modoc tubers was assessed. Symptomatic tubers in the multiple samples collected from the packaging line accounted for approximately 3%. Total RNAs extracted from one symptomatic (showing internal necrotic arcs and lines) and one asymptomatic tuber were tested by RT-PCR assay with primers specific for PMTV and *Tobacco rattle virus* (TRV). The expected band of 416 bp was amplified by the primers specific to PMTV. No amplification was observed with TRV primers. The asymptomatic tuber was negative for both PMTV and TRV. Subsequently, total RNA from four additional symptomatic tubers from the same lot were tested by RT-PCR for TRV and with a different set of primers for PMTV. The results showed that amplified products of 460 bp were obtained with PMTV primers for the four symptomatic tubers and the same tubers were negative for TRV. ELISA test results confirmed the presence of PMTV in the symptomatic tubers tested by RT-PCR assay. The amplicons of 460 bp generated from two symptomatic Modoc tubers were cloned and sequenced. The sequences were identical and the sequence was entirely (100%) identical to the corresponding sequences of PMTV isolates from North Dakota and Finland. As the internal symptoms in tubers

might go undetected, sensitive detection techniques such as RT-PCR have to be employed to restrict the disease incidence and spread by eliminating all the infected tubers rapidly (Whitworth and Crosslin 2013).

Potato mop-top virus (PMTV) spreads through infected tubers, and soilborne *Spongospora subterranea* is the natural biological vector involved in the virus transmission from infected plants to healthy plants (Arif et al. 1995). PMTV is a rod-shaped virus belonging to the genus *Promovirus* and has a tripartite genome consisting of three single-stranded positive sense RNA molecules (Torrance et al. 1999). RT-PCR assay was employed, using primers targeting the CP gene in RNA-3 of PMTV. The virus could be detected in potato seed lots and ware potato during surveillance in the USA and Canada. PMTV was detected in 4.3% of the 3221 samples tested. Confirmatory tests using two primer sets targeting gene segments in RNA-2 and RNA-3 were carried out. Amplicons generated from RNA-2 and RNA-3 were identified by RFLP analysis. In addition, results of ELISA test, immunosorbent electron microscopy (ISEM) observations, and infectivity tests on *Nicotiana debneyi* (indicator host) corroborated the results of RT-PCR assay (Xu et al. 2004).

The relative importance of infected seed tubers and infested soil as sources of inoculum for PMTV was determined using ELISA and RT-PCR assay. The rate of transmission of PMTV from infected tubers to daughter tubers ranged over 18–54%. Transmission was affected by potato cultivar and by origin of seed tuber, but not by a cultivar's sensitivity to PMTV infection. The incidence of PMTV in daughter tubers was not correlated with that in seed tubers, but appeared to be strongly associated with soil inoculum. Further, the incidence of spraing (symptom of PMTV infection) was strongly correlated with incidence of PMTV in the daughter tubers of those crops in which both virus symptoms were present (Fig. 7.8). Real-time RT-PCR assay indicated that planting PMTV-infected seed potatoes could increase the risk of introducing the virus into the field not infested by PMTV and the fungal vector *S. subterranea* (Davey et al. 2014).

Onion yellow dwarf virus (OYDV), a member of the genus *Potyvirus*, affects onion seed and garlic cloves production significantly. RT-PCR assay was developed as an

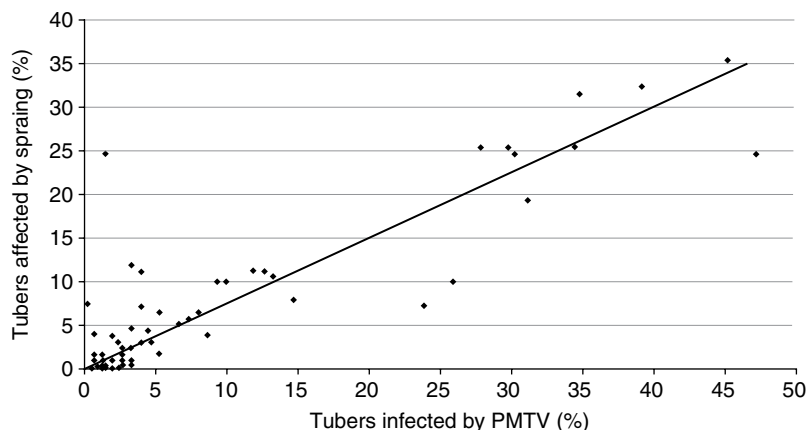


Figure 7.8 Correlation between tuber infection by *Potato mop-top virus* (PMTV) and incidence of spraing in Scottish seed potato tubers of cultivar Cara. Source: Davey et al. (2014). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

alternative diagnostic method because of the difficulty associated with the production of OYDV-specific antibody. The propagation materials generated by meristem-tip culture method employed to obtain virus-free stocks were tested by RT-PCR assay. Specific primers based on conserved region of RNA-dependent RNA polymerase (RdRp) gene and 3' UTR (untranslated region) of RNA of OYDV isolates were designed and used in the RT-PCR assay. An OYDV-specific fragment of approximately 1.1 kb was amplified from the extracts of naturally infected onion and garlic tissues. In contrast, the ELISA test failed to detect OYDV in onion samples, indicating the superiority and suitability of RT-PCR assay for providing sensitive and reliable detection of OYDV in both onion and garlic samples (Arya et al. 2006).

The incidence of *Artichoke yellow ringspot virus* (AYRSV), a member of the family *Comoviridae*, was observed in Greece where *Onion yellow dwarf virus* (OYDV) was the only previously known virus to infect onion crops. Using degenerate primers specific for the family *Comoviridae*, RT-PCR assay was employed for the detection of AYRSV in onion plant tissues. The primer pair amplified RNA-dependent RNA polymerase (RdRp) sequences. The sequence analysis and comparison with AYRSV isolates from *Cyanara scolymus* and *Vicia faba* indicated that these viruses were highly similar. The results showed that AYRSV infecting onion had a high sequence similarity to other known isolates of the virus from other host plant species (Maliogka et al. 2006).

Iris yellow spot virus (IYSV), a distinct member of the genus *Tospovirus* (family *Bunyaviridae*), affects seed and bulb production in onion in the USA. Antiserum was produced to *Escherichia coli*-expressed nucleocapsid protein of IYSV. Indirect ELISA format was found to be effective for the detection of the virus in onion tissue at a dilution of up to 1:1000. Standard RT-PCR and real-time RT-PCR assays were performed using the primer pair IYSV-465c/IYSV-239f to amplify a 227 bp fragment of N gene (coding nucleocapsid). The standard RT-PCR assay provided unambiguous results. The limits of detection for endpoint dilutions were 10^{-2} and 10^{-3} for the modified Dellaporta method and the commercial kit, respectively. The PCR product from each extraction procedure was cloned and sequenced to verify the identity of the amplicons as IYSV. Real-time RT-PCR assay was evaluated based on the threshold cycle values of diluted extracts. No difference in the limit of detection could be observed between template preparations made by using the RNeasy Kit or the modified Dellaporta method. The amplification of IYSV-specific fragment was based on the melting curves and agarose gel electrophoresis of the PCR reaction product, with a dilution limit of $1:10^6$ dilution of extracts using real-time RT-PCR format, which was more sensitive than standard RT-PCR format. The primers were highly specific and the primer sequences were highly conserved in over 20 isolate sequences with the ability to provide broad-spectrum detection of IYSV isolates. Real-time PCR allowed sensitive, rapid, and high-throughput IYSV detection (Pappu et al. 2008).

In order to investigate the distribution of *Potato virus Y* (PVY) in tubers, three potato cultivars Russet Burbank, Russet Norkotah, and Shepody tubers infected with PVY strains PVY^O, PVY^{N:O}, and PVY^{NTN} were tested from storage at 4°C at the initiation of sprouting, and then tested again at 4°C at 70 days after storage. Samples taken from eyes in the stem, middle, and bud (distal end) areas of tubers were tested using ELISA. The ELISA-negative samples were retested using RT-PCR assay. Results of ELISA alone and ELISA + RT-PCR assay were evaluated for each strain, sample location, sample time, and cultivar. ELISA tests underestimated the actual percentage of tubers infected by PVY strains. The combined results showed that PVY^{N:O} infection could be accurately

assessed and it was unevenly distributed in some cultivars. On the other hand, strains PVY^O and PVY^{NTN} were found to be evenly distributed at 78 days after initial sprouting. Based on the results, it was suggested that the protocols for PVY postharvest testing used by seed certification agencies should specify the amount of time needed before sampling, and specific protocols should be developed based on cultivar for precise detection of PVY strains and assessment of disease risk for using the seed tuber lots (Whitworth et al. 2012).

The incidence of *Potato virus Y* (PVY) suddenly increased in foundation seed potatoes in Hokkaido, northern Japan in 2008 and 2009 seasons. Molecular typing revealed that, in addition to PVY^O and PVY^{NA-N}, the most common strain identified was the recombinant PVY^{NTN} with three characteristic recombination junctions at the HC-Pro, Vpg, and CP regions. No potato tuber necrotic ringspot disease (PTNRD) was observed in foundation seed potatoes in correlation with the presence of PVY^{NTN}. An isolate with a typical PVY^{NTN} recombinant genome, namely Eu-12Jp, did not induce PTNRD in 62 Japanese potato cultivars tested in both primarily and secondarily infected plants. Based on the genome structure and the reaction of the potato N resistance genes, Eu-12Jp was classified as PVY^Z-NTN. The sudden increase in the incidence of PVY^{NTN}/PVY^Z-NTN indicated the potential risk of PTNRD emerging as a destructive disease of potato in Japan (Ali et al. 2013).

Sweet potato crops are frequently infected simultaneously by two or more viruses, resulting in mixed infection and subsequent development of complex symptoms. Detecting, quantifying, and isolating viruses directly from sweet potato plants are difficult. The viruses are generally transmitted by mechanical or graft inoculation to *Ipomoea setosa* (universal indicator plant for sweet potato viruses). Real-time PCR assays were developed for relative quantification in singleplex reactions of the potyviruses *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus G* (SPVG), and *Ipomoea vein mosaic virus* (IVMV), the crinivirus *Sweet potato chlorotic stunt virus* (SPSCV), and the begomovirus *Sweet potato leafcurl virus* (SPLCV) directly from infected sweet potato plants. The real-time PCR assay detected SPLCV-US in a higher percentage of test plants compared with conventional PCR format, particularly in the early stages of infection. Real-time PCR assay detected SPLCV-US in 17 of 20 samples tested, whereas conventional PCR assay could only detect the virus in 5 of 20 samples at 22 days after transplanting (dap). Likewise, at 35 dap, 18, and 2 of 20 samples were positive to real-time PCR and conventional PCR formats, respectively. Further, real-time PCR assay could detect lower virus titer (1000-fold) compared with conventional PCR format. No significant effect of inhibitors on PCR amplification was observed.

Virus titers of SPFMV, IVMV, and SPVG were quantified using real-time PCR and found to be lower in singly infected Brazilian Morning Glory (*I. setosa*) and *I. nil* cultivar Scarlet O'Hara plants. SPSCV was only detected in an isolated tissue culture sample and the real-time PCR protocol provided a highly sensitive detection of the virus, while conventional PCR and ELISA tests often gave ambiguous results (Kokkinos and Clark 2006). *Sweet potato leafcurl virus* (SPLCV), a DNA virus belonging to the genus *Begomovirus* (family *Geminiviridae*), has progressively assumed greater importance. In order to determine the status of infection by SPLCV, all accessions in the germplasm collection maintained by the US Department of Agriculture-Agricultural Research Service were tested using a real-time PCR assay. Of a total of 701 accessions examined, 47 were found to be positive for SPLCV. Biological indexing using the indicator plant species *Ipomoea nil* and *I. muricata* confirmed the results of real-time PCR assay.

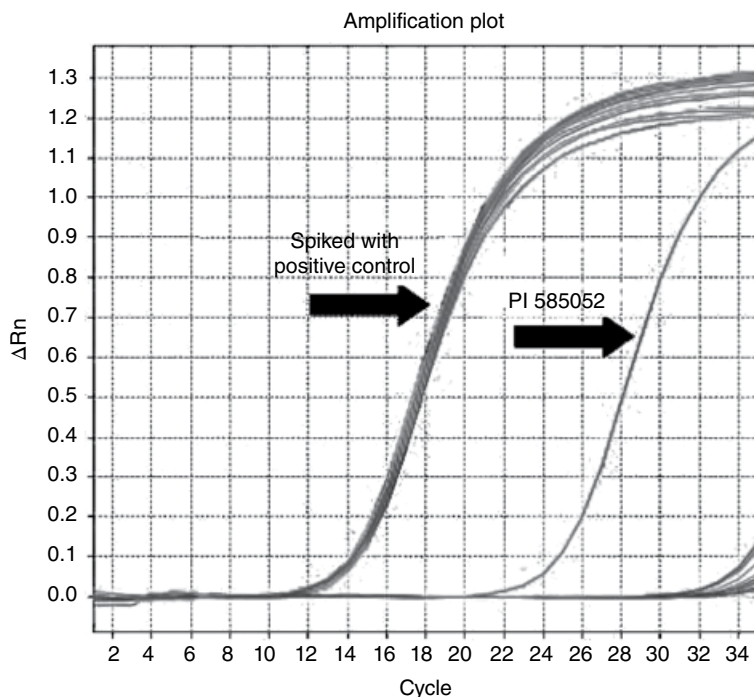


Figure 7.9 Detection of *Sweet potato leaf curl virus* (SPLCV) in in vitro plantlets of sweet potato accessions by real-time PCR assay. Amplification plot from SPLCV: samples spiked with positive control crossed the cycle threshold; the DNA extract from PI 585052 also crossed the cycle threshold. Source: Barkley et al. (2011). Reproduced with permission of the American Phytopathological Society, MN, USA.

The Ct values of accessions determined to be positive ranged from 14.78 to 29.95 with a mean and standard deviation of 21.81 and 4.03, respectively. A set of primers specific for the detection of SPLCV coat protein (CP) gene (AV1) sequence was used to amplify the total product size of 839 bp (Fig. 7.9). Several of these 47 SPLCV-positive accessions were introduced into the USA through the plant germplasm quarantine system prior to 1998, when the incidence of SPLCV was not known in the USA. Nucleotide polymorphisms among the isolates were evaluated by sequencing the AV1 CP gene from 24 SPLCV isolates from infected accessions. These SPLCV isolates shared high-sequence identity. Ten nucleotide substitutions were identified, most of which were synonymous changes. The results indicated that in vitro germplasm collections which are distributed to worldwide requestors should be regularly evaluated for viruses to avoid distribution of infected clones (Barkley et al. 2011).

Angelonia plants are infected by *Angelonia flower break virus* (AnFBV), inducing mild foliar mosaic and flower break symptoms. The genomic sequences of the Florida isolate of AnFBV were used to design primers flanking the deduced CP gene for amplification of the corresponding genome regions from the Israeli and Maryland isolates. An AnFBV-specific product was amplified from the virus-infected plants when degenerate primers of carmovirus were employed. RT-PCR assay and/or ELISA test were also used to detect AnFBV in *Angelonia* plants (Adkins et al. 2006). Likewise, degenerate primers were utilized for the detection of *Lily symptomless virus* (LSV). The primers were

designed based on the amino acid sequences of coat protein (CP) in four LSV strains. The primers amplified a 483 bp fragment from LSV-infected lily plants. RT-PCR assay was applied to detect LSV in lilies generated by repeated short meristem excision method before and during in vitro culture, and bulblets subjected to thermotherapy in vitro. The amplified banding patterns by RT-PCR revealed the absence of virus-specific band, indicating the successful elimination of LSV by adopting repeated shoot meristem excision method and thermotherapy treatment (Nesi et al. 2009).

Japanese yam mosaic virus (JYMV) and *Yam mild mosaic virus* (YMMV) infect yam plants commonly, causing significant yield losses. A duplex RT-PCR assay was developed using a tube-capture procedure followed by one-step RT-PCR format. Two primer pairs targeted a 241 bp fragment of the coat protein (CP) region of JYMV and a 174 bp fragment of the nuclear inclusion protein “b” region of YMMV, resulting in the simultaneous detection and identification of the two viruses in mixed infection on yam crops. All water yam plants showed infection only by YMMV. On the other hand, all the Japanese yam and Chinese yam plants were found to be infected by either JYMV alone or both JYMV and YMMV (Kajihara et al. 2009).

Banana crops are frequently affected by *Banana bunchy top virus* (BBTV), *Cucumber mosaic virus* (CMV), *Banana bract mosaic virus* (BBMV), and *Banana streak virus* (BSV), accounting for heavy crop losses. A quadruplex RT-PCR procedure was developed by establishing optimal PCR and RT-PCR conditions for the detection of a single virus and by employing virus-specific primers for the simultaneous detection and differentiation of all four viruses in a single reaction. Several sets of primers for each target virus were evaluated for their sensitivity and specificity by simplex and quadruplex RT-PCR formats. The protocol developed in this investigation was effective in detecting the viruses in banana samples infected with one or more viruses collected from field-grown plants and tissue-culture-generated plantlets (Liu et al. 2012).

Stone fruit trees are infected by several viruses including *Apple mosaic virus* (ApMV), *Prunus necrotic ringspot virus* (PMRSV), *Prune dwarf virus* (PDV), *American plum line pattern virus* (APLPV), *Plum pox virus* (PPV), *Apple chlorotic leaf spot virus* (ACLSV), *Apricot latent virus* (ApLV), and *Plum bark necrosis stem pitting-associated virus* (PBNPaV). A sensitive and reliable one-step RT-PCR assay was developed for detection of multiple viruses simultaneously in plants infected with two or more viruses, using a cocktail of primers specific for individual virus. The primer cocktail was used to analyze 38 stone fruit trees originating from nine different countries and six species. Amplification of a single PCR fragment in singly infected tissue using the cocktail of all primers showed the specificity of the multiplex RT-PCR format. The nine expected PCR fragments were detected when a simulated infection of eight different viruses was performed by mixing total nucleic acids extracted from single-infected tissues and also in the internal PCR control. The expected PCR-amplified viral fragments and internal control were observed in all cases. In naturally occurring mixed infection, up to four viruses (PMRSV, PDV, APLPV, and ACLSV) were simultaneously detected. The detection limit of multiplex RT-PCR varied depending on the three methods of extraction of total RNAs from infected plant tissues. Use of a cocktail of five pairs of primers did not affect the detection limit of multiplex RT-PCR, while the use of seven pairs reduced the sensitivity of the multiplex RT-PCR. However, the detection limit of multiplex was 25 times higher than that of dot-blot hybridization assay. The efficacy of multiplex RT-PCR assay and DAS-ELISA was compared for the detection of the viruses in 38 field samples which included six different hosts

(peach, almond, cherry, plum, apricot, and GF305 apple), nine geographical origins and different virus combinations. The multiplex RT-PCR assay reliably detected and identified a large number of virus combinations and up to three viruses in five fruit trees. The results indicated that the multiplex RT-PCR format was more sensitive than DAS-ELISA, in addition to its ability to detect multiple infections by two or more viruses in the same fruit tree. The multiplex RT-PCR protocol developed in this investigation represented a significant advancement for routine diagnosis of stone fruit viruses, reducing the cost, time, and handling (Sánchez-Navarro et al. 2005).

Infection of stone fruit crops by *Apricot latent virus* (ApLV) and *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) was observed in many locations. To determine the extent of virus infection, field surveys were undertaken in eastern Anatolia, Turkey. RT-PCR and nested RT-PCR formats were employed for the detection of ApLV and PBNSPaV, respectively. Of the 224 apricot samples tested, ApLV was only detected in three samples (1.3%). All infected samples yielded the expected product of 200bp following PCR amplification. In the case of 45 sweet cherry and plum samples tested, 35 samples tested positive for PBNSPaV by nested RT-PCR format. The positive samples showed the presence of virus-specific 190bp fragment. Of the positive samples, 27 sweet cherry, 1 plum, and 7 prune cultivar Italian prune samples yielded specific amplicon, indicating PBNSPaV infection (Fig. 7.10). The survey showed the infection of stone fruit crops by ApLV and PBNSPaV for the first time, indicating the necessity of monitoring the virus disease incidence and subsequent spread by employing effective diagnostic assays (Usta et al. 2007).

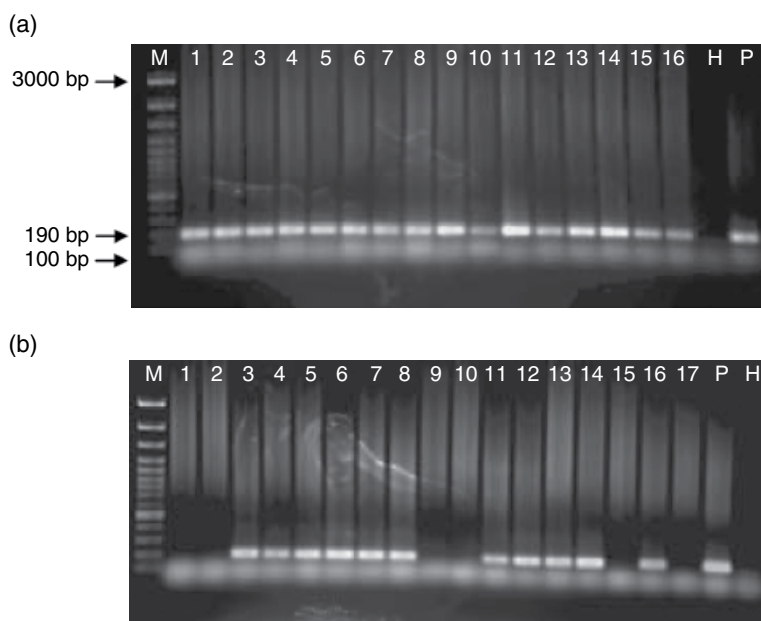


Figure 7.10 Detection of *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) by nested RT-PCR assay using ASPn1/ASPn2 primer pair. M: molecular size standards; P: positive virus control; H: healthy plant control. (a) Lanes 1–16; (b) lanes 3–18, 11–14; 16: virus positives from symptomatic and asymptomatic plant samples. Source: Usta et al. (2007). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

The sanitary status of mother plants of stone fruit trees was determined by employing the RT-PCR assay. In the case of *Apricot latent virus* (ApLV) infection, the unique fragment of 200bp specific to ApLV was amplified from the extracts of 19 of 120 apricot samples, indicating the presence of ApLV in these apricot trees. A total of 35 stone fruit samples consisting of 10 peach, 12 plum and 13 cherry samples was tested by nested RT-PCR format. The extracts from two cherry trees showed positive amplification of 190bp product specific to *Plum bark necrosis stem pitting-associated virus* (PBNSPaV). The incidence of PBNSPaV in cherry plants was detected for the first time in western Anatolia, Turkey (Gümüş et al. 2007). A survey to assess the incidence of *Prunus necrotic ringspot virus* (PNRSV) in cherry orchard of *Prunus terasus* cultivar Montomorency and *Prunus avium* cultivar Hedelfingen was undertaken in New York. The presence of PNRSV was confirmed by RT-PCR assay and amplification of the viral CP gene in total RNA extracted from infected leaves. Further, latent infection of a majority of trees (87%) by PNRSV was also revealed by RT-PCR assay. Asymptomatic PNRSV-infected trees clustered in spatial proximity of symptomatic trees (Oliver et al. 2009).

Diagnostic tests are tools that increase the knowledge of the sanitary status of a plant with respect to pathogen infection, and the possibility of reducing the probability of infection by eliminating such possible sources of inoculum. It has to be realized that there is no perfect method without false-positive and/or false-negative results, and the test selected may give a more accurate probability of the presence or absence of the target pathogen (post-test probability of infection). *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV), members of the genus *Ilarvirus* (family *Bromoviridae*), infect the majority of *Prunus* spp. They may be transmitted through pollen, seeds, and propagative plant materials. These two viruses are frequently found together in mixed infections. The use of healthy planting materials is emphasized for preventing virus spread in woody crops. Certification programs are in operation in several countries to identify healthy plant sources for propagation. RT-PCR assay provides the great advantage of simultaneous detection of several targets in a single reaction with a high level of sensitivity. Inter-laboratory evaluation involving nine European laboratories of duplex RT-PCR method was carried out, using plant crude extracts as templates for molecular diagnosis of PDV and PNRSV. A total of 576 samples from *Prunus* trees with known sanitary status, corresponding to 32 samples in 2 replicates for each laboratory, were analyzed. The level of sensitivity achieved by the method was 98.3% for PDV and 90.4% for PNRSV. The specificity of detection was 87.4% for PDV and 94.3% for PNRSV. While using crude extracts and detecting two viruses simultaneously, the specificity and sensitivity values were very high and equal to or higher than the average values published earlier for detection of single viruses using purified RNA or DNA. The inter-laboratory evaluation of the RT-PCR protocol provided very useful information on the performance and limits of the procedure tested for PDV and PNRSV (Massart et al. 2008a).

Two duplex RT-PCR methods with primers tested earlier were evaluated by nine different laboratories for the detection of four different fruit tree viruses, *Apple mosaic virus* (ApMV, *Ilarvirus*), *Apple stem-grooving virus* (ASGV, *Capillovirus*), *Apple chlorotic leafspot virus* (ACLSV, *Trichovirus*), and *Apple stem-pitting virus* (ASPV, *Foveavirus*). For this inter-laboratory evaluation, identical samples were provided as crude extract preparation to each participant laboratory. Specificity and sensitivity values for ASPV, ASGV, and ACLSV were similar to or higher than those reported earlier for specificity (82–100%) and sensitivity (38–96%). The specificity values for ASPV was low (67.9%),

reflecting the high percentages of false positives. Detection of ApMV and ACLSV was recommended to be performed separately because of difficulties encountered in discriminating the ApMV and ACLSV bands. Repeatability and reproducibility of the diagnostic test were considered as crucial characteristics but, unlike sensitivity and specificity, they were not given due attention in many investigations. This study clearly showed the usefulness of multicenter trials for giving effective guidelines and suggestions for validating new diagnostic protocols (Massart et al. 2008b).

RT-PCR assay was employed to assess the incidence of *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), and *Apple stem pitting virus* (ASPV) in Turkey. A total of 174 samples collected from apple cultivars and genotypes grown in different locations were tested. Latent infection of apple trees by these viruses was high in commercial orchards. In addition, varietal collections also showed high levels of infection by AGSV, ACLSV, and ASPV, indicating the need for development of sensitive and rapid detection technique to prevent further spread of the virus diseases quickly. Furthermore, the apple plants were propagated by budwood materials taken from mother plants whose health status had not been ascertained by diagnostic procedure(s). The infection rate of ApMV was relatively low compared to three other viruses. The RT-PCR protocol was very effective and reliable in detecting the target viruses present in the apple cultivars, facilitating the elimination of infected mother plants and selection of healthy plants for building up virus-free stocks, considered the most effective management strategy to prevent the incidence and spread of the viruses through propagative planting materials (Cağlayan et al. 2006).

Four *Plum pox virus* (PPV) strains have been identified based on differences in symptomatology of infected trees, aphid transmissibility, and disease epidemiology. PPV-M (Marcus) originating from peach trees in Greece and PPV-D (Dideron) from apricots in France are the most commonly occurring strains (Kernal and Dunez 1979). PPV-EA (El Amar) and PPV-C (Cherry) infect apricots in Egypt and sour cherry in Mouldova, respectively (Wetzel et al. 1991). Application of RT-PCR assay for the detection of PPV in cultivated plum germplasm entries and wild apricots was performed. Primers based on the sequences of PPV CP gene amplified a 243 bp fragment in the C-terminus coat protein coding region. The PPV isolates infecting plum cultivars were identified as D strain of PPV (Spiegel et al. 2004). Molecular methods of detection of PPV were employed to determine the extent of prevalence of PPV strains and to establish their identity. A total of 72 leaf samples from peaches, apricots, and plums with or without symptoms were collected from different locations. A DAS-ELISA test was applied to test all samples using polyclonal antibodies (PABs). In addition, total RNA extracted using commercial kit was used as template for RT-PCR assay to confirm the results of ELISA tests. Broad reactivity of PPV-specific primers P1/P2 was used for amplification of virus-specific DNA fragment from plant extracts. Furthermore, samples were also analyzed using PPV-M and PPV-D strain-specific primers P1/PM and P1/PD. Amplified products were resolved by gel electrophoresis and staining with ethidium bromide. Of the 72 samples tested, 42 showed positive reaction in ELISA while 56 samples were positive for RT-PCR assay, including 42 ELISA-positive samples. Using PPV generic P1/P2 primer pair, the presence of PPV was detected in 73% of peach, 88% of apricot, and 75% of plum trees. Assays with strain-specific primers showed that 49 isolates were M-type, whereas isolates from 7 peach cultivars belonged to D-type. The RT-PCR format was more sensitive and could also differentiate the PPV strains infecting the stone fruit crops in a reliable manner (Papayiannis et al. 2007).

Citrus psorosis ringspot virus 4 (CtRSV-4) was detected using RT-PCR primers, designed based on the sequences of the fragments of cDNA of the bottom component RNA of CtRSV-4. CtRSV-4 could be detected in citrus leaves at a tissue dilution of 1:12,800, representing as little as 2 µg of tissue indicating the high sensitivity of RT-PCR protocol (Garcia et al. 1997). The comparative efficacy of ELISA test and RT-PCR assay was assessed for the detection of *Citrus tristeza virus* (CTV). ELISA test successfully detected CTV in phloem-rich tissues such as petioles or midribs of citrus leaves. On the other hand, RT-PCR assay efficiently detected CTV in all tissues of citrus. In addition, RT-PCR assay was able to detect CTV at all sampling times, even when CTV concentration was below levels that were not detectable by employing ELISA tests (Mathews et al. 1997). The efficacy of RT-PCR assay was compared to that of the immunoassays ELISA and DTBIA for the detection of *Citrus tristeza virus* (CTV). Sweet orange and grapefruit trees were indexed for the presence of decline-inducing and nondecline-inducing isolates of CTV. The results showed that RT-PCR was more sensitive than both immunoassays. In addition, the decline-inducing and nondecline-inducing isolates of CTV could be differentiated precisely by employing RT-PCR assay. Both isolates could be detected in the same field-infected sweet orange and grapefruit trees (Huang et al. 2004). RT-PCR assay based on total RNA extraction method by Qiagen, consisting of crude plant sap extraction and total nucleic acid trapping on a silica bed, was found to be efficient in detecting CTV in citrus trees. RT-PCR assay provided more consistent CTV detection, especially during the summer and winter months. RT-PCR assay detected CTV in 210 of 287 samples tested, whereas ELISA could only detect the virus in 198 samples. The results from monthly tests performed in a selected number of field-grown CTV-infected trees showed that RT-PCR assay detected the virus in all trees (100%) in winter and summer. ELISA did not detect CTV consistently however, as the RT-PCR assay did. The RT-PCR protocol developed in this investigation could be used in citrus certification programs and to test the trees in nurseries and commercial orchards (Rosa et al. 2007).

Citrus tristeza virus (CTV, *Closterovirus*) and *Citrus mosaic virus* (CMBV, *Badnavirus*), infecting citrus singly or in combination, cause citrus decline in India, particularly on sweet orange. As the immunoassays were found to be inconsistent and less sensitive in detecting CTV and CMBV in all seasons, a rapid and reliable PCR-based detection procedure was essential for the detection and identification of the viruses in citrus plants. A duplex PCR assay protocol was developed using two different sets of primer pairs specific for ORF8 of CTV genome and ORF 3 of CMBV genome, to obtain amplified products of 510bp and 245bp, respectively, for CTV and CMBV. The detection limits of duplex PCR procedure were 10^6 and 10^5 , respectively. On the other hand, the simplex PCR format had the detection limits of 10^6 and 10^4 for CTV and CMBV, respectively. The duplex PCR procedure was found to be a sensitive, reliable, and rapid method for the detection of CTV and CMBV in single and mixed infections. All doubly infected field samples of citrus produced two distinct bands in the gel corresponding to the expected sizes of CTV and CMBV, indicating the possibility of detecting both viruses in a single reaction and hence considerably reducing the cost of testing. Leaf dip technique employing electron microscope confirmed the presence of nonenveloped bacilliform particles of CMBV (130 × 30 nm) and long CTV particles (2000 × 11 nm) in the infected leaves. The protocol developed in this investigation could be employed for the sensitive detection of RNA and DNA viruses infecting citrus in India (Ghosh et al. 2008).

Indian citrus ringspot virus (ICRSV) causes another important disease in citrus that negatively impacts citrus production in India. The effect of hot water and moist air treatment (40–50°C for 30 min) on the elimination of ICRSV from nodal segments obtained from mother plants was investigated by estimating the virus titer in treated plant materials. Comparative efficacy of ELISA and RT-PCR assays was assessed by detecting the virus in treated planting materials. The percentages of virus elimination varied over 40.74–60.0% by RT-PCR, whereas ELISA test could detect the virus in 48.15–73.33% of treated planting materials. RT-PCR assay could detect ICRSV in ELISA-negative samples, indicating its higher level of sensitivity in detecting planting materials containing low virus concentrations and the suitability of RT-PCR assay for ensuring virus-free nature of the planting materials (Sharma et al. 2008).

The movement of grapevine stocks is the principal mode of dispersal of viruses infecting grapevine over long distances; the secondary spread occurs through natural biological vectors. Grapevines are generally propagated by cuttings, a process that perpetuates virus infection if healthy plants are not selected for propagation. Detection of the viruses in the asexually propagated planting materials that are asymptomatic becomes difficult. *Grapevine fan leaf virus* (GFLV) was detected by employing primers that amplified a 605 bp fragment containing a part of the coat protein (CP) of GFLV. RT-PCR assay was able to detect 20 isolates of GFLV occurring in Tunisia. RFLP analysis revealed sequence variations that could form the basis for differentiation of isolates of GFLV (Fattouch et al. 2005). RT-PCR assay was applied for the detection of GFLV in 82 grapevine samples, using the primer sets S2515/A3300 or CP433V/912C. DNA fragments of 810 bp and 480 bp were amplified by these primers, respectively. The assessment of comparative efficacy of ELISA test and RT-PCR assay showed that 22 ELISA-positive samples were negative for RT-PCR assay, using one or both primers sets. No ELISA-negative samples tested positive by RT-PCR assay. The absence of GFLV in 41 samples was indicated by the results of both ELISA and RT-PCR assays. Overall, RT-PCR was found to be less robust as a diagnostic technique for the detection of GFLV in grapevine plants (Bashir and Hajzadeh 2007).

Grapevine leafroll-associated virus 1 (GLRaV-1) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) had to be eliminated from grapevine cultivar Agiorgitiko to obtain healthy planting materials. The effectiveness of virus elimination for the production of virus-free planting materials was assessed by combining in vitro thermotherapy and tissue culture methods and by RT-PCR assay to ensure the freedom of the planting materials from these two viruses. The presence of GLRaV-1 and GRSPaV was verified in treated explants cultured in woody plant medium. Meristem or shoot-tip cultures coupled with therapy were effective in eliminating GLRaV-1 and GRSPaV to a different extent, as revealed by nested RT-PCR assay. A relatively high percentage of plantlets were found to be virus-free. It seemed that GLRaV-1 could be eliminated more easily than GRSPaV. GLRaV-1 could be eliminated in 91.2% and 73.8% by meristem-tip and shoot-tip culture methods, respectively, whereas the success rates were less (61.8% and 37%) for GRSPaV. The ratio of virus elimination to survival was higher for meristem-tip culture than for shoot-tip culture (Skiada et al. 2009).

The incidence of *Grapevine fan leaf virus* (GFLV), *Grapevine leafroll-associated virus 1, 2, and 3* (GLRaV-1, -2, and -3), *Grapevine virus A* (GVA), and *Grapevine fleck virus* (GFkV) was monitored throughout one year by testing the grapevine plants by ELISA and RT-PCR assay at monthly intervals. Samples of opening bud, tips or unfolded leaves, leaf petioles, completely expanded leaves, green phloem tissues, and cortical

scrapings were used for taking tissue extracts for analysis by ELISA and RT-PCR assays. Detection percentages varied in different months depending on the tissue tested and the target virus. The highest detection percentages throughout the year were achieved for GFLV and GFKV (98% and 94%, respectively) when tested by ELISA. On the other hand, RT-PCR assay provided less efficient detection of GVA (92%) and GFLV (77%), indicating higher level of sensitivity of detection for ELISA compared with RT-PCR assay. This might be due to coextraction of a high level of phenols and polysaccharides along with total nucleic acids in grapevine tissues. The best tissue that could be used for testing by ELISA and RT-PCR assay was phloem scrapings from mature canes for detection of all viruses, giving a high level of reliability (100%; Fiore et al. 2009).

Grapevines are infected simultaneously by several viruses, resulting in complex symptoms and different combinations of viruses occurring at various locations, complicating the detection and identification of the viruses involved. Multiplex RT-PCR (mRT-PCR) protocols have been developed for detecting several viruses in a single reaction, providing quick, reliable, and cost-effective routine diagnosis of grapevine virus diseases. A mRT-PCR format was developed for simultaneous detection of nine grapevine viruses, namely *Arabis mosaic virus* (ArMV), *Grapevine fan leaf virus* (GFLV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine rupestris stem pitting virus* (GRSPaV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll-associated virus 1, 2, and 3* (GLRaV-1, -2 and -3), in combination with a plant mRNA (*Vitis* 18 rRNA) used as an indicator of the effectiveness of RNA extraction and RT-PCR assay. Primers were designed from conserved regions of each virus and their specificity was confirmed by sequencing PCR amplicons. The detection limits of single step(s) RT-PCR and two-step (m) RT-PCR format were 100 times greater than one-step system for GVA, GLRaV-3, and ArMV, and 10 times greater for all other viruses. The effectiveness of mRT-PCR format was assessed by testing 103 grapevine samples from three areas in Italy. Two-step mRT-PCR was efficient in detecting all nine viruses regardless of the plant tissue tested (in vitro plants, leaves, or phloem tissues scrapped from mature canes). In 8 samples out of 103, RT-PCR detected some viruses which were not detected by ELISA test. As these samples were all from the same vines that had mixed infections, the ELISA test failed to detect any of the components of the complex. The multiplex RT-PCR format, on the other hand, detected 1–9 fragments specific for viruses from infected samples and the viruses were identified by their specific molecular sizes in agarose gel after electrophoresis. In two-step mRT-PCR, the detection limits were 10^{-3} to 10^{-4} extract dilutions, depending on the virus. The mRT-PCR assay could detect the viruses in leaves, phloem from dormant cuttings, and in vitro plantlets from 103 naturally infected and healthy grapevines. The mRT-PCR protocol developed in this investigation was reliable in detecting grapevine viruses in mixed infections and yielded results rapidly, saving time and reducing the cost of testing (Gambino and Gribaudo 2006).

Detection of *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), and *Grapevine leafroll-associated virus 3* (GLRaV-3) was achieved by employing spot-PCR, a variant of PCR assay. Specific amplification of genomic fragments of GVA, GVB, and GLRaV-3 was carried out by RT-PCR on total nucleic acid solubilized from small pieces of charged nylon membrane, on which a drop of crude sap of infected grapevine had already been spotted. Heating the membrane at 95°C for 10 min enhanced the ease of release of viral templates from the spot on nylon membrane. The detection limit of spot-PCR was in equivalence to that of conventional PCR assay. However, spot-PCR format offered the advantage of storability of blots up to one month after spotting (La Notte et al. 1997).

Molecular variants of GVA were detected reliably and specifically by employing RT-PCR assay. Isolates of GVA obtained from various grapevines and locations were inoculated on *Nicotiana benthamiana*. The isolates of GVA could be detected by RT-PCR easily in infected *N. benthamiana* plants (Goszczynski and Jooste 2003). The effectiveness of RT-PCR for the detection of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) was demonstrated. The assay employing CP-specific primers detected GRSPaV in all grapevine samples, including those from asymptomatic plants. In contrast, the primer pair designed from the sequences of replicase gene could only detect GRSPaV in symptomatic plants, indicating the necessity of employing proper primers for virus detection in asexual propagules (Habibi et al. 2006).

Grapevine leafroll-associated viruses 1–9, belonging to the family *Closteroviridae*, are dispersed by vegetative propagation and grafting. Control of grapevine leafroll disease (GLRD) is therefore based on clonal selection and production of healthy propagating material by well-organized certification schemes. Reliable, rapid, and inexpensive methods of detecting, identifying, and differentiating the GLRaV are required. A nested RT-PCR assay that allowed generic detection of a subgroup of genetically related viruses with a distinct evolutionary history within the genus *Ampelovirus* was developed. Members of this lineage are *Grapevine leafroll-associated virus 4*, -5, -6, and -9 and two isolates (GLRaV-De and GLRaV-Pr) representing new species. The procedure involves a one-step RT-PCR for the generic detection of *Closteroviridae* species, using degenerate primers that target the HSP70h gene, followed by a nested PCR which detected all members of virus belonging to this lineage and differentiates them from other grapevine closteroviruses. The 490 bp nested PCR amplicons corresponding to the phylogenetically informative region could be sequenced directly to obtain initial genetic information for partial characterization and rapid classification. Additional primers were employed for specific detection of GLRaV-4, -5, -6, -Pr, and -De on respective single or multiplex nested PCR assays. Application of the generic assay on several field-grown grapevine plants indicated a high level of incidence of subgroup I ampeloviruses (35%) and confirmed the specificity of the protocol developed in this investigation. Further, by using the specific nested PCR assays, the sanitary status of field-grown grapevine plants could be evaluated. A relatively high incidence of GLRaV-Da (6.8%) was recorded. A high percentage (55%) of single infections were also identified using generic primers coupled with the specific nested PCR assays, indicating that this detection scheme could also be employed as a useful tool for “fishing” single infections. The multiplex RT-PCR assay could reduce the need for specific identification of each related virus, reducing the time and cost required for assessing the health status of grapevine planting materials (Maliogka et al. 2008).

Pear crops are frequently affected by *Apple stem pitting virus* (ASPV), *Apple chlorotic spot virus* (CLSV), and *Apple stem-grooving virus* (ASGV), and they induce symptomless infection in commercial cultivars. Two multiplex RT-PCR methods were employed with coamplification of an internal control from a plant mRNA to detect three viruses simultaneously. A *nad5* gene in mitochondrial mRNA of the apple was used as the internal control of coamplification to reduce the risk of false-negative results. Multiplex RT-PCR I assay successfully detected ACLSV and ASGV by amplifying 635 bp and 265 bp from the infected plant extracts specific for ACLSV and ASGV, respectively. Multiplex RT-PCR II amplified 346 bp and 256 bp bands specific to ASPV and ASGV, respectively, and the 181 bp band specific to *nad5* internal control. As the internal RNA control was coamplified with target viruses in total nucleic acid extracts from plant

tissues, purified RNA as amplification template was not required. Isolation of nucleic acids from bark chips was more successful than isolation from leaves. The four amplification products, including three target viruses and one internal control, were sequenced to demonstrate the specificity of all primers employed. Inclusion of an internal plant mRNA control and the high level of sensitivity of RT-PCR allowed the effective detection of three target viruses from bark chip samples throughout the year, reducing the risk of false-negative results (Ma et al. 2008).

Strawberry mild yellow edge virus (SMYEV), *Strawberry mild mottle virus* (SMoV), and *Strawberry vein banding virus* (SVBV) infect strawberry crops, causing significant yield losses. Total RNA and total nucleic acid extracted from infected strawberry leaves were used as templates for the detection of the viruses by single or duplex RT-PCR procedures. When total RNA and total nucleic acid were used as templates, detection results of SMYEV and SMoV were identical and the amplified band intensity was nearly the same by single or duplex RT-PCR format. On the other hand, use of pure DNA of SVBV as template provided similar detection results for single and duplex RT-PCR assays. Total nucleic acid extracted from strawberry leaves by a modified CTAB method was found to be suitable as templates for the detection of the viruses by RT-PCR assays. Extraction of total nucleic acid with the modified CTAB was easy, fast, and quite economical, taking less than 2 h to extract total nucleic acid from 6 strawberry samples. This procedure was suitable for simultaneous detection of two RNA viruses (SMYEV and SMoV) and one DNA virus (SVBV) by RT-PCR assay (Chang et al. 2007).

In another investigation, a reliable multiplex RT-PCR assay was developed for the detection of three strawberry viruses, namely *Raspberry ringspot virus* (RpRSV), *Tomato bushy stunt virus* (TBSV), and *Strawberry latent ringspot virus* (SLRSV), using a plant internal amplification control (PIAC). The multiplex PCR assay was evaluated using two or three cDNA templates at different concentrations to simulate field infections. The primers for different target viruses were employed in the multiplex PCR assay, and the primer interaction was found to be the crucial factor affecting the sensitivity and reliability of the assays. When the templates were present in similar concentrations, the detection limits for all the targets were comparable to those of individual viruses. The advantages of the multiplex PCR assay were shown to be mitigation of primer interaction, elimination of PCR inhibitors including reducing primer concentration (especially those with preferential amplification), and extension temperature. Use of bovine serum albumin (BSA), in combination with increasing the extension time and PCR cycles, resulted in the efficient detection of RpRSV, SLRSV, and TBSV in strawberry extracts by multiplex RT-PCR with the same level of specificity and sensitivity as the simplex PCR format. The multiplex RT-PCR assay also offered the advantages of saving time and a lower cost of testing compared to the single PCR format (Wei et al. 2008).

Black raspberry decline-associated virus (BRDaV) was consistently isolated from infected black raspberry (*Rubus occidentalis*) plants in Oregon, USA. The RT-PCR protocol was developed to detect BRDaV in *Rubus* plants that showed decline symptoms. *Black raspberry necrosis virus* (BRNV) shared many biological properties with BRDaV, which was identified as a strain of BRNV reported by the Scottish Crop Research Institute, UK. Double-stranded RNA extraction from symptomatic plants revealed the presence of two high-molecular-weight bands. Sequence analysis indicated that a novel virus was involved in black raspberry decline (BRD). For the detection of BRNV strain, two sets of primers were employed in tandem. Primer set 1 amplified a 417 bp fragment

of RNA dependent RNA polymerase (RdRp) region of RNA1 and primer set 2 amplified a 350 bp fragment further downstream at the 3' end of RdRp region. BRNV was detected in all samples (9) from field-grown black raspberry plants by amplifying the BRNV-specific PCR products. All sequenced BRNV amplicons were virus-specific. Adoption of Koch's postulates applicable for obligate pathogens indicated that BRNV was the causal agent of black raspberry decline disease (Halgren et al. 2007).

Infection of potatoes by *Potato virus Y* (PVY), *Potato virus X* (PVX), and *Potato leaf-roll virus* (PLRV) occurs singly or in combination in potato-growing countries all over the world, causing significant losses. Detection of these viruses in a single test is desirable to reduce the time and cost of testing required for detection of viruses individually. Three specific primer pairs were designed to amplify the full length of coat protein gene of each virus, 810 bp for PVY, 700 bp for PVX, and 548 bp for PLRV, in a multiplex (m) RT-PCR format. Specificity of the three primer pairs could be confirmed by applying nested PCR format, using three internal specific primers designed to amplify 480 bp, 360 bp, and 420 bp from the internal sequence of the CP gene of PVY, PVX, and PLRV respectively. The mRT-PCR protocol detected all three viruses in a single reaction mixture in naturally infected field-grown potato samples. The nested PCR format confirmed the identity of the virus without the need for sequence analysis. The multiplex RT-PCR followed by nested PCR assay provided higher levels of sensitivity and specificity in detecting PVY, PVX, and PLRV in a single reaction, compared to conventional RT-PCR assay. The multiplex RT-PCR format might be useful for quarantine and certification programs where results might be required rapidly (Shalaby et al. 2002).

Potato virus Y (PVY) strain PVY^{NTN} contains three major recombinant junctions (RJs) at the sites HC/Pro.P3, NIa, and the C-terminal region of coat protein (CP) gene. Specific primer pairs were designed to target the RJs so that the sense and antisense primers completely matched the nucleotide sequences at either side of the RJs. When all three primer pairs were employed simultaneously in a multiplex RT-PCR assay, three fragments of 641, 448, and 290 bp size were amplified exclusively from the recombinant PVY^{NTN} strain. On the other hand, only a single fragment of 641 bp was amplified from PVY^O isolate. Further, no amplification occurred from nonrecombinant PVY including PVY^O and North American (NA-) PVY^{NTN} strains, indicating the specificity of the primers developed in this investigation (Nie and Singh 2003).

Strains of *Potato virus Y* (PVY) were differentiated into two main groups: the strains inducing leaf necrosis on tobacco indicator plants (European PVY^N, European PVY^{NTN}, PVY^{N:O}, North American PVY^N, North American PVY^{NTN}) and the strains that do not induce leaf necrosis in tobacco. Accordingly, North American isolates were designated NA-PVY^N and NA-PVY^{NTN}, while European isolates were referred to as PVY^N and PVY^{NTN}. New PVY strains and variants emerged later, resulting in rejection of seed lots during the process of certification because the available diagnostic methods could only detect some combinations of mixed strain infections. A single multiplex RT-PCR assay that could assign PVY strain type and detect mixed infections with respect to the major strain types was therefore developed. The multiplex RT-PCR assay identified 17 samples with strain mixtures from 119 isolates characterized earlier. All the 11 PVY^O isolates produced amplicons of 269 and 689 bp, whereas 13 PVY^O isolates produced a single 328 bp product representing NA-PVY^{NTN}. A separate complementary PCR was used for verifying the NA-PVY^{NTN}. Using this multiplex assay, it was possible to detect each of the six possible two-way combinations of the major strains and also three of the four possible three-way combinations of PVY^{N:O} + PVY^{NTN} + PVY^O. The protocol

developed in this investigation might aid in the characterization of major PVY strains assay. The multiplex assay was found to be robust and inexpensive, and had been successfully transferred to and validated in four additional laboratories, revealing its usefulness for virus-free tuber production (Lorenzen et al. 2006).

A multiplex RT-PCR system was developed for simultaneous detection of five potato viruses, *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato virus A* (PVA), *Potato virus S* (PVS), and *Potato leafroll virus* (PLRV), using the 18S rRNA as the internal control which was a factor of five times more sensitive than *nad2* mRNA used in earlier investigations. The optimized multiplex RT-PCR assay was compared to DAS-ELISA test for their levels of sensitivity in detecting PVX. The sensitivity of RT-PCR assay was 100 times greater than that of DAS-ELISA test. The comparative efficiency of detection of potato viruses in 20 field potato samples by multiplex RT-PCR and DAS-ELISA procedures was assessed. DAS-ELISA detected PVY in one sample and PVS in 13 samples. PVA, PVX, and PLRV were not detected in any sample with DAS-ELISA. On the other hand, multiplex RT-PCR assay confirmed the presence of PVY and PVS in samples detected by DAS-ELISA, and also detected PVX in one sample and PVS in another sample that gave a negative result with DAS-ELISA. The optimized multiplex RT-PCR format detected as little as 0.5 pg of PVX virions, indicating its greater sensitivity (100-fold) than the DAS-ELISA test. The results showed that multiplex RT-PCR assay is a convenient and less expensive system for simultaneous detection of five economically important potato viruses in a single assay (Du et al. 2006).

The effectiveness of a multiplex RT-PCR for the simultaneous detection of *Potato yellow vein virus* (PYVV), *Tobacco rattle virus* (TRV), and *Tomato infectious chlorosis virus* (TICV) in potato plant tissue extracts, including a plant internal amplification control (PIAC), was assessed. The total RNA was extracted using an RNeasy® Plant Mini Kit as per the instructions of the manufacturer. The addition of bovine serum albumin (BSA) at a final concentration of $1\mu\text{g}\mu\text{L}^{-1}$ in reverse transcription (RT) increased the detection limit of TICV, PYVV, and TRV by a factor of 2, 4, and 8, respectively. The detection limit of the multiplex RT-PCR assay was evaluated by testing serially diluted individual and mixed cDNA templates. The multiplex RT-PCR detected the viruses at dilutions of 1:2000, 1:1800, and 1:4000 for PYVV, TICV, and TRV, respectively, when individual cDNA samples were tested using virus-specific and PIAC primers. On the other hand, the detection limits were 1:2000, 1:500, and 1:2000 for PYVV, TICV, and TRV, respectively, when three cDNA samples were mixed. In multiplex PCR assay, preferential amplification of some targets (PYVV and PIAC primers) occurred when an equal primer concentration was used, while amplification of other targets (TICV and TRV primers) was suppressed. By optimizing the primer concentrations of the targets, it was possible to obtain optimal amplification of all four targets. The multiplex PCR was found to be reliable and sensitive for simultaneous detection of three potato viruses; an internal control was used to determine the effects of inhibitors on PCR amplification and to eliminate false-negative results (Fig. 7.11). The detection sensitivity of multiplex RT-PCR was in equivalence to simplex RT-PCR format, resulting in cost-effective and time-saving detection of potato viruses (Wei et al. 2009).

Potato plantlets singly infected by *Potato virus A* (PVA), *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Potato leafroll virus* (PLRV) and mix-infected by *Potato virus M* (PVM), PVS, and PVY were grown in the MS medium amended with different concentrations ($0\text{--}200\text{mgL}^{-1}$) of ribavirin, an antiviral chemical. Adverse effects of ribavirin on plant height and fresh weight were progressively increased with



Figure 7.11 Detection of *Potato yellow vein virus* (PYVV), *Tomato infectious chlorosis virus* (TICV), and *Tomato rattle virus* (TRV) by the multiplex PCR assay using serially diluted individual and mixed DNA templates. Lanes 1–7, 8–14, and 15–21: PYVV at original 1:10, 1:100, 1:500, 1:1000, 1:2000, and 1:4000. TICV: original, 1:10, 1:100, 1:200, 1:400, 1:800, and 1:1600. Lanes 22–29: serially diluted samples of three mixed cDNA species: original, 1:10, 1:100, 1:500, 1:1000, 1:2000, 1:4000, and 1:8000. M: molecular size standards (100bp). Source: Wei et al. (2009). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

an increase in ribavirin concentration up to 150 mg L^{-1} , and the plantlets could not survive when the maximal concentration of 200 mg L^{-1} was used. Ribavirin was more efficient in eliminating PVA, PVM, PVS, and PVX than PVY and PLRV as revealed by ELISA tests. Healthy plantlets could be generated with high frequency (up to 100%) by culturing the plantlets in MS medium containing $75\text{--}150 \text{ mg L}^{-1}$ ribavirin after 2–3 subcultures. On the other hand, PVY- and PLRV-free plants could be obtained by using $75\text{--}150 \text{ mg L}^{-1}$ ribavirin in the medium for growing plantlets after 3 subcultures. Quantitative RT-PCR (qPCR) analysis showed that virus content could be reduced by ribavirin. Healthy seedlings were generated from infected stocks at the first or end of propagation and no viruses could be detected at the post-eradication stage. Ribavirin treatment did not seem to induce any genetic variation in potato plantlets. The results indicated that ribavirin treatment *in vitro* could be an effective approach for elimination of viruses in the propagation of potato (Yang et al. 2014).

Garlic crops are infected by *Onion yellow dwarf virus* (OYDV) and *Garlic latent virus* (GarLV) (synonym for *Shallot latent virus* (SLV)), capable of causing significant losses. A duplex RT-PCR assay was developed for the simultaneous detection of OYDV and GarLV in total RNA extracts obtained from garlic leaves or cloves using virus-specific primer pairs. The assay detected both viruses in all ten selections of garlic plants grown in the field. GarLV was detected in all samples while OYDV, although detected in all ten selections, could be detected in only four of five samples from two selections. No amplification occurred in samples from healthy control garlic plants. As the viruses were not present in equal concentrations in infected plants, a two-fold increase in GarLV primers improved its detection by duplex RT-PCR assay. The sensitivity of duplex RT-PCR assay of OYDV and GarLV was not reduced, as detection of both viruses could be achieved to the same level as in simplex RT-PCR format. Duplex RT-PCR assay was efficient in detecting both viruses in samples from leaves and cloves, saving time and cost of testing compared to standard RT-PCR format (Majumder et al. 2008).

Black pepper crops are seriously infected by stunt disease with which *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) are associated. A single-tube multiplex (m) RT-PCR assay was effective in simultaneously detecting both CMV and PYMoV in the mother plants. Two amplicons of expected sizes – 650 bp for CMV and 450 bp for PYMoV – were amplified from the total nucleic acids extracts of infected plants used as templates. No amplification of the virus-specific fragments was seen in the extracts from healthy pepper plants. The mRT-PCR format was found to be

effective in detecting both viruses in the nursery and field-grown plants and was amenable for large screening of pepper samples rapidly (Bhat and Siju 2007).

A multiplex RT-PCR assay was developed for the detection of *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TuMV), *Konjac mosaic virus* (KoMV), and *Zantedeschia mild mosaic virus* (ZaMMV) in the calla lily plants in Taiwan. Based on the sequences of 3' terminal region of respective viruses, specific primers were designed. In addition, a primer pair specific to plant mitochondria *nad5* mRNA was used to produce a 185 bp fragment as an internal control to prevent false-negative results. The primer pairs TuRD/TuCPF1 for TuMV, ZUNR1/ZUNF2 for ZaMMV, DRO/DF2 for DsMV, ZRD/ZF3 for KoMV, and mtR1/mtF2 for *nad5* mRNA employed in this protocol amplified fragments of 1077, 792, 457, 260, and 185 bp, respectively, when the individual clones were tested. They were specifically amplified by the primer mixture of TuMV, ZaMMV, DsMV, KoMV, and mt control. Generally the yield of amplicons for the viruses DsMV, KoMV, and mt was less in multiplex PCR compared with simplex PCR format, and all tested primers were specific to their targets. Optimization of primer concentration of individual viruses was therefore carried out for amplification of expected RT-PCR products for four viruses and mt target to the desired level. Total RNAs purified from virus-infected plants were used directly or mixed in different combinations. The specificities of primers were verified by employing simplex and multiplex PCR formats. Samples of fully expanded leaves of calla lily randomly collected from fields were tested by multiplex RT-PCR assay. Calla lily samples showed single-virus infection to an extent of 72% (35/50), while two-thirds (23/36) of plants were infected by two or more viruses. Using indirect-ELISA format, virus infection in only 50% of plants could be detected by employing potyvirus-specific antibodies. Multiplex RT-PCR protocol developed in this investigation was suitable for sensitive, rapid, and reliable detection of potyviruses infecting calla lily plants, particularly for producing virus-free stocks by certification programs (Hu et al. 2010).

The results of multiplex RT-PCR indicated that the expected RT-PCR products could be specifically amplified and identified on the basis of their molecular sizes. Sensitivity of detection by RT-PCR assay was greater (25–625 times) than that of indirect ELISA test depending on the virus. During field surveys multiplex RT-PCR assay was able to detect more single- as well as mixed-infection samples than indirect ELISA format. The multiplex RT-PCR protocol was found to be simple, rapid, and reliable for simultaneous detection of the four potyviruses infecting calla lily plants, and the assay could save cost and time when testing large numbers of samples (Hu et al. 2010).

Cymbidium mosaic virus (CymMV) and *Odontoglossum ringspot virus* (ORSV) are important viruses infecting orchids, inducing considerable adverse impacts on production. A multiplex RT-PCR assay was developed for the detection of CymMV and ORSV. These primers amplified the respective viral coat protein (CP) genes. In addition, a primer pair derived from the plant mitochondrial NADH dehydrogenase gene (*nad5*) was tested for the amplification of *nad5* mRNA as internal control in the multiplex RT-PCR. The fragment of internal control mRNA was consistently amplified from total RNA of healthy and infected plant extracts. The primer pair for CymMV CP-F1/CymMV CP-R1 and the primer pair for ORSV CP-F1/ORSV CP-R1 amplified a 669 bp fragment and 474 bp fragment in the RT-PCR assay, respectively. The amplification efficiency of RT-PCR assay was not affected when the same amount of CymMV and ORSV RNAs were added alone or together to the plant total RNA. The specificity of three designed primer pairs for CymMV, ORSV, and *nad5* mRNA was confirmed by simplex

and multiplex RT-PCR assays. The detection sensitivity of multiplex RT-PCR was 1 pg for CymMV and 10 pg for ORSV, regardless of the presence of one or two viruses in the samples. The presence of CymMV and ORSV in *Phalaenopsis* orchids sold in a flower market was detected in three infected plants. The expected cDNA fragments of the viruses and internal control were amplified in infected samples, indicating the applicability of the multiplex RT-PCR assay for detection of viruses infecting orchid plants (Lee and Chang 2006). The RT-PCR assay was applied to determine the involvement of *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV) and tospoviruses in leaf yellowing of *Phalaenopsis* orchid plants. ORSV and CymMV were efficiently detected simultaneously by RT-PCR from total RNA extracts, using commercial kits, the simple direct-tube (SDT) technique, and the primers designed in this investigation. The presence of ORSV and CymMV was detected in 55% and 34% of plants, respectively. Severe symptoms were observed in plants infected with both viruses. Using SDT technique, RNA could be extracted effectively and the RT-PCR protocol was cost-effective for screening for disease incidence in commercial orchards intended for production of high-quality virus-free plants. Furthermore, RT-PCR protocol detected the presence of *Orchid fleck virus* (OFV) in Australian orchids by employing virus-specific primers. None of the samples showed infection by the tospovirus *Capsicum chlorosis virus* (CaCV) reported earlier on *Phalaenopsis* orchid plants (Yamane et al. 2008).

As sugarcane is a vegetatively propagated crop, screening for viruses infecting sugarcane is important to restrict the introduction and/or prevention of virus disease spread during germplasm exchange and seed cane production. In order to assess the incidence of *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV), and *Sugarcane streak mosaic virus* (SCSMV) on cultivated sugarcane and noble canes, a survey was undertaken. In the one-step RT-PCR assay, virus-specific primers generated amplicons of expected size of 900 bp and 5850 bp for SCMV and SrMV, respectively. The absence of an amplicon specific for SCSMV indicated that the virus was absent in the samples tested. The amplified RT-PCR products 33 and 10 for SCMV and SrMV CPs, respectively, were directly sequenced to confirm the specificity of the assay. The nucleotide sequences of the RT-PCR products shared more than 76% homology with CP gene of the corresponding virus. RT-PCR data indicated that most of the hybrid and noble canes in southern China were commonly infected by either SCMV or SrMV singly or doubly with both viruses (Xu et al. 2008a).

In a later investigation, a quadruplex RT-PCR assay was developed to detect *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV), *Sugarcane streak mosaic virus* (SCSMV), and *Sugarcane yellow leaf virus* (SCYLV). Virus-specific primers were designed for simultaneous detection and differentiation of SCMV-F1/R1, SrMV-F/R, SCSMV-F/R, and SCYLV-F/R of these viruses. The primers specific for SCMV, SrMV, SCSMV, and SCYLV amplified DNA fragments of 720 bp, 860 bp, 1160 bp, and 512 bp, respectively, and each of the RT-PCR bands produced using these primers was observed clearly following gel electrophoresis. No significant primer pair interactions or primer-dimer formation was observed. The best cycling conditions for the quadruplex RT-PCR assay were one cycle of 50°C for 30 min and 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min in a quadruplex assay. All four viruses could be detected using sample dilutions up to 10^{-7} , 10^{-8} , 10^{-4} , and 10^{-5} for SCMV, SrMV, SCSMV, and SCYLV, respectively, and virus-specific bands were visualized after gel electrophoresis. All four target viruses were detected in 202 field samples collected throughout the entire growing season. Most of the positive samples showed the presence

of single virus and 29 samples contained mixed infection by 2 viruses. The multiplex RT-PCR may be preferred because of reduced cost, labor, and time required compared to simplex RT-PCR assays (Xie et al. 2009).

7.2.3.3 Combination of RT-PCR with Other Diagnostic Assays

The sensitivity and specificity of RT-PCR assay may be enhanced substantially by combining antibody binding with virus protein followed by PCR amplification. Immuno-PCR is a highly sensitive procedure in which a DNA fragment is linked to an antigen–antibody complex using protein A (linking antibody) and streptavidin (attached to DNA). Protein A and streptavidin have a strong affinity to each other. This complex is then bound to a biotin-labeled DNA sequence which is subsequently amplified by PCR. The sensitivity of immuno-PCR assay may be about 10^5 times more than that of ELISA test (Sano et al. 1992). In immunocapture (IC), the virus is captured (concentrated) by using specific antibody and the specific RNA sequence of the captured virus particles is amplified. Woody plants contain large quantities of phenolic compounds or polysaccharides that can interfere with nucleic acid template preparation, resulting in inhibition of PCR amplification. The virus particles may be selectively concentrated by applying the immunocapture procedure, prior to PCR amplification (Nolasco et al. 1993). The availability of antigen-specific antibody is the only requirement for performing immuno-PCR assay, whereas nucleic acid sequence data is also needed for immunocapture (IC-) RT-PCR procedure. These techniques have been demonstrated to be effective for sensitive and reliable detection of several plant viruses in symptomatic and/or asymptomatic planting materials.

Plum pox virus (PPV) could be efficiently and reliably detected by employing IC-RT-PCR assay. The sensitivity of this technique was about 250 times greater than the standard PCR format (Wetzel et al. 1992). In a later investigation, the higher level of sensitivity of detection of PPV by IC-RT-PCR was revealed by the positive results for the presence of PPV in 31% of ELISA-negative leaf samples and in 23% of ELISA doubtful samples from field-grown plants, collected during field surveys taken up to assess the extent of PPV incidence. In addition, the identity of majority of isolates was determined as M-serotype of PPV analysis of PCR products by random fragment length polymorphism (RFLP) (Varveri and Boutiska 1998).

In another study, the presence of PPV was detected in 92–100% of root samples from plum trees by IC-RT-PCR assay. However, ELISA test could only detect PPV in 36–65% of the root samples, indicating that bulked samples from fibrous roots were more suitable tissues for the detection of PPV since the frequency of detection by IC-RT-PCR format was not significantly different from that of ELISA test, probably due to uneven distribution of PPV in aerial plant tissues (Adams et al. 1999).

Plum pox virus (PPV) was detected in wild apricot and cultivated plum maintained in a germplasm collection in Kazakhstan using triple-antibody sandwich (TAS-) ELISA format and IC-RT-PCR assays. The universal PPV-specific MAb5B was used as the secondary antibody and the primers P1 and P3 were employed for amplification of a 243bp fragment of the coat protein (CP) coding region. The 836bp fragment in the P3-6K1 region of the genome was amplified using the primers PC1 and PP3. Clarified plant extracts were incubated in microtubes precoated with the PAbA1PPV. The IC-RT-PCR reaction was carried out providing the conditions essentially as for RT-PCR assay. The PPV-specific 243bp fragment and the 836bp product were amplified from samples using RT-PCR assay. In several samples, the 243bp product appeared as weak

ethidium bromide-stained band. IC-RT-PCR assay targeted the PPV CP region, allowing the amplification of 243 bp fragment in nine wild apricot accessions, including eight ELISA-negative samples and one ELISA-positive ASR2. RFLP analysis of the amplified 243 bp product following treatment with endonucleases *AluI* and *RsaI* was performed in plum and apricot samples. An amplified 836 bp cDNA fragment derived from the P3-6K1 coding region of both plum and apricot isolates had restriction profiles typical of strain D. Nucleotide identities of 99–100% were observed for the 243 bp fragment of the Kazakhstan isolates when compared with the corresponding regions of strain D, and 94–95% identity with strain M (Spiegel et al. 2004).

Apple and pear cultivars are frequently infected by *Apple stem pitting virus* (ASPV) and *Pear vein yellows virus* (PVYV), which are closely related. These viruses were sap-transmitted to the assay host *Nicotiana occidentalis* ssp. *oblique*, which responded with characteristic symptoms early. The effectiveness of IC-RT-PCR assay was evaluated for its sensitivity, specificity, and reliability, as the standard RT-PCR format did not provide reproducible results. IC-RT-PCR was also unable to provide detection of the viruses to the desired level. A bacterially expressed protein employing ASPV CP gene as chimerical protein was used as an immunogen. Antiserum (647) produced against this protein was utilized for capturing the ASPV virions followed by RT-PCR procedure. When used as a trapping antibody, the antiserum (647) effectively trapped the virus particles and reacted specifically in immunosorbent electron microscopy (ISEM) and in Western blot. The antibodies positively reacted with ASPV- and PVYV-infected leaf extracts of *N. occidentalis* in PTA-ELISA format. The results indicated that the sensitivity of IC-RT-PCR assay employing antiserum 647 was high enough for its application for detection of ASPV in apple and related viruses in pear in a certification program for producing virus-free planting materials (Jelkmann and Keim-Konrad 1997).

The effectiveness of application of ribavirin (1- β -ribofuranosyl-1, 2, 4-triazole-3-carboximide) with antiviral activity on the elimination of *Plum pox virus* (PPV), causing sharka disease from *Prunus domestica* cultivar Cacanska shoots (grown under tissue-culture conditions), was assessed. The DAS-ELISA test and IC-RT-PCR assay were employed to assess the health status of the shoots two years after treatment with ribavirin. The percentages of PPV-free plants increased with increase in the concentration of ribavirin used for treatment. The DAS-ELISA test was unable to detect low concentrations of PPV present in the treated plants, and higher percentages of plants had to be considered as PPV-free. However, IC-RT-PCR assays showed that only 15.38% and 16.5% of plants treated with 40 and 60 mg L⁻¹ of ribavirin, respectively, were PPV-free, whereas 76.9% and 100% of treated plants were indicated to be virus-free by the results of DAS-ELISA tests. The results clearly revealed the higher level of sensitivity of IC-RT-PCR assay, facilitating the detection of low virus titers in plants treated with the antiviral chemical. The results of IC-RT-PCR assay could be relied upon for the programs aiming to produce virus-free stocks in vitro (Paunović et al. 2007).

Immunocapture (IC-) RT-PCR assay was applied for the detection of *Citrus tristeza virus* (CTV), the citrus decline strain. This format combines the specificity of PCR and simplicity of ELISA test and consequently sensitivity of detection was enhanced significantly. The sequences of the CP gene of CTV strains were used to design the primers and fluorescent probes. Intensity of fluorescence was assessed after performing PCR amplification, instead of electrophoretic analysis, after carrying out PCR amplification. The IC-RT-PCR assay could detect CTV strains in all ELISA-positive samples and also in additional 20% of the samples that were ELISA-negative, indicating the greater

sensitivity of this protocol (Nolasco et al. 1997). In a later investigation, primers corresponding to both ends of the CP gene were designed for use in the IC-RT-PCR assay for the detection of CTV in planting materials. The extracts from the bark tissues of infected citrus plants were tested. The presence of CTV was detected in all samples collected from orchards in the coastal regions of Croatia-Montenegro and Albania (east Adriatic region), indicating the sensitivity and specificity of IC-RT-PCR format. Single-strand conformation polymorphism (SSCP) analysis revealed the coexistence of different genomic variants within isolates of CTV. The results suggested that mixed infections due to variants might result in variation of biological characteristics of the isolates. Regardless of absence of visible symptoms in field trees, severe CTV variants appear to be widespread and present for a long time in the Adriatic region, making it a reservoir of severe strains of CTV (Cerni et al. 2009).

Grapevine plants are infected by several virus diseases, of which grapevine leafroll disease complex caused by genetically distinct viruses designated *Grapevine leafroll-associated virus 1-9* (GLRaV-1-9), was found to be economically most important. These viruses could be detected by PCR assays. Immunocapture (IC-) RT-PCR procedure was applied for efficient and sensitive detection of *Grapevine leafroll-associated virus 1* and 3 (GLRaV-1 and -3) by using specific primers. The presence of these two viruses was detected both in infected grapevine plants and infective mealy bug (*Planococcus ficus*) (Acheche et al. 1999). In another investigation, specific primers were designed based on the sequences of conserved heat shock protein (HSP 70) region of Closteroviruses for the detection of GLRaV-1. No amplification occurred in extracts of grapevine tissues infected by GLRaV-2, -3, and -4, indicating the specificity of the primers for GLRaV-1. This protocol was about 125 times more sensitive than ELISA test (Sefc et al. 2000).

The usefulness of IC-RT-PCR assay for the detection of other viruses infecting grapevines has been reported. *Arabis mosaic virus* (ArMV) and *Grapevine fan leaf virus* (GFLV) were detected simultaneously by IC-RT-PCR procedure with higher level of sensitivity and specificity. A single pair of degenerate primers designed from the sequences coding for movement proteins of the target viruses was employed in the procedure developed in this investigation. The IC-RT-PCR format was 10 times more sensitive than ELISA tests (Wetzel et al. 2002). Isolates of *Grapevine virus A* (GVA) belonging to *Vitivirus* (*Flexiviridae*) were detected and differentiated based on the type of symptoms induced in the indicator host *Nicotiana benthamiana* inoculated by mechanical transmission. DAS-ELISA test detected GVA in 14.2% of the grapevine samples tested. Using RT-PCR or IC-RT-PCR formats, a fragment of 430bp of the coat protein (CP) gene was amplified from infected plant extracts by employing the primer pair H587/C995. The identity of the virus isolates could be established by cloning and sequencing of amplicons, which showed that GVA isolates shared 100% nucleotide homology with each other and they were identical at a molecular level (Misbeh et al. 2007).

ELISA formats have been extensively applied for the detection of several viruses in planting materials; however, the levels of sensitivity for detection of certain strains of viruses that are present in low concentrations, especially in dormant trees, were not found to be satisfactory. In such cases, PCR-ELISA (PCR-microplate hybridization) combination was effective and more sensitive in detecting virus strains that were undetectable by standard ELISA format. PCR-ELISA combination was employed for the simultaneous detection of *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV). Multiple alignments of PNRSV and ApMV CP genes were made to select PCR primers in the regions conserved between two viruses. Virus-specific

oligonucleotides were used as capture probes in the PCR-ELISA method. The selected primers were effective for the detection of both PNRSV and ApMV, as indicated by the analysis of a range of isolates of both viruses. Simultaneous application of the specific capture probes of both viruses resulted in the improvement of levels of sensitivity of detection of PNRSV and ApMV by PCR-ELISA combination (Candresse et al. 1998). Detection of *Plum pox virus* (PPV), *Cherry leafroll virus* (CLRV), and *Citrus tristeza virus* (CTV) could be effectively accomplished by using PCR-ELISA combination (Olmos et al. 1997; Rowhani et al. 1998). *Grapevine fan leaf virus* (GFLV) transmitted by the nematode vector *Xiphinema index* is one of the economically important viruses with the potential for ruining grape production. *Chenopodium quinoa*, the assay host plant, was inoculated by sap transmission for rapid multiplication of the virus. GFLV was purified from *C. quinoa* and the antiserum positively reacted with Tunisian grapevine samples in DAS-ELISA tests. Oligoprobe-RT-PCR was employed for the detection of GFLV in ELISA-positive samples. The assay amplified a 606bp region of GFLV CP gene sequence. PCR products amplified by H2042 and C2647 primers were digested with endonuclease *AluI*. Two strains of GFLV were precisely identified based on the restriction profiles. RT-PCR-RFLP combination of techniques has the potential for providing reliable data for epidemiological and resistance-breeding investigations (Fattouch et al. 2005).

The TitanTM one-tube RT-PCR System (Boehringer Mannheim, Germany) was applied as per the manufacturer's recommendations, using the primer pair ASGV4F/ASGVR for the detection of *Apple stem grooving virus* (ASGV). The ASGV viral replicase gene sequences were directly amplified from diluted plant sap of either ASGV-infected *Chenopodium quinoa* (assay host) or sap from leaves or bark tissues of apple. The RT-PCR system was used for the first strand synthesis and a mixture of *Taq* and PWO DNA polymerase for the PCR step. The RT-PCR products were hybridized with both a biotin-labeled capture probe linked to streptavidin-coated microtitre plate and a digoxigenin (DIG-) labeled detection probe. The complex was detected with an anti-DIG-conjugate-labeled with alkaline phosphatase. It was possible to detect as little as 400fg of ASGV in plant tissue extracts spiked with purified virus preparation. Using the protocol developed in this investigation, the primers ASGV4F/ASGV4R specifically amplified the virus in ASGV-infected apple trees from different locations. There was no signal when the amplification products obtained from primers targeting viral coat protein (CP) region of ASGV genome or with primers specific for *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV) were used. The diagnostic procedure employed in this investigation combined the power of PCR to increase the number of copies of the target gene sequence, the specificity of DNA hybridization, and the ease of colorimetric detection and sample handling in microplates. This protocol is suitable for large-scale application in indexing planting materials as required for certification programs, since about 100 samples could be processed simultaneously in one microplate (Marinho et al. 2003).

Detection of *Potato leafroll virus* (PLRV) directly in dormant potato tubers by ELISA was found to be unreliable. Furthermore, performing ELISA tests on leaf extracts of sprouted tubers was labor-intensive and required at least 5 weeks to obtain results. A gel-free reverse-transcription (RT-) based fluorogenic detection method was therefore designed for PLRV detection in dormant potato tubers. A PLRV sequence-specific oligonucleotide (Roche Molecular Systems Inc., CA) containing a 5'-terminal reporter fluorescein and a 3'-terminal rhodamine "quencher" was specifically degraded during

amplification, resulting in a relative increase in reporter-associated fluorescence. This system was coupled to immunocapture (IMC) of PLRV from tuber sap by paramagnetic beads coated with an antiserum against the virus. The addition of cell-wall-degrading enzymes, cellulose, and macrozyme to tuber sap was essential to make detection of PLRV possible on tubers after a storage period. By combining IMC with RT-PCR, the detection limit of PLRV in tuber sap was at least 100 times lower compared to conventional ELISA test. Another advantage of IMC/RT-PCR over ELISA test is the possibility of assessing the intact virus, whereas ELISA tests give positive results for the presence of virus protein. The IMC/RT-PCR format was found to be fast and easy to perform and also robust. It showed potential for development into a fully automated system requiring only minimum laboratory manipulations. With this assay procedure, the inspection time of seed potatoes for PLRV could be reduced from 5 weeks to 1 day (Schoen et al. 1996).

Accurate and rapid methods of detection of strains of *Potato virus Y* (PVY) in dormant potato tubers are primarily required for the production of virus-free and high-yielding seed tubers. Although ELISA tests were adopted widely for virus detection, the level of sensitivity of ELISA formats was considered as a limiting factor. An immunocapture (IC) step prior to RT-PCR concentrates virions present in the dilute plant extracts, resulting in enhancement of detection sensitivity. The comparative efficiency of DAS-ELISA and one-step immunocapture (IC-) RT-PCR assay for the detection of PVY was assessed. Both methods detected all isolates tested and showed sensitivities around the expected detection levels attained by them. DAS-ELISA detected PVY up to 10 ng mL^{-1} ; IC-PCR provided a 1000-fold increase in sensitivity over that of DAS-ELISA test (Fig. 7.12). The combination of PCR and ELISA procedures was employed to detect the amplicons generated by PCR amplicons, followed by their adsorption on a microplate wells. The IC-PCR mixture was diluted 200-fold into microplate well and PCR-ELISA was performed to obtain absorbance values from 100 fg mL^{-1} of virus for the IC-PCR product. The IC-PCR-ELISA procedure offered an overall 100,000-fold increase in the sensitivity over DAS-ELISA test. The purified PVY from tobacco extract

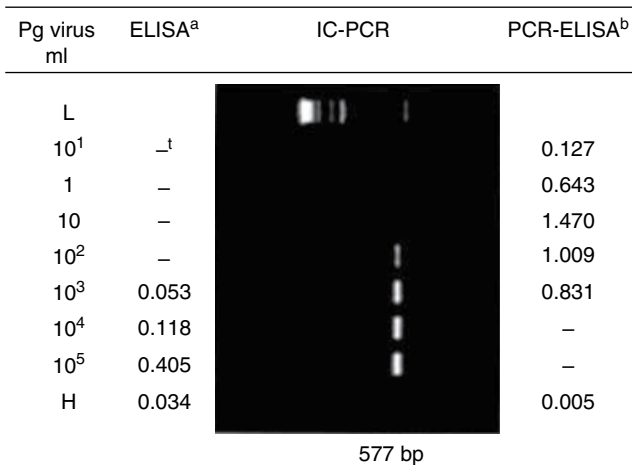


Figure 7.12 Comparative levels of sensitivity of DAS-ELISA, IC-PCR and IC-PCR-ELISA techniques for the detection of *Potato virus Y*. IC-PCR-ELISA was more sensitive than other two techniques tested. Source: Varveri (2000). Reproduced with the permission of Springer Science, Heidelberg, Germany.

was detectable at 100 fg mL^{-1} and PVY was detected in potato sample at a dilution endpoint of 10^{-7} equivalent to 1 ng tissue. Furthermore, this highly sensitive procedure coupled with the print-capture (PC) modification to avoid specific sample preparation was found to be a most practical and useful tool for the detection of PVY in plant samples as well as in single aphid vector of PVY (Varveri 2000). A multiplex assay for the detection of *Tobacco rattle virus* (TRV) and *Potato mop-top virus* (PMTV) in potato tubers was developed by combining PCR with tube fluorescent product detection (TaqMan®). This protocol could be employed in place of two separate assays (an RT-PCR for TRV and an ELISA for PMTV). Forty isolates of TRV and PMTV could be reliably detected with high sensitivity (100–10,000-fold). In addition, the IC-RT-PCR assay offered the advantages of reduction in contamination and elimination of electrophoresis step, in addition to time and cost savings of post-PCR manipulations as compared to standard RT-PCR format (Mumford et al. 2000a).

Potato mop-top virus (PMTV), the type member of the genus *Pomovirus*, causes symptomless infections, making detection and identification of the causal virus difficult. PMTV has a three-partite, single-stranded RNA genome constituted by RNA-1, RNA-2, and RNA-3. The coat protein (CP) gene and read-through domain of RNA-2 and 8K gene and 3'untranslated region of RNA-3 were characterized from 37 PMTV isolates detected in potato tubers from field-grown plants in Finland and a screen house in Latvia. Sequencing and RFLP analysis of PCR amplicons indicated that the majority of isolates infecting tubers comprise restrictotypes RNA-2II and RNA-3B. The infections in 5 lots of 100 asymptomatic minitubers were tested by DAS-ELISA and IC-RT-PCR assays. Fourteen tubers were positive for both assays. These minitubers were grown from in vitro plants of cultivar Saturna. Analysis of PMTV in 37 naturally infected potato tubers revealed 2 distinguishable types of RNA-2 and RNA-3. The combination of RNA-2II and RNA-3B was more common than any other combination, and comprised the typical genomic composition of PMTV strains infecting potato in Finland. The genetic diversity of PMTV was largely due to the existence of two alternative types of RNA-2 and RNA-3. Indexing tubers for PMTV with anti-CP antibodies or using CP gene-specific primers in IC-RT-PCR assay was evaluated for reliable and sensitive detection of PMTV in dormant seed tubers, which were mostly asymptomatic. By pooling 50 sprouts in one sample, large-scale indexing of tuber lots by IC-RT-PCR with higher detection sensitivity could be achieved (Latvala-Kilby et al. 2009).

The detection of plant viruses in cultivated plants as well as in weed plants present in the field and adjacent areas may be useful to reduce the incidence of virus diseases in the principal crops. Plants belonging to 21 different weed species were inoculated with *Potato virus Y* (PVY) by aphids and sap inoculation. DAS-ELISA and IC-RT-PCR assay were employed for detecting PVY in the inoculated weed plants. The presence of PVY in *Erodium cicutarium*, *Geranium pusillum*, *Lactuca serriola*, and *Lamium purpureum* was detected by ELISA test. IC-RT-PCR tests performed on *G. pusillum* and *L. purpureum* failed to detect PVY directly, but PVY could be detected by IC-RT-PCR only in samples picked from tobacco plants back-inoculated with the inoculum of PVY from *G. pusillum* and *L. purpureum*. The failure of IC-RT-PCR assay in detecting PVY in these two weed species might be associated with the difficulty in obtaining quality templates for amplification (Kaliciak and Syller 2009).

Potato virus Y (PVY), considered a serious problem for the seed potato industry, has a maximum allowable infection level of 2% in certified seed tubers. The emergence of genetically recombinant and serologically distinct strains of PVY led to the development

of diagnostic assays to determine strain identity and to detect mixed strain infections more easily, sensitively, and precisely. An assay procedure incorporating the advantages of ELISA and multiplex RT-PCR assay was developed for detection of PVY in single or mixed infections. The virus titer was enhanced by ELISA and then virions were lysed by heating to release the viral RNA for the action of reverse transcriptase. The cDNA product was used as the template for detection of PVY infection by multiplex PCR format, eliminating the need for RNA extraction and handling. The IC-RT-PCR assay was more sensitive in detecting PVY in infected plant sap than multiplex RT-PCR or ELISA alone, while retaining the ability to differentiate strains of PVY that infected potato crops. The IC-RT-PCR protocol developed in this investigation was found to be particularly useful for potato seed tuber certification and diagnostic laboratories as a confirmatory test in conjunction with ELISA tests (Mallik et al. 2012).

Garlic crops are commonly infected by *Onion yellow dwarf virus* (OYDV) along with other viruses belonging to the genera *Potyvirus*, *Carlavirus*, and *Alexivirus*. In order to identify garlic isolates of OYDV-G, causing yellow stripes in garlic plants, a PAb antiserum was used in indirect ELISA format for the detection of OYDV-G in infected plant tissues. IC-RT-PCR assay was performed using the one-tube single-step method to confirm the specificity of virus detection. The virions of OYDV in infected garlic tissues and purified preparations were immunocaptured by specific OYDV-IgG, resulting in the release of viral RNA for use as the template when primers capable of amplifying the central region of OYDV CP gene were employed. A single fragment of c. 601 bp was amplified and recognized as a single band following agarose gel electrophoresis and staining with ethidium bromide. No amplification in extracts from healthy plants was observed. The distribution of OYDV in garlic plants was studied. OYDV concentration was low in young apical leaves and increased up to sixth leaf below the apex. OYDV titer was higher in older leaves compared with apical leaves. Cloves of garlic were found to be more suitable for detection of OYDV than leaf initials of the dormant plantlets (Mahmoud et al. 2007).

7.2.4 Real-Time PCR Assay

Real-time PCR assay overcomes the limitations of conventional PCR format. PCR products are detected by real-time PCR as the reaction is progressing, and therefore post-PCR steps can be avoided. Real-time PCR assay using specific instruments and fluorescent probes provides the advantage of combining the amplification, detection, and quantification of target DNA fragments in a single step. Specific methods employ oligonucleotide probes labeled with a donor fluorophore and an acceptor dye (quencher) that produce a light signal according to the fluorescence resonance energy transfer (FRET) chemistry. The specific methods are TaqMan, molecular beacons, and scorpion PCR procedures. Nonspecific methods involve the use of dyes such as SYBR Green I that emit fluorescent light when intercalated into ds-DNA. Fluorescence is increased in proportion to the amount of total ds-DNA present in the reaction. Real-time PCR permits the increase of specific PCR amplicons to be monitored during successive amplification cycle. A pair of PCR primers and an internal hybridization oligonucleotide probe provided dual specificity. TaqManTM (PE Biosystems) chemistry utilizes the 5' → 3' nuclease activity of *Taq* DNA polymerase to cleave specific probe which is labeled with a 5' reporter (FAM) and 3' quencher (TAMAR) fluorescent dye during strong elongation of target sequence. A detectable fluorescent signal is generated during amplification.

The PCR assay involves annealing of the probe to a complementary strand of an amplified product, followed by cleaving of the probe by *Taq* polymerase during extension of one of the primers and the dye molecules are displaced and separated. After separation, the electronically excited reporter cannot be suppressed by the quencher dye. This leads to variation in the green emission intensity, depending on the concentration of PCR amplicons in the reaction. The PCR cycle number at which the fluorescent signal (emission) exceeds a certain background fluorescence level is known as the threshold cycle (Ct), and is directly proportional to the amount of target DNA present in the sample. Fluorogenic probes may be labeled with different distinguishable reporter dyes to amplify and detect two or more distinct sequences in a single PCR reaction tube without the need for melting curve analysis. Real-time PCR systems are fully automated with “closed tube” product detection, eliminating the risk of cross-contamination to the maximum extent. Another advantage of real-time PCR assay is the possibility of testing as many as 96 samples at a time without the need for post-PCR manipulations. However, the requirement of expensive fluorescent probes and instruments limit the feasibility of using the real-time PCR procedure in many laboratories (Belgrader et al. 1999). Real-time PCR technique has also been applied for the detection of fungal and bacterial plant pathogens in seeds and propagules.

Real-time PCR assay was shown to be very efficient and precise in detecting grapevine viruses in planting materials in Italy. A 1157 bp fragment of *Grapevine fan leaf virus* (GFLV) RNA-2 coat protein gene was amplified. A fluorescent scorpion probe based on the highly conserved CP region was employed for the detection of GFLV (Finetti-Sialer and Ciancio 2005). Different isolates of *Grapevine leafroll-associated virus 1, 2, 3, 4, and 9* (GLRaV-1, -2, -3, -4, and -9) were collected from South Africa, Europe, Australia, Asia, Latin America, and the USA. TaqMan® probes and primers that targeted the regions with 100% sequence identity to the GFLV isolates were designed. Real-time TaqMan® and conventional RT-PCR assays were compared for their efficiency in detecting GLRaVs in purified total RNA and crude extracts from test plants. TaqMan® RT-PCR format was more sensitive than the conventional one-step RT-PCR for detecting different isolates of GLRaV-1, -2, -3, -4, -5, and -9 in purified total RNA preparations and also in crude extracts of infected grapevine tissues from different locations. TaqMan® real-time RT-PCR format provided sensitive, reliable, and specific detection and differentiation of the isolates of grapevine leafroll viruses (Osman et al. 2007).

Mixed infections of grapevines by viruses and phytoplasmas causing grapevine yellows disease (GYs) became a serious threat to grapevine production in Italy. A method was developed for multiple detection of *Grapevine leafroll-associated virus 1* and *2* (*Ampelovirus*) and *Grapevine virus A* (GVA) (*Vitivirus*) as well as Flavescence dorée (FD) and Bois noir (BN) phytoplasmas using the same crude extract as template. Real-time RT-PCR assay was applied for the detection of FD, while real-time nested PCR assays following RT-PCR was used for detection of BN, GLRaV-1 and -3, and GVA. The sap was prepared by semi-automatic maceration requiring minimal time and effort, consisting of a tissue grinding step in carbonate buffer and boiling step in glycine buffer. Real-time RT-PCR for FD phytoplasma detection was five orders of magnitude more sensitive than RT-PCR method used previously. For the detection of BN, the real-time RT-PCR assay required a nested step to achieve high sensitivity because of possible low concentration of the template in the host plant tissue. The TaqMan fluorogenic assay developed for the detection of FD and BN was optimized for GLRaV-1, -2, and -3 and GVA detection by designing a real-time nested PCR format

following a one-tube RT-PCR. As grapevine viruses had RNA genomes and were strictly localized to the phloem (as for phytoplasmas), sampling could be done from the same tissue and RT-PCR allowed use of the same tissue and extent without the need to perform separate nucleic acid extractions. The real-time nested PCR was more sensitive than ELISA and real-time RT-PCR assays previously employed for virus detection (Margarita et al. 2009).

Infected budwood is considered as the primary source of inoculum for the spread of *Citrus tristeza virus* (CTV) over long distances or to different countries, and natural biological vectors (aphids) are responsible for the secondary spread of the disease. TaqMan real-time RT-PCR assay was shown to be effective in detecting and quantifying CTV in plant materials using purified RNA targets or coupled to tissue print and squash procedure. In addition, the efficacy of DAS-ELISA and IC-RT-PCR assays was determined using purified RNA and compared with real-time RT-PCR format. The detection limit of ELISA was $1:10^3$, whereas IC-RT-nested PCR format could detect CTV targets up to 1.5×10^6 . Real-time RT-PCR assay was the most efficient in detecting CTV in field-grown trees. Of the 432 trees tested, 235 were found to be CTV-positive for tissue-print ELISA format. The results of tissue-print ELISA were confirmed by the conventional and tissue-print real-time PCR formats. Tissue-print ELISA indicated the differences in the number and intensity of immunoprecipitates localized in vascular area of imprints. Real-time RT-PCR assay was effective and efficient in detecting and quantifying CTV isolates from different hosts and origins. With this protocol, it was possible to consistently detect as few as 17 targets of purified CTV transcripts. The sensitivity of real-time RT-PCR assay was 1000 times higher than IC-RT-nested PCR and 10^6 times higher than the ELISA test. The quantitation limit ranged from 1.7×10^2 to 1.7×10^9 transcript copies. The tissue-printing and squash real-time RT-PCR formats exhibited high sensitivity, specificity, feasibility, and reliability for detection and quantification of CTV in immobilized plant materials. The results of the assay could be useful for epidemiological investigations (Bertolini et al. 2008).

The severe isolates of *Citrus tristeza virus* (CTV), including seedling yellows (SY) and/or stem pitting (SP) in grapefruit or sweet orange, are a major threat to the citrus industry worldwide. CTV isolates are composed of a population of sequence variants resulting from the error-prone nature of RNA polymerases, repeated inoculations of field trees by aphid vectors carrying different CTV variants, frequent recombination events between diverged sequences, and different selection pressures. Some CTV populations have a predominant sequence and a pool of closely related variants and others contain highly divergent variants; their ratio could alter the biological properties of the isolates. The precise identification of the CTV variants was accomplished by quantitative real-time RT-PCR, using a general primer set and three TaqMan-locked nucleic acids (LNAs) probes targeting sequences characteristic of severe, mild (nonSY, nonSP) and T36-like isolates. Specific amplification occurred in fresh or silica-desiccated CTV-infected samples, and all isolates except one reacted with one or more probes. Detection in fresh tissues was generally more suitable than in desiccated tissue. Specificity of the product amplified was checked by melting curve analyses and by agarose gel electrophoresis. For each sample, the melting curve showed a single peak, indicating a specific PCR product with no significant primer-dimer or nonspecific amplification products. Electrophoresis analysis confirmed amplification of a single DNA species for 186 bp for most isolates or 204 bp for T36-like isolates. Standard curves using RNA transcripts homologous to the three probes allowed a reproducible quantitative assay, with a wide

dynamic range of detection starting with 10^2 copies. RT-PCR assay with homologous and heterologous transcript RNA mixes demonstrated that each probe reacted only with its cognate sequence which was detected even at ratios below 2.5%. Analysis of 56 pathogenically distinct CTV isolates from 20 countries showed that mild isolates reacted with mild probe, whereas severe SP and SY isolates reacted with the severe SP or T35-like probes and often with the second probes. This protocol included three separate real-time RT-PCR reactions (one with each TaqMan LNA probe) and was found to be more rapid, sensitive, and specific than previously used methods. This method enabled estimation of the ratio of different genotypes of CTV present in infected trees in different locations (Ruiz-Ruiz et al. 2009).

The establishment of quarantine and certification programs is vital for avoiding the entry of and preventing materials infected by severe strains of *Citrus tristeza virus* (CTV) being planted to maintain and sustain zero-tolerance policies in all citrus-growing countries. In order to develop sensitive, specific, and reliable CTV-detection methods, tissue-print (TP) real-time RT-PCR assay was compared with the validated TP-ELISA format using CTV-specific MABs 3DF1 and 3CA5 for CTV detection. As there could not be any perfect diagnostic test with no false-positive and false-negative results, the diagnostic parameters of a technique (sensitivity, specificity, and likelihood ratios (LRs)) were calculated to characterize the efficacy of a diagnostic test. Samples (1395) from healthy and CTV-infected nursery and mature trees were analyzed by both methods. The total agreement between the detection methods was quite high. The diagnostic parameters of each technique were evaluated in a second test involving 658 *Citrus macrophylla* nursery plants. For TP-ELISA, a sensitivity of 0.8015, a specificity of 0.9963, and a positive and negative likelihood ratio of 216.42 and 0.199, respectively, were estimated. For TP real-time RT-PCR, a sensitivity of 0.9820, a specificity of 0.8519, and a positive and negative likelihood ratio of 6.63 and 0.021, respectively, were recorded. These comparative estimates indicated that TP real-time RT-PCR was the most sensitive technique and could be employed to monitor the sanitary status of commercial citrus trees and assist in the prevention of CTV disease spread in the nursery blocks. The combination of both techniques could accurately substitute for the bioindexing method using Mexican lime plants for detection of CTV, which previously required a long time to provide the results (Vidal et al. 2012).

The exchange and transport of plum plant materials for vegetative propagation has been the principal mode of dispersal of *Plum pox virus* (PPV). The presence of PPV in planting materials has to be tested in all kinds of planting materials intended for distribution to farmers and germplasm exchange. Detection protocol for PPV was developed for large-scale testing of field-grown peach trees (*Prunus persica*). Crude supernatants were obtained for use in a direct real-time PCR (drtPCR) procedure. The leaves from peach trees were macerated in a specially prepared buffer, referred to as direct pathogen extract buffer. Specific TaqMan primers and probes were designed for PPV detection: two sets specifically to PPV-D and PPV-M strains individually, and one for universal detection of PPV strains D, M, C, EA, and W. Using *Prunus* spp. tissue infected with 30 known fruit tree viruses, the universal primer and probe set correctly identified PPV-infected and PPV-free samples with no nonspecific cross-reaction. A threshold cycle value of 36 was suggested as the maximum threshold to declare a sample positive for PPV, based on endpoint analysis of the drtPCR reaction. Comparative efficacy of drtPCR and ELISA for detection of PPV in 12,200 field samples was assessed. The results showed that drtPCR assay was approximately 100- to 1000-fold more sensitive

than ELISA test and drtPCR assay could detect PPV at earlier stage of infection than ELISA, facilitating eradication of infected plants and identification of infected planting materials (Kim et al. 2008).

Nursery plants and planting materials are generally commercialized in winter, during the dormant period when the virus titer is low. Use of diagnostic methods which were not sensitive enough to detect the low concentrations of *Plum pox virus* (PPV) led to the certification of infected planting materials as virus-free and consequent introduction of PPV into new areas. A real-time multiplex PCR assay was applied for the detection and differentiation of PPV strains. SYBR Green I employed in this simple procedure was inexpensive and provided more reliable results rapidly, since the use of electrophoresis was not needed (Varga and James 2005). In a later investigation, four sample preparation methods, namely dilution, spot, squash, and tissue-print coupled with real-time RT-PCR assay, detected all described PPV strains using either TaqMan or SYBR Green protocols. The dilution and spot real-time RT-PCR methods were slightly more sensitive than the tissue-print and squash methods (Fig. 7.13). As PPV was unevenly distributed in infected woody hosts, the use of plant extracts was more reliable than single tissue-print or squash procedures. The dilution and spot procedures had the same detection limits, but they were 10 and 100 times more sensitive than cooperational-PCR (Co-PCR) and indirect DAS-ELISA techniques, respectively. The nylon and Whatman membranes were found to be suitable for immobilizing target nucleic acids. Spot real-time RT-PCR was validated as a reliable technique for detection of PPV in plants during the dormant period. Adult *Prunus* trees (405) were evaluated for their health status using different techniques with samples collected in the winter. Of the 405 trees tested, 201 showed symptoms of sharka disease in the following spring, indicating the prevalence of 49.6% infection by PPV. The highest sensitivity of PPV detection in winter was provided by conventional real-time RT-PCR which detected PPV in 97.5% of trees that developed sharka symptoms, the false negatives (5 of 405) being extremely low. Spot real-time RT-PCR format also had high sensitivity (93.6%), followed by IC-RT-PCR (91.5%), and indirect DAS-ELISA (86.6%). Spot real-time RT-PCR was compared with validated indirect-ELISA format in a large-scale analysis of 2919 *Prunus* samples by using the same plant extracts for both tests. Similar corroborating results were obtained from both tests in 90.8% samples (Table 7.8). The results validated the use of immobilized PPV targets and spot real-time RT-PCR as a screening method for large-scale analysis, which might facilitate production of PPV-free *Prunus* plants (Capote et al. 2009; Section 7.5.4).

Viruses infecting potatoes have been detected by RT-PCR assays for which different extraction procedures, specific RT-PCR reagents, amplification protocols, and/or virus-specific primers have been used. Standardized RT-PCR procedures were developed and validated for the detection of eleven viruses, namely *Alfalfa mosaic virus*, *Impatiens necrotic spot virus*, *Tobacco rattle virus*, *Tomato spotted wilt virus*, *Potato leafroll virus*, *Potato mop-top virus*, *Potato virus A*, *Potato virus M*, *Potato virus S*, *Potato virus X*, and *Potato virus Y* and one viroid *Potato spindle tuber viroid*. Nucleic acids were extracted from infected and healthy potato plant tissues using a modified procedure of Dellaporta. One-tube RT-PCR format was used for amplification on employing virus-specific oligonucleotide primers and the amplified PCR products were resolved by electrophoresis in 1.5 agarose gel and stained with ethidium bromide. The RT-PCR protocol provided a robust, convenient, cost-effective, and sensitive detection of 11 potato viruses, useful for potato seed tuber certification agencies and commercial diagnostic laboratories.

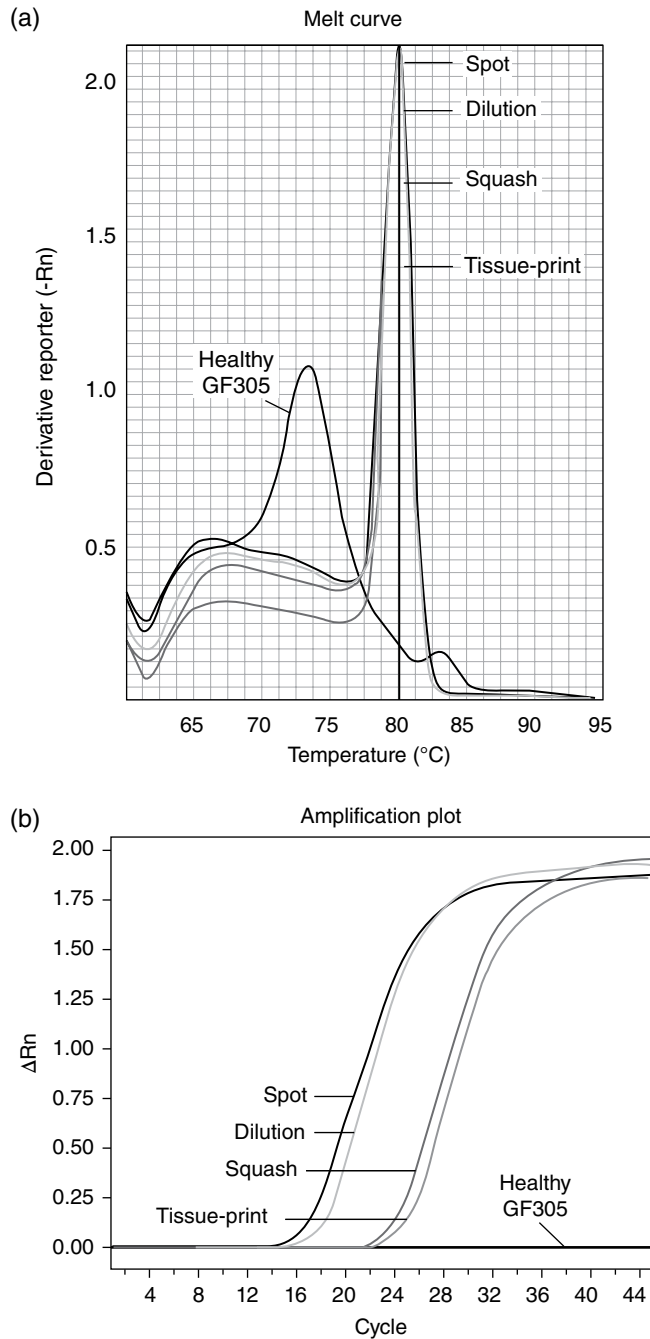


Figure 7.13 Detection of *Plum pox virus* (PPV) by real-time RT-PCR with (a) SYBR Green and (b) TaqMan chemistries, employing four direct sample preparation methods—dilution, spot, tissue-print, and squash methods—along with healthy GF305 peach seedlings as negative control. Dilution and spot real-time RT-PCR methods were slightly more sensitive than tissue-print and squash methods. Source: Capote et al. (2009). Reproduced with permission of International Microbiology.

Table 7.8 Comparison of levels of sensitivity and specificity of methods for detection of *Plum pox virus* (Capote et al. 2009).

Detection method	Sensitivity + SE ^a	Specificity + SE ^a
DASI-ELISA	0.866 ± 0.017	0.990 ± 0.005
IC-RT-PCR	0.915 ± 0.014	0.961 ± 0.01
Spot real-time RT-PCR	0.936 ± 0.012	0.980 ± 0.01
Conventional real-time RT-PCR	0.975 ± 0.010	0.892 ± 0.01

^a Standard error

The RT-PCR protocol involving the use of standardized conditions could detect and identify the O, European-N, North American-N, N:O, and NTN strains of PVY. The PSTVd-specific primers detected PSTVd in dry tomato tissue stored for over 30 years (Crosslin and Hamlin 2011). *Potato virus Y* (PVY) present in dormant potato tubers was detected by employing TaqMan real-time assay and its efficiency was compared with other diagnostic methods. Three pairs of primers and probes were designed and evaluated for their suitability to facilitate the real-time RT-PCR detection of PVY strain groups, including PVY^O, PVY^N, PVY^{N:O} (= PVY^{N-Wi}), and PVY^{NTN}. Among the primer and probe combinations tested, the combination of PVY-1 produced the lowest threshold cycle (Ct) value of 25.75. Various parameters including primer/probe combination, reaction volume, amplification cycles, and master mixes from different sources were adjusted for further optimization. The real-time RT-PCR was applied for the detection of PVY in dormant tubers of different cultivars and potato fields. Out of 1069 single-virus (PVY-) infected tubers tested, both RT-PCR formats detected PVY in 52 samples, ELISA on sprouts in 45 samples, ELISA on leaves in 54 samples, and visual observations in 53 samples. However, when the 61 multiple-virus-infected tubers were tested, both RT-PCR formats and ELISA test were found to be equally sensitive as they detected the viruses in both sprouts and leaves, giving similar number of positives. Nevertheless, real-time RT-PCR provided the advantage of offering results rapidly for large-scale application for detecting PVY in dormant potato tubers, whereas the grow-out test required 6–8 weeks to obtain results and remained the least-favored technique for the detection of PVY in dormant tubers (Singh et al. 2013).

7.2.5 Molecular Beacon

A novel fluorescence-based nucleic acid pathogen method of detection involves the use of a probe referred to as a molecular beacon, comprising a single-stranded DNA (oligonucleotides) with a stem-loop structure. The loop portion has a probe sequence that is complementary to a target sequence. The stem portion is formed by the annealing of the 5' and 3' arm sequences which are not related to the target sequence. A fluorescent moiety is attached to the 5' arm terminus, while a quenching moiety is attached to the 3' arm at the opposite end. The molecular beacon is added to the solution containing the target nucleic acid and the mixture is heated to 80°C, followed by cooling to 20°C. The arms associate to form a stem-loop conformation when there is no target molecule. Under this condition, the fluorescence emitted from the fluorophore is quenched by

fluorescence resonance energy transfer (FRET) via the quencher due to their close proximity to each other. On the other hand, if the target is present, the probe forms a hybrid with the target via its complementary sequence within the loop region, resulting in the displacement of the fluorescent moiety from the quenching moiety and leading to emission of fluorescent signal.

The emission of a fluorescent signal is only produced by molecular beacons when they hybridize with their complementary target nucleic acids. As the unhybridized molecular beacons do not fluoresce, there is no need to remove them from the reaction mixture. By tagging each molecular beacon with fluorescent moieties possessing different emission wavelengths, it is possible to employ multiple molecular beacons for detection of different targets. RT-PCR reaction may be performed with primers that amplify specific genomic sequences of interest, yielding targets complementary to their respective molecular beacons for subsequent detection. In addition, large number of samples up to 96 can be analyzed simultaneously, the efficiency paralleling that of RT-PCR and ELISA tests (Tyagi and Kramer 1996).

Cymbidium mosaic virus (CyMV) and *Odontoglossum ringspot virus* (ORSV), infecting orchids, were detected efficiently by employing the molecular beacon technique. Two pairs of primers were designed to target the RNA-dependent RNA polymerase (RdRp) genes and coat protein (CP) gene of CyMV and ORSV, respectively, for their simultaneous detection. RT-PCR was performed using the above primer pairs to amplify the genome targets from total RNA extracted from CyMV- and ORSV-infected *Oncidium* orchid leaves. 6-carboxyfluorescein (FAM) was used as a molecular beacon specific to CyMV, whereas ORSV was labeled with tetra-chloro-6-carboxyfluorescein (TET). The universal quenching moiety 6-carboxy-tetra methyl-rhodamine (TAMRA) was attached to the 3' ends of all molecular beacons employed. The detection limit of assay using molecular beacons was as little as 0.5 ng of viral RNA of both CyMV and ORSV simultaneously in 100 mg of coinfecting *Oncidium* orchid leaves. The tubes containing total RNA isolated from CyMV- and ORSV-infected leaves generated significant increases in fluorescence intensities upon the addition of both sets of molecular beacons. No fluorescence signal appeared in healthy control samples. The molecular beacon technique was found to be rapid and specific in providing sensitive detection of orchid viruses, and showed potential for screening plants for the production of virus-free plants by certification and quarantine programs and for selection of disease-resistant plants by screening germplasm collections (Eun and Wong 2000).

Strawberry vein banding virus (SVBV), belonging to the genus *Caulimovirus* (retroid virus), has a double-stranded circular DNA genome and is included as a quarantine pathogen by the European and Mediterranean Plant Protection Organization (EPPO/OEPP 1978). Biological indexing on indicator plants was the only method accepted by inspection services during certification procedures. It was necessary to develop a sensitive and rapid assay due to the limitations of bioindexing and immunoassays. An improved technique for the detection of SVBV in *Fragaria* spp. based on nucleic acid sequence-based amplification (NASBA) and real-time detection using molecular beacons (real-time NASBA) was developed. The coupling of NASBA and molecular beacons enabled a simultaneous amplification and detection of the specific amplicons in a sealed tube under real-time conditions. The NASBA assay was designed to amplify part of the RNA coding of the CP of SVBV. Variability within this region was determined among six different American and European isolates and the primers and molecular beacons were designed based on the conserved segments (431 bp) within this region.

The detection limit of real-time NASBA was 101 molecules of RNA per reaction in vitro, with no apparent reduction in sensitivity due to incorporation of molecular beacons into the NASBA system.

Real-time NASBA was employed for the detection of SVBV in 317 field-grown plants from the Czech Republic, the Slovak Republic, Poland, Latvia, and Italy. None of the strawberry plants was found to be infected by SVBV and the results were confirmed by a concurrent SVBV-specific RT-PCR assay. The assay was specific and sensitive for detection of SVBV and tested negative for the other common aphid-borne strawberry viruses, *Strawberry crinkle virus* (SCV) or *Strawberry mottle virus* (SMoV). The results of the NASBA assay were in good agreement with those of biological indexing. Reproducibility of the results throughout the year showed that the applicability of the assay might not be limited to the period of active plant growth. The real-time NASBA assay showed good potential for accurate, fast, and sensitive detection of *Strawberry vein banding virus* in strawberry plants. Real-time NASBA offers several advantages over standard PCR format such as reduction in contamination rates, nonrequirement of post-PCR manipulations, possibility of automation and high-throughput application of NASBA, and less expensive instruments. In addition, NASBA could be performed in standard ELISA format used commonly by several countries (Vašková et al. 2004).

7.2.6 Single-Strand Conformation Polymorphism Analysis

Differences in the genomic DNA may be determined reliably by employing single-strand conformation polymorphism (SSCP) analysis. When separated, the + and – strand of ds-DNA become metastable sequence-specific folded structures with distinct electrophoretic mobilities in nondenaturing polyacrylamide gels. Single nucleotide changes may be detected under such conditions (Magome et al. 1999). The SSCP analysis provided rapid results requiring less time than RFLP analysis and was particularly effective in detecting and differentiating serologically related strains of viruses, as in the case of isolates of *Beet necrotic yellow vein virus* (BNYVV) which could be assigned to A, B, or P groups (Koenig et al. 1995).

Apple stem grooving virus (ASGV) isolates from apple, European pear, and Japanese pear were found as at least two to four sequence variants within a single tree and were characterized by employing a combination of an IC-RT-PCR and SSCP analysis of the PCR products from ASGV-infected trees. Two to four bands were detected in samples from all trees, indicating ASGV existed as a mixture of sequence variants. The composition of sequence variants (the number of bands and their relative quantity) differed among leaf samples from different branches, showing that each sequence variant might be distributed unevenly within an individual tree. Since ASGV was spread only by graft transmission, sequence variation within a single tree might occur by means of successive accumulation of nucleotide substitutions into the pre-existing genome and/or by mixed infection of each sequence variant originally contained in the rootstock or scion by graft transmission. In contrast, the SSCP analyses of P-209 and Li-23-infected *C. quinoa* leaves showed only a single band in each isolate, indicating that the genomes of these isolates appeared to be homogenous. Repeated passages of these isolates by sap transmission changed the composition of sequence variants originally contained in ASGV isolates, indicating that some sequence variants rapidly multiplied and dominated, whereas other variants decreased to undetectable concentration in leaves of *C. quinoa* (Magome et al. 1999).

The SSCP analysis was applied for the detection and differentiation of isolates of *Citrus tristeza virus* (CTV). The CTV isolates collected from diverse locations were investigated to assess the differences in their coat protein (CP) gene sequences. The amplicons from CP genes of 17 CTV isolates after RT-PCR amplification were cloned and sequenced. Sequences of the clones showed sequence homology between 91.7% and 99.8%. After clone amplification and denaturation of PCR products, they were compared based on the results of SSCP analysis in 8% polyacrylamide gels. The patterns of 16 or 17 clones exhibited variations under two different electrophoretic conditions. The SSCP analysis with combination of two electrophoretic conditions and restriction of eight clones with *Eco9II* allowed discrimination between 21 of 22 CP gene clones selected for comparison. SSCP analysis, not dependent on the availability of full sequence data for comparison, was found to be rapid and inexpensive. In addition, the result of SSCP might be used as a sound basis for identification and differentiation of several genes or gene regions of viral genomes (Rubio et al. 1996).

Biologically characterized isolates (19) of *Citrus tristeza virus* (CTV) covering a wide range of symptom expression and geographic origin were compared by SSCP analysis of cDNA of the four 3' terminal genes, *p13*, *p18*, *p20*, and *p23*. There was no profile of any of the four genes that was conserved in all isolates within the biogroup. Many of the mild isolates of CTV (biogroups 0–2) showed just two DNA bands, whereas the most severe isolates (biogroups 4–5) usually showed four or more bands. The electrophoretic profile of cloned DNA fragments sometimes showed three instead of two bands, due to the presence of two stable conformations for one of the two strands. However, the presence of multiple DNA bands in the SSCP profile of an RT-PCR product generally indicated that the cDNA analyzed contained more than one sequence variant. The set of SSCP profiles of the four genes allowed identification of individual isolates, but no profiled characteristic of a geographic location was observed. Sweet orange plants singly inoculated with a mild or severe isolate yielded the SSCP profile characteristic of each isolate. On the other hand, the SSCP profile of plants successfully inoculated with both isolates (mild and severe) was a composite of the two individual profiles. The SSCP profile of singly inoculated plants remained constant, but the profile of doubly inoculated plants varied with time. Changes in the SSCP profiles of plants preinoculated with mild isolate T11 or T32 and challenge-inoculated with severe isolate T318 were monitored. The plants in which the p20 SSCP profile characteristic of T318 became predominant showed symptoms, whereas plants in which the predominant SSCP profile was characteristic of the mild isolate remained symptomless. The virulent RNA population usually out-competed and reached higher titer than the pre-existing mild RNA populations. The results indicated the usefulness of SSCP analysis to study the changes in the RNA populations of doubly inoculated plants and to monitor the effectiveness of cross-protection between mild and severe isolates of *Citrus tristeza virus* (Sambade et al. 2002).

7.2.7 Reverse-Transcription Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification procedure developed by Eiken Chemical Co., Tokyo, Japan. Because of its simplicity, rapidity, specificity, and cross-effectiveness, RT-LAMP method is likely to replace PCR-based assays. RT-LAMP technique is based on the principle of standard displacement reaction and stem-loop structure that amplifies the target with high specificity, selectivity, and rapidity under isothermal conditions, and this technique does not

require an expensive thermocycler. The amplification efficiency of LAMP method is extremely high due to continuous amplification under isothermal conditions, producing a large amount of the precipitate of magnesium pyrophosphate as a byproduct leading to turbidity. Using four specially designed primers that include six short sequences matching the target DNA fragment, the DNA is amplified under isothermal conditions within a short period of time. The reactions can be monitored unambiguously by detecting the turbidity, using an inexpensive turbidimeter. In addition, the higher amplification efficiency of the LAMP technique enables simple visual observation of amplification via the naked eye by using a UV-lamp in the presence of an intercalating dye such as SYBR Green I or ethidium bromide (Notomi et al. 2000; Parida et al. 2004). The reaction is carried out in a single tube by mixing of the buffer, primers, reverse transcriptase, and DNA polymerase, and incubating the mixture at 63°C for 60 min. The increase in the turbidity of the reaction mixture correlates with the amount of DNA synthesized. The measurement of turbidity is extremely simple and no expensive equipment is required for the LAMP procedure, as in real-time PCR format. In addition, the time required for the RT-LAMP assay is just 30 min compared with 3–4 h needed for RT-PCR based assays (Notomi et al. 2000; Parida et al. 2004).

Detection of *Japanese yam mosaic virus* (JYMV) in infected Japanese yam plants was achieved by employing reverse-transcription loop-mediated isothermal amplification (RT-LAMP) technique which was simple and provided results rapidly. Neither elaborate RNA extraction steps nor precise thermal cycling and gel electrophoresis was needed as in standard and other PCR formats. JYMV was effectively detected in leaves, propagules, and roots of Japanese yam plants. The intensity of turbidity determined using a turbidimeter indicated the virus concentration in the infected plant extracts (Fukuta et al. 2003). For the detection of *Potato virus Y* (PVY), a set of four primers matching a total of six sequences of CP gene of PVY was designed in such a way that a loop could be formed and elongated during DNA amplification. PVY CP complementary DNA clones were employed as templates and the LAMP reaction was optimized by adjusting the concentrations of $MgSO_4$, dNTPs, and Bst DNA polymerase. A positive reaction is indicated by the formation of a visible precipitate of insoluble magnesium pyrophosphate as a byproduct of LAMP reaction. Absorbance values recorded at 405 and 600 nm using a microplate reader and/or a spectrophotometer reflected the virus titer in the plant tissue samples. Assessment of turbidity visually and spectrophotometrically could save time and significantly reduce the cost of data analysis. The determination of comparative efficacy of the one-step RT-LAMP and two-step RT-PCR assays showed that both methods provided equally efficient detection of PVY in leaf and tuber samples, each one diagnosing PVY infection in 234 of 240 samples tested. The advantages of one-step RT-LAMP technique were the relative simplicity in reaction setup and detection of amplified products with high efficiency. In addition, isothermal conditions required for performing RT-LAMP analysis could be obtained easily using a water bath, whereas an expensive thermocycler would be needed to obtain the accurate temperature conditions required for RT-PCR assays. Furthermore, use of hazardous chemicals such as ethidium bromide for staining gels after electrophoresis as required for PCR-based assays could be avoided, and the positive reaction could be inferred by observing the formation of a precipitate in RT-LAMP assay (Nie 2005).

A one-step, accelerated reverse-transcription loop-mediated isothermal amplification (RT-LAMP) procedure was developed for the detection and identification of *Plum pox virus* (PPV). Six primers based on the sequences of a conserved region in the

C-terminus of the CP coding region of PPV were designed and employed in RT-LAMP assay. The isolates of five strains of PPV including the strains D, M, EA, C, and W were detected and precisely identified. The virus could be detected reliably in both herbaceous and woody host plant species infected by PPV. The relative sensitivity levels of RT-LAMP and real-time RT-PCR with SYBR Green I and melting curve analysis were evaluated using serially diluted total RNA extracts. RT-LAMP assay was as sensitive as the real-time RT-PCR formats except at lower template concentrations, when the latter technique gave more consistent results. A longer incubation period was required for detecting lower template concentration of PPV by RT-LAMP assay. The optimal temperature of incubation was 60°C for 30 min for successful detection of PPV by RT-LAMP procedure. RT-LAMP assay was found to be a highly sensitive and cost-effective tool with potential for large-scale application for assessing the disease incidence and restricting the subsequent spread of *Plum pox virus* (Varga and James 2006).

The RT-LAMP assay was developed for the reliable and sensitive detection of *Banana streak virus* (BSV), a significant constraint impacting banana production. Four primers matching a total of six sequences of the concerned ORF III polyprotein genes were designed. DNA extracts from field-infected banana plants were used as template for the detection of BSV. The LAMP assay could detect as low as 1 pg μL^{-1} template DNA. Test results of all field samples collected from different regions of South China showed that LAMP was found to be more sensitive than PCR assay. This relatively simple procedure could be applied for the detection of BSV in field-grown banana plants and showed potential for routine screening of tissue culture materials raised for the production of virus-free stocks (Peng et al. 2012).

Potato virus Y (PVY) exists in the form of many strains and recombinant variants with differing levels of virulence on potatoes. Potato seed certification systems operating in different countries require efficient methods for accurate identification and quantification of the strains of PVY. Immunoassays and RT-PCR assays have limitations that affect the cost and time required for diagnosis of PVY infection. Methods based on reverse-transcription (RT-) isothermal amplification (LAMP) of DNA facilitate fast, sensitive, and specific detection of target RNA. A closed-tube real-time RT-LAMP assay was developed for fluorescent detection of PVY in potato. A set of primers for RT-LAMP assay was designed by aligning 40 sequences of the coat protein genes of different isolates of PVY to amplify the conserved region of CP gene. The specificity of the designed primers was confirmed by amplifying RNA extracted from leaves of virus-free plants inoculated with PVY, *Potato virus M* (PVM), *Potato virus S* (PVS), and *Potato virus X* (PVX). The primers only amplified the expected amplicons from plants infected by PVY, but not from plants infected by other viruses tested. The RT-LAMP assay provided specific and sensitive detection of PVY in a single tube at 65°C. The closed-tube format eliminated the risk of carry-over contamination. The RT-LAMP format provided very fast detection of PVY in 11–30 min, depending on the virus concentration. Under the conditions of experimentation, the RT-LAMP assay was 1000-fold more sensitive than RT-PCR format. Further, RT-LAMP assay might be applied for quantitative or semi-quantitative estimation of viral RNA, as for real-time RT-PCR format. The effectiveness of RT-LAMP assay was equivalent to that of DAS-ELISA and RT-PCR techniques for testing field-grown potato plants. RT-LAMP assay combines the high-throughput advantage of DAS-ELISA test and sensitivity of RT-PCR format. RT-LAMP procedure therefore has the potential to become an alternative to methods currently employed for routine detection of *Potato virus Y* in potatoes (Przewodowska et al. 2015).

7.2.8 DNA Array Technology

DNA array technology is a promising multipathogen detection system, useful for detecting two or more pathogens (viruses) present in propagules. DNA technology aims to miniaturize traditional bioanalytical detection system so that hundreds of biomolecules with unique identity can be detected simultaneously in one single experiment. In a microarray platform (glass slide), thousands of diagnostic probes can be spotted and hybridized with labeled targets such as nucleic acids extracted from specimens and chemically modified with a fluorescent or other reporter (compound/molecule). DNA microarray provides a medium for hybridization of the known with unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. Microplates or standard blotting membranes may be used in the assays, based on the size of the deposited sample spotted designated as microarrays (<200 µm in diameter) or macroarrays (>300 µm in diameter) (Wang et al. 2003; Morais et al. 2006). The microarray has been applied less frequently for the detection of plant viruses compared to fungal or bacterial pathogens infecting crop plants. The DNA arrays contain gene sequences from each target virus in a single assay. The microarray is exposed to fluorescently labeled cDNA from the sample to be tested, followed by scanning with a microarray scanner to detect the targets in the test samples. Commercial kits such as Qiagen are available, variously referred to as DNachip, Biochip, genechip, and gene array (Shi et al. 2003).

Compact discs (CDs) made from a 1.2 mm thick disc of polycarbonate (PC) plastic coated with a 100 Å reflective layer of aluminum, silver, or gold protected by a transparent polymeric layer were used to detect *Plum pox virus* (PPV) and also for the analysis of single nucleotide polymorphisms (SNPs) in PPV. Samples from different serogroups SER1, SER2, and SER3 of PPV were used in macroarray assay. Identification of virus types and SNP tests were carried out on polycarbonate CD. Samples of PPV type Dideron (D) were specifically hybridized with both universal (P21) and D-specific probes. On the other hand, type Marcus (M) hybridized with both universal and M-specific probes which implied a full score for discriminating different types of *Plum pox virus*. The match/mismatch ratio reached was 17 and 18 for type M and D, respectively. The results indicated that the CD array could be an effective alternative technique for rapid and reliable detection of plant viruses. The SNP analysis showed that SER1 samples hybridized with probes SNP3B and SNP1A and SER2 samples hybridized with SNP1B and SNP3A, while samples of SER3 specifically hybridized with SNP1B and SNP3B. The match/mismatch ratios were 3.7 and 4.5 for SER1 and SER3, respectively. On the other hand, SER2 was better scored with a match/mismatch ratio of 11.8. These values confirm the ability to analyze nucleotide polymorphisms which were associated with different serotypes within PPV type D. This investigation revealed the advantage of using compact disc (CD) surfaces as high-throughput screening platforms to perform a wide range of experiments for detection and differentiation of plant viruses and their strains (Morais et al. 2006).

The microarray technique was found to be effective in detecting *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato virus A* (PVA), and *Potato virus S* (PVS) in plants infected by individual or a mixture of viruses. Closely related viruses or strains of these viruses could be detected and differentiated even when the sequence identity was less than 80%. Sequence variants with greater than 90% sequence identity could be selected and distinguished by the microarray method. Discrimination of virus species within the same genus was also achieved and discrimination could be effected where the sequence

identity was less than 80%. The potyviruses PVY and PVA were effectively discriminated, as were the quite distinct strains of PVS (the ordinary and Andean). The results of microarray were in good agreement with those of ELISA format with equivalent sensitivity (Boonham et al. 2003).

Short synthetic single-stranded oligomers (40-nt) were used as capture probes instead of PCR products. Using a microchip array, *Potato virus X*, *Potato virus A*, *Potato virus S*, *Potato virus Y*, *Potato virus M*, and *Potato leafroll virus* were efficiently detected in both single and mixed infections. The oligonucleotide-based microarray was able to efficiently detect and differentiate the principal strains of PVY and PVS (Bystricka et al. 2005). Potato-infecting viruses *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV), and *Cucumber mosaic virus* (CMV) were detected using nonradioactive microarray system. Oligonucleotide (70-mer) probes specific to the target virus were immobilized on nylon membranes. Total plant RNA extracts were used for cDNA and second-strand synthesis, employing an anchor primer sequence with random pentamers coupled at the 3' end. Subsequent synthesis by PCR using the anchor primer alone led to a relatively unbiased amplification of plant and viral RNAs. After chemically labeling the cDNAs, the product was used as a target in hybridization. CMV, PVY, and PLRV were detected by employing this protocol. Viral RNAs were amplified by a factor of 100–1000 and could therefore be detected in single and multiplex infections. The labeled target DNA hybridization was specific to the infecting virus and there was no cross-hybridization to probes for heterologous viruses. The limit of detection of the macroarray was similar to that of DAS-ELISA test. The macroarray protocol developed in this investigation was less sensitive by 10^3 and 10^5 times relative to conventional RT-PCR and real-time RT-PCR assays respectively (Agindotan and Perry 2007).

Multiple pathogen detection of 11 potato viruses and a viroid *Potato spindle tuber viroid* (PSTVd) was achieved by employing a macroarray procedure. The viruses commonly occurring in North America include *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Potato mop-top virus* (PMTV), *Potato leafroll virus* (PLRV), *Potato latent virus* (PotLV), *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Tobacco rattle virus* (TRV) for which seed tubers were tested for the presence of the virus(es) in potato seed tuber certification programs. Oligonucleotide probes (70-mer) and labeled targets were prepared in a random primer amplification method. Samples from 12 reference virus stocks and 36 field isolates from potato plants were analyzed. All eleven viruses were consistently detected by the macroarray procedure and the results were similar to those obtained with standard ELISA tests. Four isolates of PSTVd in mixed infections with one or more viruses were also detected in the array, although strong hybridization signals could only be obtained with viroid-specific primers in combination with anchored-random primers. Up to four viruses, or a viroid plus two viruses, were detected in individual plants with no apparent competition or inhibition. The macroarray procedure might compliment available diagnostic methods such ELISA, and holds promise in the reliable and sensitive detection of viruses in dormant and sprouted tubers. The cost-effectiveness of the macroarray approach for simultaneous detection of as many as 11 viruses and one viroid in infected potatoes could be an important factor when choosing diagnostic tests for large-scale application (Agindotan and Perry 2008).

A complementary DNA (cDNA) macroarray was developed for the simultaneous detection of 12 viruses infecting potatoes since there was cross-hybridization between

viruses in the previously available macroarray, making the interpretation of results difficult. In addition, the procedures required expensive tools such as an imaging system for chemiluminescence. Virus-specific capture probes and biotin-labeled target amplification were therefore used to enhance sensitivity and to reduce cross-hybridization. A suitable region in the viral genome was selected for each of following viruses to be detected: *Alfalfa mosaic virus*, *Cucumber mosaic virus*, *Potato acuba mosaic virus* (PAMV), *Potato leafroll virus*, *Potato mop-top virus*, *Potato virus A*, *Potato virus M*, *Potato virus S*, *Potato virus X*, *Potato virus Y*, *Tomato ringspot virus* (ToRSV), and *Tomato spotted wilt virus* (TSWV). The viruses and their respective cDNAs were cloned into plasmid vectors. Capture probes for each virus ranging from 290 to 577 bp were generated by PCR and immobilized on a nylon membrane. Total RNAs were extracted from plants infected by each virus and cDNAs were synthesized from the RNA extracts using a random 9-mer primer. Subsequently PCR reactions were performed, using one primer pair specific to each of the 12 potato viruses. During PCR, amplified cDNAs were labeled with biotin and used as a target for hybridization analysis on a macroarray membrane. Hybridization signals between capture probes for the 12 viruses and their respective target cDNAs were observed using chemiluminescent or colorimetric detection. Hybridization signals with capture probes were only detected when homologous virus targets were examined and no hybridization to healthy plant extract was observed, facilitating identification of each virus. For example, when an AMV-infected plant was used as template, PCR was performed using primer pair AMVF2/AMV1R and biotin-labeled cDNA was hybridized on a macroarray. Clear signals were observed in the positions of capture probes for AMV and there was no signal from other viruses in chemiluminescent detection, indicating the absence of cross-hybridization with any other viruses tested. The results of colorimetric detection were in complete agreement with those obtained using chemiluminescence detection, indicating that inexpensive colorimetric procedure could be used with no compromise in the sensitivity of detection. The microarray protocol was 5×10^2 to 4×10^6 times more sensitive than ELISA test and 5 to 5×10^4 times more sensitive than RT-PCR assay except for AMV detection. The efficacy of the macroarray technique was demonstrated to have practical application by testing field samples. Further, the protocol showed potential for use in breeding, quarantine, and foundation seed potato production programs, contributing to effective management of virus diseases affecting potatoes (Maoka et al. 2010).

7.2.9 Small-RNA Deep-Sequencing Analysis

Universal detection of several viruses in infected plants is possible by analyzing virus-derived small interfering RNAs (siRNAs) that accumulate in infected plant tissues due to the activation of antiviral defense mechanism based on RNA silencing (Llave 2010). The occurrence of a severe grapevine vein clearing and vine decline syndrome was observed in grapevine orchards in the Midwest USA. In order to establish the nature of the possible virus causing the disease, deep sequencing of sRNAs enriched from symptomatic and asymptomatic grapevine plants was performed. A large number of viral small RNAs that share identical nucleotide sequences with the genomes of 27 plant viruses was profiled. Two cDNA libraries were constructed from a symptomatic and an asymptomatic grapevine plants. Deep sequencing of the two cDNA libraries showed that the most abundant viral small RNAs aligned with the genomes of viruses in the genus *Badnavirus*, family *Caulimoviridae*. Amplification of the viral DNA by PCR

allowed the assembly of the whole genome sequence of a grapevine by a DNA virus for the first time in the USA, closely associated with vein clearing symptoms; the virus was provisionally designated *Grapevine vein clearing virus* (GVCV). RT-PCR assays were performed on samples from grapevine cultivar Chardonnay in a commercial vineyard using the primer set GVCV-F1/GVCV-R1. The results indicated that the GVCV-specific fragment of 530bp was present in the extracts of infected young leaves with vein clearing symptoms. Other grapevine cultivars infected by GVCV included Chardonnay, Vidal Blanc, and Chardonnay in Illinois and Indiana. The results showed that GVCV was present in six cultivars in three states in the Midwest USA and these cultivars exhibited vein clearing symptoms (Zhang et al. 2011).

Infection of cherry trees in Italy and plum trees in Azerbaijan by a new virus was observed. Double-stranded RNAs purified from the infected trees were analyzed by deep sequencing. Contigs showing weak, but significant, identity with various members of the family Betaflexiviridae were constructed. A comparison of nucleotide sequences led to the conclusion that viral isolates present in the *Prunus* plants belonged to the same viral species. The genomic organization of the isolates was similar to that of some members of Betaflexiviridae, with three overlapping open reading frames (RNA polymerase, movement protein, and capsid protein). Phylogenetic analyses of the deduced encoded proteins showed a clustering with the sole members of the genus *Tepovirus*, *Potato virus T* (PVT). Based on the results, the new virus designated *Prunus virus T* (PrVT) was considered as a new member of the genus *Tepovirus*. An RT-PCR assay was developed for the detection and identification of two other PrVT isolates and the prevalence of the PrVT isolates was estimated to be 1% in the large *Prunus* collection (Marais et al. 2015).

The approach based on small-RNA deep-sequencing (SRDS) was applied to detect viruses in a single sweet potato plant inoculated with *Sweet potato chlorotic stunt virus* (SPCSV-EA) and *Sweet potato feathery mottle virus* (SPFMV) (Kreuze et al. 2009). Analysis of its siRNA revealed the presence of three additional previously unknown viruses which included the strains A and B of *Sweet potato pakakuy virus* (SPPV, genus *Badnavirus*, family *Caulimoviridae*) and *Sweet potato symptomless virus 1* (SPSV1, genus *Mastrevirus*, family *Geminiviridae*). SRDS was shown to be a novel technique capable of detecting both RNA and DNA viruses. This technique was applied for field surveys for assessing the incidence of grapevine virus associated with grapevine vein clearing and vine decline syndrome (Zhang et al. 2011).

Small-RNA deep-sequencing (SRDS) procedure was applied to determine the nature of viruses infecting sweet potato grown in Honduras and Guatemala, which are within the main center of domestication of sweet potato, and the Galapagos Islands (Ecuador), which are within the secondary center of domestication. SRDS on the pooled RNA (10 µg) from 11 sweet potato plants from Guatemala and Honduras resulted in 4,155,175 reads (size range 10 to 27-nt) and a total of 132,965,600 bases. The viruses detected by SDRS procedure included *Sweet potato feathery mottle virus* strain RC and *Sweet potato virus C* (*Potyvirus* spp.), *Sweet potato chlorotic stunt virus* strain WA (SPCSV-WA, *Crinivirus* sp.), *Sweet potato leaf curl Georgia virus* (*Begomovirus* sp.), and *Sweet potato pakakuy virus* strain BC (= *Sweet potato badna virus* B). The identity established by SRDS method was confirmed by PCR assay and sequencing of the amplicons. Four viruses were detected in a sweet potato sample from Galapagos Islands. NCM-ELISA and TAS-ELISA formats using the virus-specific antibodies detected SPFMV and SPCSV-WA, respectively, and other viruses were not detectable by these immunoassays.

This investigation showed that 4 million small RNA reads might be readily obtained by SRDS, using 10µg of total RNA from each sweet potato sample. SRDS might be widely applicable and useful for simultaneous detection of several viruses in crop plants infected with a wide range of unrelated viruses, about which little information may be available (Kashif et al. 2012).

7.3 Detection of Viroids in Seeds

Among the disease-causing pathogens of plants, viroids are the smallest in size and simplest in structure; however, they are capable of inducing distinct symptoms when they gain entry into the susceptible host plant tissues. They have small, single-stranded covalently closed circular RNAs with a highly base-paired rather stiff rod-like conformation. None of the viroids has the ability to code for any viroid-specific protein, and they have to depend on the host’s enzyme systems entirely for the replication of their genomic RNAs. It has been suggested that the viroids have shed all the dispensable processes of viral pathogens to make use of the host in the most sophisticated manner to become highly gifted modern RNA parasites (Tabler and Tsagris 2004). Viroid RNAs carry the essential information for their independent replication when introduced into cells/tissues of susceptible plants, attaining required concentrations for production of characteristic symptoms or remaining latent in seeds and/or propagules. The infected symptomatic or asymptomatic seeds and propagules are the primary sources of inoculum for their dispersal to subsequent generations and to different geographical locations. Contaminated tools and propagation practices may also aid in the viroid transmission from infected to healthy plants.

7.3.1 Classification of Viroids

The genomes of viroids are GC-rich and consist of 260–402 nucleotides. Biological, biochemical, and structural characteristics have been used for the classification of viroids into two families (Góra-Sochaka 2004; Mayo and Brunt 2007) as detailed in Table 7.9. Viroids with a central conserved region (CCR) and without hammerhead ribozymes are included in the family of *Pospiviroidae*. In contrast, viroids lacking CCR

Table 7.9 Viroids belonging to different families and genera transmitted through seeds/propagules of infected plants (Mayo and Brunt 2007).

Family	Genus	Viroid
Pospiviroidae	<i>Pospiviroid</i>	Potato spindle tuber viroid, Citrus exocortis viroid, Tomato planta macho viroid
	<i>Hostuviroid</i>	Hop stunt viroid
	<i>Cocaviroid</i>	Citrus viroid IV, Hop latent viroid
	<i>Apscaviroid</i>	Apple scar skin viroid, Australian grapevine viroid, Citrus bent leaf viroid, Citrus viroid II, Citrus viroid III
Avsunviroidae	<i>Avsunviroid</i>	Avocado sunblotch viroid
	<i>Pelamoviroid</i>	Peach latent mosaic viroid

have the ability to self-cleave through hammerhead ribozymes and are assigned to the family *Avsunviroidae*. Furthermore, the site of viroid replication is different for the members of these two families. The nucleus is the site of replication for the members of *Pospiviroidae*, whereas the members of *Avsunviroidae* replicate in the chloroplasts (Flores et al. 2003, 2005). Similarity to viruses in the type of symptoms induced and modes of transmission of viroids has contributed to the failure of viroids being recognized as a separate group of disease-causing entities until the second half of the twentieth century. Diener (1971) demonstrated for the first time that potato spindle tuber disease was caused by a single-stranded RNA with no protein coat as in the case of viruses, and the causative agent was designated *Potato spindle tuber viroid* (PSTVd).

7.3.2 Biological Methods

Symptoms induced by viroids vary depending on the host-plant–viroid combinations after varying periods of incubation. However, specific and characteristic symptoms with diagnostic value are observed in particular organs of infected plants. *Potato spindle tuber viroid* (PSTVd) causes abnormally elongated tubers which assume a spindle or cylindrical shape with prominent eyes (Diener 1979). Apple fruits exhibit recognizable symptoms induced by *Apple scar skin viroid* (ASSVd) (Koganezawa et al. 2003). Citrus viroids, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CvD-III), and *Citrus viroid IV* (CvD-IV), produce distinguishable symptoms in citrus plants (Duran-Vila et al. 2000). However, most viroids induce symptoms in their natural hosts which are not clearly distinguishable, and it is difficult to identify the viroids based on the type of symptoms caused in their primary host plant species. Biological indexing by grafting infected citrus samples (scions) onto Arizona 861-S1 Etrog citron plants was useful for detecting *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), and *Citrus viroid III* (CvD-III) (Malfitano et al. 2005). Diagnostic/assay/bioindicator plants that exhibit clear symptoms rapidly have been used for differentiating viroids, and include tomato cultivar Rutgers, *Citrus medica* cultivar Etrog, *Gynura aurantica*, *Chrysanthemum morifolium* cultivar Mistletoe, *Cucumis sativa* cultivar Sporu, and *C. sativus* (Narayanasamy 2011).

The grow-out method has been applied for the detection of viroids in seeds as in the case of viruses under greenhouse conditions. Specific numbers of seeds collected from infected plants are sown in pots, the development of symptoms is recorded in growing seedlings, and percentages of infected seeds are calculated. The transmission of *Potato spindle tuber viroid* (PSTVd) via pollen, true seeds of potato (Fernow et al. 1970), and also through tomato seeds (Benson and Singh 1964) was observed. *Peach latent mosaic viroid* (PLMVd) was shown to be transmitted through pollen by pollinating healthy plants with pollen from infected plants. Two peach, cultivars Velvel C and Vivian C, were infected when pollinated with pollen from infected peach cultivar Nectaross (Barba et al. 2007). *Citrus exocortis viroid* (CEVd) was shown to be transmitted through seeds of *Impatiens walleriana* and *Verbena* x hybrid by visual examination of developing seedlings emerging from seeds collected from commercial sources. All 19 samples showed infection by CEVd, with a seedling transmission rate of 66% in *I. walleriana* and 28% in *V x hybrida*. The presence of viroid in nongerminated seed was 21%. Storage of seeds for 2 years significantly reduced the percentage of seed transmission in these host plant species (Singh et al. 2009). *Pepper chat fruit viroid* (PCFVd) belonging to the genus *Pospiviroid* was detected in tomato seed in Australia. Other viroids detected

in tomato seed were *Potato spindle tuber viroid* (PSTVd), *Citrus exocortis viroid* (CEVd), and *Tomato chlorotic dwarf viroid* (CDVd). PCFVd causes disease in *Capsicum*, potato, and tomato. It is known to be transmitted through seed of *Capsicum* (Verhoeven et al. 2009; Reanwarakorn et al. 2011).

7.3.3 Physico-Chemical Methods

The presence of viroids in seeds/propagules has to be detected unambiguously and rapidly to eliminate the infected plants. The biological diagnostic methods require a long time to obtain results, in addition to being cumbersome and inconsistent; alternative diagnostic procedures based on physico-chemical properties of the viroids have therefore been developed. Nucleic acid molecules could be separated based on the differential mobility in an electrical field. The viroid molecules differ distinctly from normal host RNAs in their electrophoretic mobility in nondenaturing and partially denaturing gels. Polyacrylamide gel electrophoresis (PAGE) technique utilizes this property of viroid nucleic acids. *Potato spindle tuber viroid* (PSTVd) was detected in potato and tomato plants by extracting the cellular nucleic acids from healthy and infected potato plants by employing PAGE procedure. PSTVd only appeared as a distinct band in the extracts from infected plants, but not in healthy plant extract. Further, PAGE technique was applied to differentiate mild and severe strains of PSTVd. PAGE procedure was employed to eliminate infected seed tubers of several elite or basic potato seed stocks and viroid-free seed stocks were developed in certification programs (Morris and Wright 1975). The PAGE technique was shown to be efficient in detecting viroid and viroid-like RNAs in grapevine plants.

A band with faster mobility than that of *Citrus exocortis viroid* (CEVd) under non-denaturing conditions was detected by PAGE procedure. Two bands were visualized under fully denaturing conditions, one co-migrating with circular forms of CEVd and a second band migrating at a faster rate than the linear forms of this viroid. This RNA species did not hybridize with cDNA probe to CEVd. The identity of the viroid was confirmed by its reaction on the bioindicator *Gynura aurantica* plants (Flores et al. 1985). The detection of *Potato spindle tuber viroid* (PSTVd) in true seeds of potato was more effectively achieved by return gel electrophoresis. This procedure was comparable with or superior to the nucleic acid hybridization method (Singh et al. 1988). Return gel electrophoresis provided more sensitive detection of *Apple scar skin viroid* (ASSVd) in apple leaf tissue, *Hop stunt viroid* (HSVd) in plum bark, and *Citrus exocortis viroid* (CEVd) in citrus leaves (Asai et al. 1998). *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), and *Citrus viroid III* (CV-III) were most widely distributed in the citrus orchards of Campania region in southern Italy. Sequential (s)PAGE analysis of nucleic acid extracts of 112 citrus field-grown trees revealed that 8 source trees did not contain any viroid-like RNA, whereas 12 trees contained single RNA band with the molecular size of HSVd (7 samples), CEVd (2 samples), or CVd-III (3 samples). The remaining source trees showed the presence of 2 or more viroid-like RNAs with the electrophoretic mobility of CEVd, *Citrus bent leaf viroid* (CBLVd, formerly known as *Citrus viroid I*), HSVd, CVd-III, and/or CVd-IV, revealing the occurrence of mixed infections by two viroids in the same tree. The results of sPAGE procedure were consistent with those of Northern and dot-blot analyses using DIG-labeled probes specific to CEVd, CBLVd, HSVd, CVd-III, and CVd-IV. The results of sPAGE procedure performed on 112 field trees over 3 years were confirmed by molecular hybridization analyses. Infection by

CEVd, HSVd, and CV-III accounted for 68%, 87%, and 85% respectively, whereas CVd-IV and CBLVd infections were detected in 24% and 13% of the field trees tested. The results emphasized the urgent need to establish effective certification programs to prevent further spread of the viroid diseases affecting citrus plants (Malfitano et al. 2005). Return (R-) PAGE technique was employed as an adjunct procedure to reverse-transcription (RT-) PCR assay for the detection of viroids. RT-PCR assay was employed for the detection of *Potato spindle tuber viroid* (PSTVd) and *Chrysanthemum stunt viroid* (CSVd) in 6 of 891 potato tubers and 2 of 154 chrysanthemum samples, respectively. The amplified product of 199bp was detected by R-PAGE technique. The virus-specific band could be visualized by R-PAGE procedure. The expected band location for PSTVd and CSVd in R-PAGE gel was adjacent to each other (Bostan et al. 2010).

7.3.4 Nucleic Acid-Based Techniques

Nucleic acid hybridization techniques using viroid-specific probes and reverse-transcription (RT) polymerase chain reaction (PCR) assay, using appropriate primers targeting specific regions of the viroid genome, have been applied for the detection of viroids present in seeds and propagules. Different hybridization procedures have been shown to be useful in detecting the viroids in seeds. Dot-blot hybridization using digoxigenin (DIG-) labeled cRNA probes was found to be effective for routine detection of *Chrysanthemum stunt viroid* (CSVd) (Mumford et al. 2000b). Northern and dot-blot analyses using viroid-specific DIG-labeled probes were useful for efficient detection of *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd = *Citrus viroid I*), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV). The results obtained with hybridization assays corroborated those of sPAGE procedure (Malfitano et al. 2005). Simultaneous detection of four viroids infecting grapevine was achieved by employing polyprobe and nonisotopic dot-blot hybridization technique. A polyprobe was constructed by cloning tandem full-length sequences of *Hop stunt viroid* (HSVd), *Australian Grapevine viroid* (AGVd), and *Grapevine yellow speckle viroid-1* and *-2* (GYSV-1, -2) into a single vector. The cRNA probe detected all four viroids with sensitivity similar to that obtained using individual probes. The grapevine samples collected during a field survey in Beijing, Xinjiang, China from 78 varieties showed the presence of grapevine viroids in 56 varieties (71.8%). The protocol developed in this investigation was found to be rapid, reliable, and cost-effective for detection of infection by four viroids in grapevine (Zhang et al. 2012b).

Peach latent mosaic viroid (PLMVd) infection was found to be widely distributed in the peach germplasm from Europe, Asia, North America, and South America. The cherry isolate of PLMVd has 377 nucleotides (nts) which are 91–92% homologous to PLMVd isolates of peach. Dot-blot and Northern blot hybridization analyses of total nucleic acids extracted from plum, apricot, cherry, and peach samples resulted in hybridization signal only from infected stone fruit samples. PLMVd from peach had an electrophoretic mobility similar to the viroid from plum (Hadidi et al. 1997). Dot-blot hybridization procedure using DIG-labeled viroid-specific probes was effective in detecting PLMVd and *Hop stunt viroid* (HSVd) infection in peach trees. The single or mixed infection by PLMVd and HSVd were high, reaching 77% and 69% respectively, in different orchards of Czech Republic (Hassan and Ryšánek 2004).

The natural mode of transmission of *Peach latent mosaic viroid* (PLMVd) was investigated since a high incidence of field infections and rate of natural spread (5–10% new infections/year) were observed. Under experimental conditions, a low percentage of transmission by the aphid *Myzus persicae* was recorded (Flores et al. 1992), but natural spread of PLMVd was not documented. Pollen from 9 peach trees of 3 cultivars (3 plants/cultivar) infected with an Italian isolate of PLMVd were used to pollinate 18 virus-free 5 year old peach trees of 6 cultivars (3 plants/cultivar). Pollen collected from pollinated trees was tested for the presence of PLMVd by spot-blot hybridization and RT-PCR procedures. Both tests detected PLMVd in all samples (100%) tested from peach trees used as donors. Positive signals from hybridization and RT-PCR were obtained both in the supernatant of pollen and in washed pollen of PLMVd-infected trees, indicating that PLMVd was located both externally and internally. Of the 18 trees pollinated with pollen from infected donors, five trees showed the presence of PLMVd. PAGE procedure confirmed the presence of the viroid by revealing viroid-specific band. Seed transmission of PLMVd was investigated by testing 1000 seeds from PLMVd-infected plants of 10 cultivars (100 seeds/cultivar) using dot-blot hybridization and RT-PCR techniques. The viroid was detected in the teguments of 242 of 500 (c. 49%) seeds tested and in 224 of 500 (c. 45%) peeled kernels. The seedlings from seeds of PLMVd-infected plants were negative by RT-PCR and hybridization techniques, indicating a lack of transmission of PLMVd through seeds. The results showed that PLMVd was pollen-borne but was not transmitted from seeds to seedlings, although the viroid was present in the seed (probably PLMVd could not infect the embryo of the seed). Further, PLMVd could be detected in peach roots. However, root infection may not have a significant role in the spread of the viroid from infected plant to healthy plants (Barba et al. 2007).

Hop latent viroid (HLVd) could be readily detected by RT-PCR in hop anthers from small buds and practically mature anthers. However, HLVd was eliminated from hop pollen during the process of pollen maturation and germination. Transmission of HLVd through pollen was investigated during different stages of pollen and seed development. HLVd was readily detectable in mature pollen, but the intensity of amplified cDNA dramatically decreased in pollen maturation stages and no viroid was detected in germinating pollen. HLVd could propagate in uninucleate microspores and was still abundant in young pollen. However, in later stages during pollen vacuolization and maturation, HLVd was effectively eliminated. Traces of HLVd were still detectable by RT-PCR in mature pollen, but disappeared during pollen germination. It appeared that viroid RNA was intensively degraded by endonucleolytic cleavage of circular RNA and subsequent linear RNA degradation during pollen development. The interval when rapid viroid degradation occurred was therefore determined. HLVd monomeric circular and linear forms could still be detected by Northern blotting in stages following postmitotic young bicellular pollen during initial vacuolization, but was detectable only by RT-PCR in mature pollen. HLVd cleavage products, including RNA fragments shorter than unit-length HLVd, were detected in young bicellular pollen. HLVd degradation in hop pollen was found to be a selective process that efficiently eliminated HLVd during pollen maturation and germination. The majority of the degraded HLVd RNA in mature pollen included discrete products in the range of 100–230 nucleotides which did not correspond to siRNAs (small interfering RNAs). HLVd eradication from pollen correlated with developmental expression of a pollen nuclease and specific RNases. Activity of the pollen nuclease HBN1 was maximal during the vacuolization step and decreased in mature pollen. Total RNase activity increased continuously up to the final

steps of pollen maturation. The developmentally activated HBN1 and pollen ribonucleases could participate in the mechanisms of HLVD recognition and degradation (Matoušek et al. 2008). Pollen transmission of *Hop stunt viroid* (HSVd) was investigated by collecting closed flowers of plum trees and the pollen grains were mechanically separated from anthers and stored in petridish. RT-PCR assay was used to detect HSVd in pollen from parental donor plants of cultivars Black Sunrise and Black Glow. HSVd was detected both inside and outside the granules, suggesting the possibility of transmission of HSVd to seed and subsequently to plants (Luigi et al. 2010).

Citrus exocortis viroid (CEVd) infects *Impatiens walleriana* and *Verbina x hybrida* plants, seriously affecting production of flowers. RT-PCR assay was applied to detect CEVd in the seeds of these flower crops. The viroid was detected in the nongerminated seeds and also in the seedlings growing from the infected seeds. The rate of detection of CEVd in the seeds was at a lower level than in the seedlings because the seeds had a smaller amount of tissue compared to the seedlings which could allow virus replication after seed germination, attaining a detectable level of viroid titer. The seedlings were grown in separate plots. In three RT-PCR tests, CEVd was detected in leaves and petals of *Impatiens* and *Verbena* from all CEVd-positive plants, but not from healthy plants. The presence of CEVd in commercially distributed seeds and its seed transmission in these two species of ornamental plants could be conclusively established by the RT-PCR assay (Singh et al. 2009).

A viroid disease observed in the glasshouse crop of sweet pepper (*Capsicum annuum*) in the Netherlands was investigated to establish the identity of the viroid pathogen. The viroid induced vein necrosis and reduced fruit and tuber size in tomato and potato, respectively. The viroid designated *Pepper chat fruit viroid* (PCFVd) was detected in pepper plants cultivar Yolo Wonder at 6 weeks after inoculation, by employing RT-PCR assay using the primer pair AP-FW1/RE2. Based on the biological and molecular properties of the viroid infecting sweet pepper, the pathogen was named *Pepper chat fruit viroid* and proposed as a new species in the genus *Pospiviroid*. The presence of PCFVd in inoculated plant species (7) including tomato and potato was also detected using the primer pair. The seeds (72) collected from pepper fruits in artificially inoculated plants were sown and at 2 weeks after emergence the seedlings were tested by RT-PCR assay in groups of 6 plants. Groups that reacted positively were tested individually, and RT-PCR products of 3 plants were sequenced. The predominant nucleotide sequence of the viroid had not changed during seed germination and seedling formation. The results indicated that PCFVd was transmitted through seeds of infected pepper plants. The genome of PCFVd consisted of 349 nucleotides and the primary structure showed high similarity to the members of *Pospivirus* genus (Verhoeven et al. 2009).

7.4 Detection of Viroids in Propagules

7.4.1 Nucleic Acid-Based Techniques

In addition to seeds, propagules form the primary sources of inoculum, spreading the disease to different geographical locations as well as to subsequent generations of crops. Viroids may also spread through contaminated tools and propagation practices, whereas the involvement of biological vectors as in the virus disease is only known in a few cases such as *Peach latent mosaic viroid* (PLMVd) to a limited extent (Flores et al. 1992).

The importance of sensitive, reliable, and rapid techniques for detection of viroids in propagules must therefore be emphasized. As conventional biological methods are time-consuming and inconsistent, modern molecular techniques seem to be the choice of researchers in accomplishing the task of producing viroid-free stocks to restrict the initial incidence and subsequent spread of the viroid diseases. However, nucleic acid-based techniques have to be validated by testing them at several locations other than the laboratory where the protocol was developed for their reliability, reproducibility, and cost-effectiveness. Further, the feasibility of the technique for large-scale application under field conditions and for the certification and quarantine programs also has to be demonstrated.

7.4.1.1 Nucleic Acid Hybridization

Citrus viroid V (CVd-V) has a G + C-rich genome of 293–294 nucleotides and its predicted secondary structure of minimum free energy is a rod-like conformation with 68.7% paired nucleotides. A new variant of CVd-V was detected in California, Spain, and Nepal. The viroid-like RNA was inoculated onto Etrog citron, which was used as a secondary bioamplification host. Northern blot hybridization procedure was applied using DIG-labeled viroid-specific probes. Northern blot hybridization with full-length CVd-V-specific probe corroborated the sequence similarity of CVd-V with the viroid-like RNA, but not with HSVd or CVd-V included as controls. Nucleic acid extracts from Etrog citrons graft-inoculated with 62 field isolates collected in different citrus-growing countries and one sweet orange sample from Nepal were analyzed by Northern hybridization with CVd-V-specific DIG-labeled DNA probe. CVd-V was identified in a single source of Seminoletangelo (*C. paradise* x *C. tangerine*) from Spain, in the sweet orange from Nepal, and in six isolates of sweet orange from Sultanate of Oman. CVd-V could also be detected by slot-blot hybridization with CVd-V-specific probe. RT-PCR assay using CVd-V-specific primers was able to provide confirmatory results for the presence of the new variant of CVd-V in *Citrus* spp. (Serra et al. 2008).

Citrus plants are natural hosts of several viroids belonging to the family *Pospiviroidae*. Detection of the viroids directly in citrus plants by diagnostic methods yielded erratic results. Etrog citron had to be used as a secondary bioamplification host, which allowed rapid multiplication of the viroids to reach detectable concentrations. To avoid the use of Etrog citron, an easy-to-use Northern hybridization protocol was developed for the detection of *Citrus exocortis viroid* (CEVd) directly in samples collected from field-grown *Citrus* spp. and cultivars. The Northern hybridization protocol involved: (i) use of nucleic acid preparations from bark tissue samples collected from the field-grown trees, regardless of the growing season and storage conditions; (ii) separation on 5% PAGE or 1% agarose blotting membrane and fixing; and (iii) hybridization with viroid-specific DIG-labeled probes and detection with anti-DIG-alkaline phosphatase conjugate and autoradiography with the chemiluminescence substrate disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2-(5-chloro) tricyclodecan)-4-yl) phenyl phosphate (CSPD) (Roche). The protocol was tested with viroid-infected trees of sweet orange, lemon, mandarin, grapefruit, sour orange, Swingle citrumello, Tahiti lime, and Mexican lime (Fig. 7.14). The Northern hybridization protocol did not require the use of Etrog citron for bioamplification of the target viroid and provided an alternative for reliable viroid indexing. The procedure and synthesis of probes were easy for viroid detection in *Citrus* spp. in commercial orchards. The Northern hybridization protocol was highly specific and sensitive for viroid detection (Murcia et al. 2009).

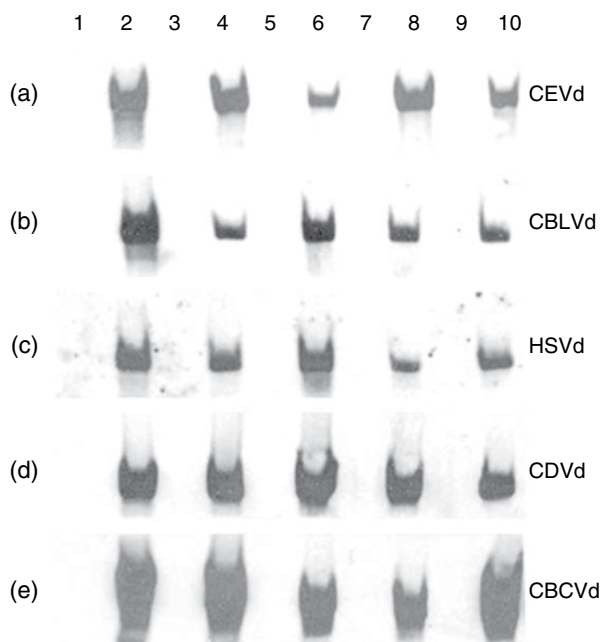


Figure 7.14 Detection of (a) CEVd, (b) CBLVd, (c) HSVd, (d) CDVd, and (e) CBCVd in mixed infections in citrus plants by Northern hybridization with viroid-specific probes. Lane 2: Fino lemon; lane 4: Common mandarin; lane 6: Tahiti lime; lane 8: Foster grapefruit; lane 10: sour orange; lanes 1, 3, 5, 7, and 9: corresponding healthy control. Source: Murcia et al. (2009). Reproduced with permission of Elsevier BV, USA.

Potato spindle tuber viroid (PSTVd) is an A2 quarantine pathogen as declared by EPPO and is transmissible by true seed, pollen, and seed tubers, as well as through contaminated tools. DIG-labeled cDNA probes were found to be effective in detecting PSTVd in the extracts of potato tubers and leaves (Nakahara et al. 1997). The incidence of PSTVd in ornamental solanaceous plants was assessed in Italy. *Solanum jasminoides* and *S. rantonnetti* showed symptoms of infection by viroid-like agents. In order to evaluate the possibility of detecting PSTVd in the infected ornamental Solanaceae, the simple, fast, and economic detection procedure of tissue-printing hybridization (TPH) was employed. Tissue prints were prepared by directly pressing freshly cut herbaceous stems of *S. jasminoides* and *S. rantonnetti*. Strong signals that perfectly matched those obtained with dot-blot hybridization were generated, while healthy controls showed consistently negative reaction. PSTVd diagnosis in potato using DIG-labeled probes should be confirmed by another technique such as RT-PCR and sequencing of the generated amplicon (Di Serio 2007).

Hybridization techniques have been applied for the detection of viroids infecting potato and fruit crops. The usefulness of labeled cDNA specific to *Peach latent mosaic viroid* (PLMVd) was demonstrated for detection of PLMVd efficiently in stem pieces and dormant buds of peach (Skrzeczowski et al. 1996). The sanitary status of the Clonal Genebank of stone fruit in Canada was evaluated by applying molecular methods. Tissue-printing hybridization (TPH) technique was employed to test a total of 336 trees comprising 116 peach and nectarine, 84 sweet and sour cherries, 54 plums, and 44 apricots for the presence of *Peach latent mosaic viroid* (PLMVd) and *Hot stunt viroid* (HSVd).

Infection of 28 of 30 peaches and nectarines by PLMVd and two apricots by HSVd was revealed by TPH assay. Viroid-free mother trees were identified by TPH procedure and were used for vegetative propagation (Michelutti et al. 2005). Tissue-printing hybridization (TPH) assay was employed to detect *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd), and *Pear blister canker viroid* (PBCVd) in about 1000 trees in the commercial orchards and nurseries located in Bosnia Herzegovina, Malta, Lebanon, Morocco, and Turkey. High rates of infection by PBCVd (5.4–13%) were observed. Of the 398 pear trees assayed by TPH, 35 tested positive for PBCVd. The Northern blot hybridization assay and/or RT-PCR procedure also confirmed the results of TPH method. By cloning and sequencing of the amplified cDNA products, the identity of PBCVd was confirmed for some isolates. TPH procedure was employed for testing samples from more than 800 trees during a survey undertaken in different countries indicated above. ASSVd was not detected in any of the apple trees in Morocco, Lebanon, and Bosnia Herzegovina or in pear trees (194) in Morocco and Lebanon. PBCVd was detected in one quince tree. The results of the investigation using TPH assay indicated that viroid-free germplasm should be imported and viroid-indexed mother trees alone have to be used for producing propagation material. TPH was found to be an easy and valuable tool for identifying viroid-free mother plants of pome fruit crops (Di Serio et al. 2010).

The viroids *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd), *Apple fruit crinkle viroid* (AFCVd), *Pear blister canker viroid* (PBCVd), *Hop stunt viroid* (HSVd), and *Peach latent mosaic viroid* (PLMVd) are well distributed worldwide, causing significant adverse impact on fruit production. These six viroids belong to two different families; with the exception of PLMVd, which is included in *Avsunviroidae*, five belong to *Pospiviroidae*. A simple and sensitive dot-blot hybridization assay using a digoxigenin (DIG-) labeled cRNA polyprobe was developed for the simultaneous detection of these six viroids infecting pome and stone fruit trees. The poly6 probe was constructed by cloning sequentially partial sequences of each viroid into a single vector, with run-off transcription driven by the 17 promoter. All six viroids could be detected with a dilution range of 5^{-3} to 5^{-4} in total nucleic acid extracts from infected trees. Two viroids PBCVd and PLMVd were detected in the 5^{-4} diluted samples. The other four viroids –ASSVd, ADFVd, AFCVd, and HSVd– were detected in the 5^{-3} diluted samples (Fig. 7.15). The respective detection limits of the poly6 probe for detecting each of the six viroids were similar to those of the single probes, indicating that the poly6 probe was reliable for the detection of its homologous viroids in the dot-blot hybridization assay. Nucleic acid extracts were prepared from the three apple and three pear trees inoculated with ADFVd, AFCVd, ASSVd, and PBCVd approximately 1 year after grafting. The dot-blot hybridization assay using the poly6 probe yielded intense hybridization signal in all six trees, and the identity of each viroid was confirmed using the viroid-specific primers. Combinations of the three apple viroids ASSVd, ADFVd, and AFCVd were found in the inoculated apple trees, and all four viroids were detected in the inoculated pear trees. Poly6 probe was found to be able to detect in six peach and six plum plants coinoculated with PLMVd and HSVd at different periods after inoculation. Of the 12 pooled samples, mixed infections of PLMVd and HSVd were present in 6 GR305 pooled peach samples and HSVd alone was detected in 5 plum samples. Of the remaining pooled sample, a myrobalan plum was not infected by either PLMVd or HSVd. Field samples (725) representing a diverse collection of germplasm accessions from throughout the world and commercial orchards were tested by dot-blot hybridization using poly6 probe. Most of the samples were free of the six target viroids. The hybridization

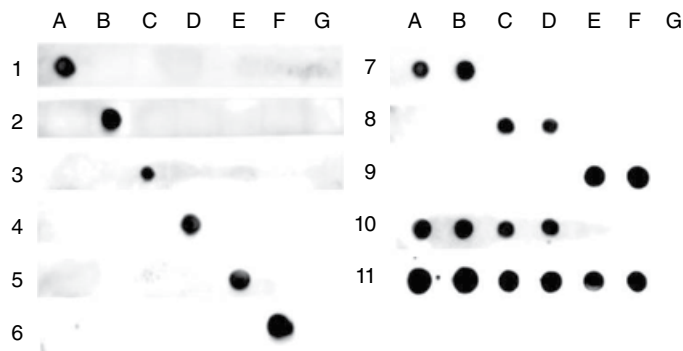


Figure 7.15 Detection of six viroids in mixed infections by dot-blot hybridization assay using DIG-labeled probes in extracts of pome and stone fruit trees. A1–11: extracts from plants infected by *Apple scar skin viroid* (ASSVd); B1–11: extracts from plants infected by *Apple dimple fruit viroid* (ADFVd); C1–11: extracts from plants infected by *Pear blister canker viroid* (PBCVd); D1–11: extracts from plants infected by *Apple fruit crinkle viroid* (AFCVd); E1–11: extracts from plants infected by *Hop stunt viroid* (HSVd); F1–11: extracts from plants infected by *Peach latent mosaic viroid* (PLMVd); G1–11: extracts from healthy plants. Single probes tested: row 1 (ASSVd); row 2 (ADFVd); row 3 (PBCVd); row 4 (AFCVd); row 5 (HSVd); and row 6 (PLMVd). Polyprobes tested: poly2-A (ASSVd and ADFVd, row 7); poly2-B (PBCVd and AFCVd, row 8); poly2-C (HSVd and PLMVd, row 9); poly4-AB (ASSVd, ADFVd, PBCVd, and AFCVd, row 10) and poly6 (row 11). Source: Lin et al. (2011). Reproduced with permission of Elsevier BV, USA.

assay using poly6 probe capable of detecting six viroids infecting pome and stone fruit trees in a single test could be particularly beneficial as an alternative to the single-probe assays, reducing cost, time, and labor requirements for diagnostic assays, especially for quarantine and certification programs (Lin et al. 2011).

7.4.1.2 Reverse-Transcription (RT-) PCR Techniques

Reverse-transcription (RT-) PCR-based methods have been applied for the detection of several viroids infecting a range of crop plant species, asexually propagated by different plant parts obtained from mother plants whose freedom from viroid infection has to be ensured. The effectiveness of detection of *Chrysanthemum stunt viroid* (CSVd) and *Potato spindle tuber viroid* (PSTVd) by dot-blot hybridization, RT-PCR, and TaqMan assays was compared using a wide range of isolates obtained from various locations and cultivars. Dot-blot hybridization and TaqMan assays efficiently and specifically detected CSVd and CSVd/PSTVd, respectively. On the other hand, RT-PCR alone was unable to differentiate CSVd and PSTVd. However, a combination of RT-PCR assay and RFLP analysis could be employed to differentiate these two viroids. The detection limits of dot-blot hybridization, RT-PCR, and TaqMan assays for CSVd were 1:100, 1:1000, and 1:100,000 respectively. When CSVd-primer-probe set and PSTVd-primer-probe set were employed, TaqMan amplification of the viroid genomes resulted in two distinct patterns based on which respective viroids could be detected in the RNA extracts from infected plants (Fig. 7.16). The TaqMan assay provided a high level of sensitivity, reduction in the risk of false-negative results, and elimination of contamination by using closed-tube format (Mumford et al. 2000b).

RT-PCR assay was effective in detecting *Potato spindle tuber viroid* (PSTVd) in tree potato pollen and seeds. Total nucleic acid (TNA) extracts or Gene ReleaserTM-treated extracts of plant tissues were subjected to PCR amplification. An amplified full-length

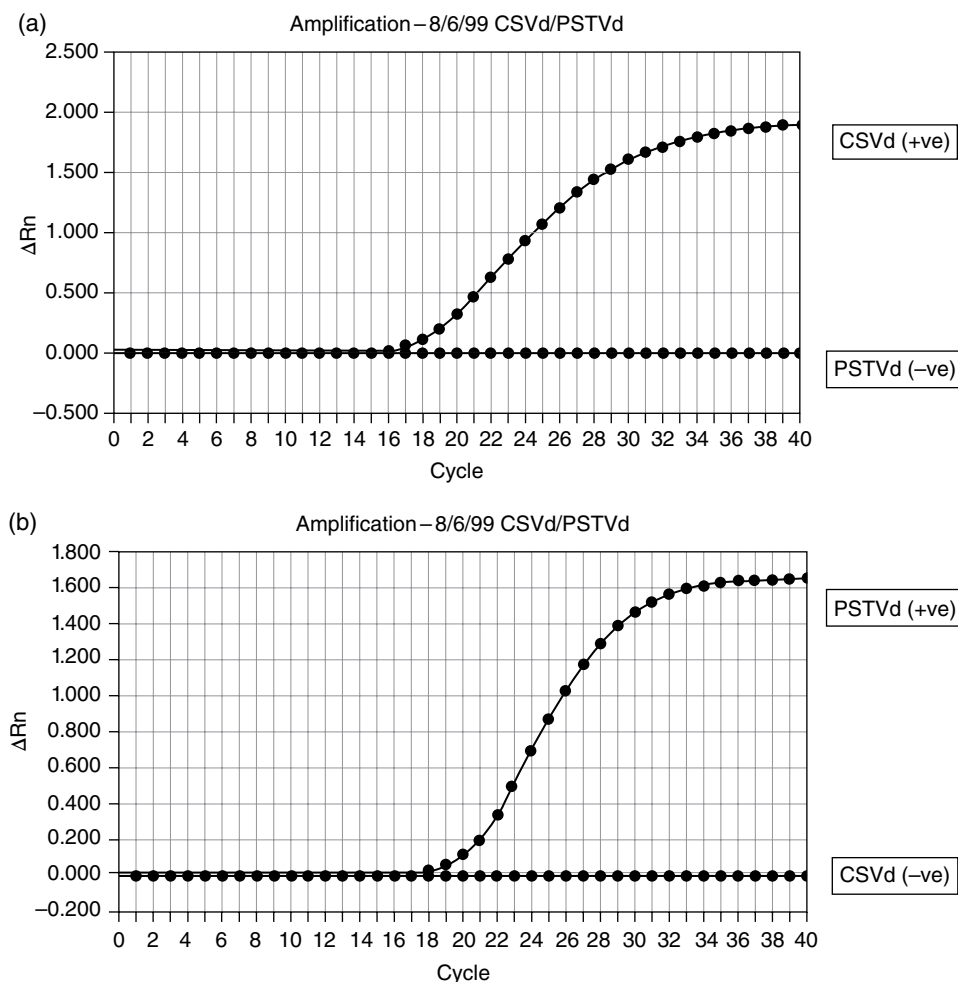


Figure 7.16 Detection of (a) *Chrysanthemum stunt viroid* (CSVd) and (b) *Potato spindle tuber viroid* (PSTVd) by real-time TaqMan assay using viroid-specific probes. TaqMan amplification plots for specific detection of CSVd and PSTVd. Source: Mumford et al. (2000a). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

PSTVd cDNA could be produced by treating as few as five pollen grains with Gene ReleaserTM (Shamloul et al. 1997). RT-PCR assay was employed to detect PSTVd using the primer pair PSTVd-321/PSTVd-323 which were complementary and identical to positions 156–179 and 180–203, respectively, of the PSTVd reference variant. The region covered by these adjacent primers was highly conserved in most known PSTVd variants. Using this primer set, a cDNA of about 360 bp corresponding to the full-length PSTVd RNA could be amplified from infected samples. PCR analyses showed that all samples testing positive by dot-blot hybridization generated an RT-PCR amplicon of this size, indicating the infection of the sample by PSTVd (Di Serio 2007).

Efficient methods have to be employed for the successful detection of viroids in plant tissues. In order to recover viroids, different extraction procedures and RT-PCR formats and other molecular techniques have been employed. Four extraction protocols were

compared for their efficiency to obtain RNAs of high concentration and purity from bark tissues sampled from *Citrus limon* plants infected by *Citrus exocortis viroid* (CEVd). The concentration of CEVd in the bark was 10 times higher than in the leaves of Etron citron, which was used as a bioamplification host for CEVd. RT-PCR assay provided a sensitive detection of CEVd by amplifying the entire viroid genome (Umaña et al. 2001). Detection of three citrus viroids, namely *Citrus exocortis viroid* (CEVd), *Citrus viroid II* (CVd-II) (*Hop stunt viroid*), and *Citrus viroid III* (CVd-III), in field-collected citrus plants in Florida was accomplished by employing RT-PCR assay. The PCR amplification products were analyzed by agarose gel electrophoresis. CEVd, CVd-II, and CVd-III were consistently detected in tissues from experimentally infected orange, lemon, and lime cultivars, but detection of viroids in the tissues of grapefruit and mandarins was less consistent, possibly due to low concentration and uneven distribution of the viroids in this host plant species. Bark tissues from woody, budwood-sized twigs were the best tissue sources for viroid detection. Samples collected in warm weather yielded better results than those obtained in winter. A collection of 55 viroid isolates derived from 44 different sources representing different hosts and locations in Florida was tested by RT-PCR assay. CEVd was detected in 22 sources infected by 20 isolates of CEVd, whereas 45 isolates of CVd-III were found in different source plants. The correlation between results of RT-PCR assay and biological indexing exceeded 90%. Several isolates causing moderate symptoms in Etron citron were not amplified by the primers specific to CEVd, CVd-II, and CVd-III. One of the isolates was identified as *Citrus viroid IV* which was found for the first time in Florida. The protocols developed in this investigation were successfully used in three different laboratories (Garnsey et al. 2002).

Citrus cuttings collected from field-grown plants were forced to sprout in a growth chamber in sterilized agriperlite and grown under artificial illumination (1000 lux and photoperiod for 16h) at a temperature of 27–32°C. Leaves from forced cuttings were tested at 15 or 30 days after sprouting by one-step RT-PCR. In forced cuttings, *Hop stunt viroid* (HSVd) and *Citrus viroid III* (CVd-III), but not CEVd, were readily detected in the leaves collected at 15 days after sprouting. However, leaves collected at 30 days after sprouting yielded amplicons of expected size for CEVd (371 bp), HSVd-cit (300 bp), and CVd-III (296 bp). Forced cutting method allowed citrus viroid detection when field conditions were unfavorable for their detection and identification from samples directly from the groves. Forcing cuttings to sprout in growth chamber at temperatures around 30°C seemed to promote viroid replication without the need for Etron 861-S1 citron for their amplification. The forced cuttings technique was applied routinely for indexing citrus plants along with sequential (s-) PAGE and bioindexing methods for the presence of citrus viroids in Italy (Guardo et al. 2005).

A one-tube RT-PCR assay was developed for the detection of *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd) in citrus samples (105) collected during the survey in citrus fields in central Italy. The assessment of frequency of viroid presence in this region showed that CEVd and HSVd infections were present in 71% and 19% of the citrus samples. Both viroids were more widespread in citrus germplasm from Lazio than from Tuscany regions. Of the 21 cultivars belonging to 9 *Citrus* spp. and 1 hybrid, 12 cultivars and the hybrid were infected with CEVd, one cultivar was infected with HSVd, and 3 cultivars showed infection by both viroids. The cultivars that showed no visible symptoms were grafted onto tolerant rootstocks. HSVd-positive samples were also asymptomatic. The one-tube RT-PCR format in a single PCR reaction detected both CEVd and HSVd, widely distributed and economically important viroids, in citrus

germplasm collections. The one-step RT-PCR protocol provided an earlier and more rapid detection and identification of the citrus viroids compared with the procedures employed earlier. Biological tests confirm the results obtained using one-step RT-PCR format developed in this investigation. Use of the one-step RT-PCR might reduce the number of biological tests required, since these tests need to be carried out only on viroid-negative samples (Ragozzino et al. 2005).

The yellow corky variant of *Hop stunt viroid* (HSVd-sycV) and *Citrus exocortis viroid* (CEVd) infecting Washington Navel orange was detected using a combination of RT-PCR and dot-blot hybridization (DBH) techniques. The RT-PCR products were subjected to DBH assay. Southern blot hybridization could detect HSVd only at a dilution of 1:100. RT-PCR assay was 10-fold more sensitive than Southern blot hybridization. Detection of DIG-labeled HSVd and CEVd cDNA products by probe capture hybridization was 100 to 1000 times more sensitive than detection by PAGE analysis. RT-PCR-DBH combination was sensitive and specific for the detection of targeted viroids (Fig. 7.17). RT-PCR-DBH showed two levels of specificity based on internal hybridization probe. RT-PCR-DBH assay, with a higher level of sensitivity, specificity, visual indication, adaptation to automation, and rapidity of providing results, could become a preferred diagnostic tool for diagnosis of viroids infecting citrus, especially for certification and quarantine programs (Bagherian et al. 2009).

RT-PCR assay was applied to assess the sanitary status of citrus varieties and/or clones with respect to the extent of viroid infection, in order to select elite mother plants of citrus for vegetative propagation. The results of RT-PCR assay indicated that all mother plants tested were infected by *Citrus exocortis viroid* (CEVd) and *Citrus bent leaf viroid* (CBLVd), although they were free of *Citrus tristeza virus* (CTV) and *Citrus variegation virus* (CVC). The viroids could be successfully eliminated from 3 lemon, 6 mandarin, and 20 “Jaffa” orange plants by adopting micrografting technique. These virus- and viroid-free plants formed the nuclear stock for government plantations, private nurseries, and growers in Cyprus (Kaparia-Isaia et al. 2007).

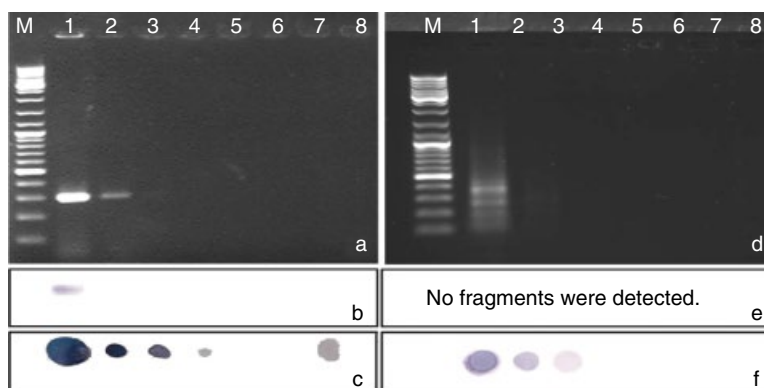


Figure 7.17 Comparative levels of sensitivities of electrophoresis, Southern blot hybridization, and RT-PCR dot-blot hybridization (DBH) for the detection of *Hop stunt viroid* (HSVd) and *Citrus exocortis viroid* (CEVd) in citrus. (a, b) electrophoresis; (b, e) Southern blot hybridization; (c, f) RT-PCR-DBH. Lanes: M: molecular standards (100 bp); 1–3: serially diluted positives (10^{-1} – 10^{-4}); 4: negative control without template; 5: RT product; 6: extracted RNA. Source: Bagherian et al. (2009). Reproduced with permission of Iranian Society of Virology, Iran.

The presence of citrus viroid in citrus collections from eight provinces maintained in the greenhouse of National Citrus Virus Exclusion Center, China was tested. A simple, one-step multiplex RT-PCR protocol was applied for the detection of five viroids, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV). Total nucleic acids (TNAs) were extracted using a micro and rapid nucleic acid extraction procedure. A cocktail of primer pairs specific to all five viroids was employed for simultaneous detection of these viroids by the one-step multiplex RT-PCR assay. Most of the plants showed infection by more than one viroid species, and a few of them were found to be infected by up to four citrus viroids. The protocol developed in this investigation revealed the extensive incidence of citrus viroids in many provinces of China, and the need for careful selection of viroid-free mother plants for vegetative propagation to establish viroid-free nuclear stock by employing sensitive and reliable diagnostic technique(s) (Wang et al. 2009).

The possibility of citrus budlings originating in the Middle East as the potential sources of citrus viroid inoculum was investigated. Citrus budlings (101) belonging to 9 citrus cultivars which originated from 5 countries collected from commercial nurseries were tested by the multiplex RT-PCR assay for the detection of *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus dwarfing viroid* (CDVd), and *Citrus bark cracking viroid* (CBCVd). Multiplex RT-PCR analysis, followed by sequencing of representative isolates, indicated that 29, 28, 54, 68, and 79% of the citrus budlings from Oman, Lebanon, Syria, Egypt, and Jordan showed positive reactions for CEVd, CBLVd, HSVd, CDVd, and CBCVd, respectively. Nucleotide sequence similarity of viroids from Oman was high (94–100%) with the citrus viroids from other countries. The results revealed that the citrus viroids could spread through budlings to other geographical locations (Al-Harthi et al. 2013).

Infection levels of *Hop stunt viroid* (HSVd), *Hop latent viroid* (HLVd), and *Apple fruit crinkle viroid* (AFCVd) in commercial hop (*Humulus lupulus*) plantings in three regions of intensive hop production in Washington State were assessed using RT-PCR formats. A one-tube duplex RT-PCR assay was employed for the detection of HLVd and HSVd, using viroid-specific primers, and a separate RT-PCR assay was applied for the detection of AFCVd using primers that reacted with a wide range of ascapiviroids. RT-PCR performed using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, CA). Higher-molecular-sized bands frequently appeared in assays for HSVd and HLVd. HSVd was detected in 10 of 33 hop gardens sampled. Samples in which HSVd was detected in 2004 originated from all three regions of hop production. The results suggested that HSVd could have remained undetected for a significant period of time, leading to its widespread incidence. HLVd was also widely distributed in Washington hop production, as it was detected in all three regions and in all gardens except one sampled. None of the plants tested yielded positive results for AFCVd, reported in hops in Japan. *Hop latent viroid* was frequently associated with high frequency (98 of 126 hop plants tested), indicating the need for use of sensitive and reliable techniques for testing the hop plant materials (Eastwell and Nelson 2007).

The association of *Hop stunt viroid* (HSVd), and other viroids and viruses, with plum dapple fruit and fruit rugosity was investigated using RT-PCR assay. Among the viruses, *Apple chlorotic leaf spot virus* (ACLSV) was the only virus that could be detected in the plants tested; *Plum latent mosaic viroid* (PLMVd) was also detected by RT-PCR assay. HSVd was detected in all symptomatic samples, but asymptomatic samples did not show the presence of HSVd. Sequencing of the cloned HSVd isolates showed a high

homology (99–100%) among clones except F20.2 with 92% homology. The parental pollen donor plants “Black sunrise” and “Black glow” were found to be infected with the same HSVd isolate of the fruit seedlings. The pollen collected from the parental plants was also positive for the RT-PCR assay, indicating the presence of the viroid both inside and outside of pollen granules. The results indicated the possibility of infected pollen being the reservoir of HSVd inoculum, in addition to the infected plum plants (Luigi et al. 2010).

The extent of incidence of *Apple scar skin viroid* (ASSVd) in Xinjiang Province of China was assessed by applying RT-PCR and in situ RT-PCR assays. ASSVd-specific primers amplified fragments of about 330bp from extracts of samples from infected apple, pear, apricot, and peach trees. The RT-PCR assay detected 15 strains of ASSVd from apple, 13 strains from pear, 11 strains from peach, and 12 strains from apricot samples. Sequence analysis of the selected clones showed that the full-length of ASSVd contained 330-nt. The detection rates for ASSVd were 2.1%, 2.1%, 2.8%, and 3.3% in apple, pear, peach, and apricot, respectively. The extent of incidence of ASSVd in different countries varied over 0.4–8.5%. The in situ RT-PCR assay revealed the blue-purple positive signals in the nucleus of palisade tissues of ASSVd-infected plants, but not in healthy plant tissues. The presence of ASSVd-specific plant tissues and ASSVd-specific nucleotide sequences was detected in infected tissues confirming the infection by *Apple scar skin viroid* (Wang et al. 2012).

A duplex RT-PCR assay in combination with a plant mRNA as internal control was developed to evaluate the quality of RNA extracted from a dormant potato tuber, and the effectiveness of detection of *Potato spindle tuber viroid* (PSTVd) by the duplex RT-PCR was also assessed. Furthermore, a noninfectious RNA based on the PSTVd sequence, was generated in vitro as a readily available positive control to prevent false-negative results. This noninfectious RNA was distinguishable from the true positives in PSTVd infection. This protocol provided an accurate and reliable system that could be useful for routine application in plant quarantine and phytosanitary certification programs (Hataya 2009).

The members of the genus *Pospiviroid* infect potato, tomato, and ornamental plants and nine species, namely *Potato spindle tuber viroid* (PSTVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Mexican papito viroid* (MPVd), *Tomato planta macho viroid* (TPMVd), *Chrysanthemum stunt viroid* (CSVd), *Citrus exocortis viroid* (CEVd), *Tomato apical stunt viroid* (TASVd), *Iresine viroid* (IrVd), and *Columnnea latent viroid* (CLVd) (Hadidi and Candresse 2003). RT-PCR assay was employed to detect the viroids in a total of 1766 samples consisting of 891 potato tubers, 156 potato leaves, 258 tomato leaves, and 461 ornamental plants. PSTVd was detected in 4 of 891 tubers, and CSVd in 2 of 154 chrysanthemums grown in greenhouses. Samples of tomato and ornamental plants did not show infection by any viroids belonging to *Pospiviroid* in Turkey, except CSVd which was detected for the first time. Amplification of a 199bp fragment for both PSTVd and CSVd occurred in the infected plants, but not in healthy control plants. The amplified RT-PCR product was visualized as the distinct band by return (R-) PAGE procedure. The results indicated that potato seed tuber and ornamental plant should be rigorously tested by sensitive diagnostic assays to prevent the introduction of *Pospiviroid* spp. which may be transmitted through seeds and vegetatively propagated planting materials (Bostan et al. 2010).

As potato is infected by several viruses and viroids, the need for standardization of RT-PCR procedures was realized; a standardized amplification protocol was therefore

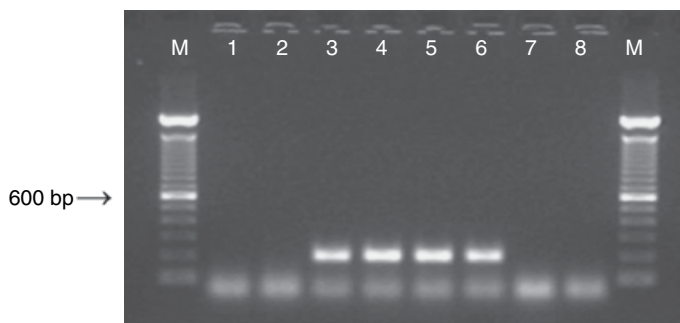


Figure 7.18 Detection of *Potato spindle tuber viroid* (PSTVd) in potato, tomato, and petunia by RT-PCR assay. M: DNA size standards; lanes 1, 2, and 7: healthy tomato, petunia, and potato; lane 3: dry tomato infected with Cornell severe PSTVd strain; lane 4: freshly infected petunia; lanes 5 and 6: dry tomato infected with PSTVd-S; lane 8: water control. Source: Crosslin and Hamlin (2011). Reproduced with permission of Springer Science, Heidelberg, Germany.

developed for the detection of PSTVd and 11 important viruses of potatoes. Further, the same RT-PCR conditions could be effectively adopted for the detection and differentiation of major strains of *Potato virus Y* (PVY). The validated primers and the standardized RT-PCR conditions could provide a robust, convenient, and cost-effective detection useful for potato seed tuber certification agencies and commercial diagnostic laboratories. The ability to test for the presence of these diverse pathogens with the same reagents, thermal cycling parameters, and gel electrophoretic conditions might simplify the diagnostic testing for these important potato pathogens (Fig. 7.18) (Crosslin and Hamlin 2011).

Peach latent mosaic viroid (PLMVd), belonging to the *Avsunviroidae* family, remains latent in orchards for several years and it is transmitted via propagation materials and the cutting and grafting tools commonly used in production areas (Flores et al. 2004). Rapid, reliable, and sensitive techniques are therefore required to avoid the uncontrolled spread of PLMVd and certification of false-negative planting materials/plants. Use of endpoint RT-PCR and molecular hybridization assays were found to be insufficient to avoid false negatives. A complete detection procedure based on reverse transcription (RT) followed by a quantitative real-time PCR format was developed. Initially dimeric PLMVd clones were employed to avoid the difficulties of reverse transcription and inhibitory effects of total extract. Five reverse primers were selected in conserved regions of the PLMVd highlighted by a multiple sequence alignment of 119 PLMVd sequences. In order to avoid selecting nonspecific primers amplifying plant DNA or RNA, the reverse primers R14, R12, R7, R3, and R2 combined with the forward primer (F-PLMVd) were tested by RT-PCR on crude sap extract from peach tree leaves. Three PLMVd-specific primers identified in this manner yielded amplicons of 65 to 135 bp, as recommended for real-time RT-PCR (qPCR). Based on the higher determination coefficient of primers, the F-PLMVd/R7 pair ($R^2 = 0.991$) was selected for further optimization. The comparative effectiveness of reference RT-PCR and RT-qPCR assays was determined. RT-PCR only detected PLMVd at a dilution of 1:100. On the other hand, RT-qPCR assay proved to be 10^5 times more sensitive than the conventional RT-PCR format. The reproducibility of the qPCR assay was checked by independent amplifications from the same extract. The melting curve confirmed specific

amplification of PLMVd even in the most diluted samples (10^{-7}). In order to determine the suitability of RT-qPCR assay for certification programs, the assay was performed using the extracts from two young peach seedlings inoculated at three months after emergence. The reference RT-PCR format did not detect PLMVd in the samples harvested until 8 months postinoculation. The optimized RT-qPCR assay detected PLMVd from 3 months after postinoculation and quantified the viroid concentration in the infected seedlings. The optimized RT-qPCR protocol developed in this investigation incorporates quick, low-cost extraction procedure and efficient primers, making the procedure suitable for routine testing in certification and quarantine programs (Parisi et al. 2011).

7.4.1.3 Reverse-Transcription Loop-Mediated Isothermal Amplification

Reverse-transcription (RT-) loop-mediated isothermal amplification (LAMP) technique is a simple, robust, and rapid method that has been applied for the detection of different kinds of microbial plant pathogens in infected plants. Because of its isothermal nature, RT-LAMP method has the potential for deployment in the field tests. RT-LAMP method was developed and validated for the detection of *Potato spindle tuber viroid* (PSTVd) in potatoes and tomatoes along with RT-PCR format in order to assess the relative sensitivity, rapidity, and reliability of the methods. RT-LAMP procedure was employed to detect PSTVd in total nucleic acid (TNA) extract from infected tubers and tomato leaves using the primers PSTVd-F3, -B3, FIP, -BIP, -LE, and -LB. An increase in the turbidity (formed as white precipitate in the RT-LAMP mixture) began to appear in about 30 min and reached the highest concentration in about 45–60 min. The specific amplifications of the target PSTVd RNA were confirmed by agarose gel electrophoresis. The detection sensitivity of RT-LAMP was about 10 times greater than that of RT-PCR format. RT-LAMP assay was applied to detect PSTVd in the extracts of a potato tuber, 400 potato seeds, and 400 tomato seeds. The viroid was detected stably in all infected samples. PSTVd was detected in the clarified crude sap from PSTVd-infected potato leaves and tomato leaves within 60 min. Further, PSTVd could be detected in the extracts of one tomato seed, but not in any of the 400 potato seed samples tested by RT-LAMP method. The RT-LAMP procedure was simplified by using clarified crude sap and an insect pin. Potato leaves and tomato leaves were struck with insect pins (No. 5) several times and the pins were dipped into 25 μ L of RT-LAMP reaction mixture. PSTVd could be stably detected from infected leaves of tomato. However, detection of PSTVd in potato leaf extracts was inconsistent. The time needed for detection of PSTVd for RT-LAMP method was about one-third of the time required for performing the RT-PCR assay, since PSTVd could be detected by RT-LAMP method under isothermal conditions without gel electrophoresis. Consequently, RT-LAMP method could detect PSTVd rapidly and with high sensitivity compared with RT-PCR assay with these advantages. RT-LAMP procedure might become an alternative for efficient and reliable detection of PSTVd in potatoes and tomatoes (Tsutsumi et al. 2010; Section 7.5.5).

In order to improve the effectiveness of detection of *Potato spindle tuber viroid* (PSTVd) in a single potato tuber, real-time reverse-transcription (RT-) loop-mediated isothermal amplification (RT-LAMP) technique was developed. Amplification was followed in real-time and the specificity of the final product was confirmed by its melting temperature. Total RNA was extracted from fresh leaves, tuber tissue, or lyophilized plant material using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions with minor modification. The sequences of 173 PSTVd isolates were

aligned and the consensus sequence was used for LAMP primer design. The analytical specificity of RT-LAMP was tested experimentally against a collection of 16 PSTVd isolates and 42 isolates belonging to 6 other species of the genus *Pospiviroid*. The specificity of the RT-LAMP assay was compared with that of the single-step real-time RT-PCR format. Both methods detected PSTVd and *Tomato planta macho viroid* (TPMVd), which are closely related. However, the signals from RT-LAMP assay could be distinguished from the PSTVd signal by melting temperature analysis. The specificity of the RT-LAMP assay was also compared with that of Tsutsumi RT-LAMP procedure (Tsutsumi et al. 2010) which was able to detect only 14 of 16 PSTVd isolates, 2 of them only with late signals (after 31 min). The analytical sensitivity of RT-LAMP assay for PSTVd was estimated to be between 100 and 1000 viroid copies per reaction, a 10-fold greater sensitivity than that of the RT-PCR and 100 times lower than that of real-time RT-PCR assays. Further, RT-LAMP assay was more sensitive (10-fold) than the Tsutsumi RT-LAMP method.

Another format of the assay was developed, based on formation of turbidity in the PCR reaction mixture. RT-LAMP assay required about 10 min less than that needed for the optimized Tsutsumi RT-LAMP format. The one-step RT-LAMP assay could be a simple first-line screening of potato PSTVd RNA, allowing real-time detection of this pathogen of quarantine importance. A few cross-reactions observed in the RT-LAMP assay might be resolved from the signals observed. *Tomato chlorotic dwarf viroid* (TCDVd), *Tomato planta macho viroid* (TPMVd), *Chrysanthemum stunt viroid* (CSVd), and *Columnea latent viroid* (CLVd) could be differentiated from PSTVd based on melting curve analysis. This RT-LAMP protocol could be employed to detect PSTVd in potato tuber and leaves and also in tomato and ornamental hosts of PSTVd. The speed, lower screening time, and cheaper reagents and consumables required for the RT-LAMP assay may significantly reduce the cost of testing (Fig. 7.19) (Lenarčič et al. 2012).

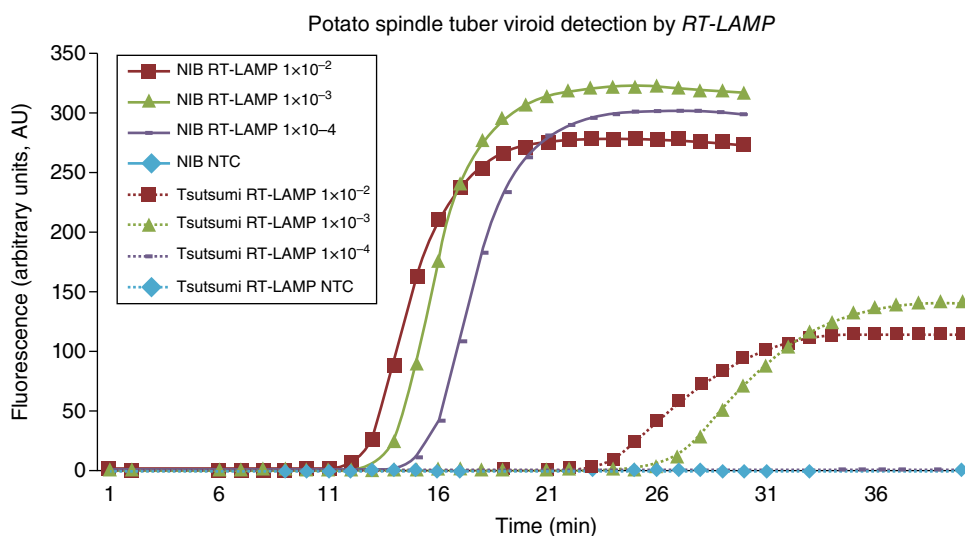


Figure 7.19 Comparative sensitivity of RT-LAMP and Tsutsumi RT-LAMP assays for detection of *Potato spindle tuber viroid* (PSTVd) using different dilutions of total potato plant RNA. Dilutions of total RNA: 1×10^{-2} ; 1×10^{-3} ; 1×10^{-4} ; control: water (without template). Source: Lenarčič et al. (2012). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

An outbreak of *Potato spindle tuber viroid* (PSTVd) occurred in commercial glasshouse-grown tomato crops in Queensland, Australia. Phylogenetic analysis revealed that the genotype of this isolate grouped in a cluster of PSTVd genotypes from tomato and *Physalis peruviana* and had a mutation ($U_{257} \rightarrow A$) that was earlier linked to lethal symptom expression in tomato. The viroid could be mechanically transmitted from crushed fruit sap but not from undamaged fruits. A low rate of asymptomatic infection was recorded among the affected plants in the glasshouse. None of the solanaceous weed species present outside the glasshouse was infected by PSTVd, indicating that the weeds were not likely to have a role in PSTVd epidemiology. Monitoring and subsequent testing of new tomato crops grown in the glasshouse showed that viroid infection had been successfully arrested. The trace-back analysis linked the outbreak of PSTVd to an infected tomato seed lot, indicating that PSTVd was transmitted internationally through contaminated seeds (van Brunschot et al. 2014).

7.4.1.4 Single-Strand Conformation Polymorphism Analysis

Six isolates of *Peach latent mosaic viroid* (PLMVd), inducing three different symptoms under field conditions, were examined by using single-strand conformation polymorphism (SSCP) and nucleotide sequence analyses. Each of the PLMVd isolates was found to be composed of a population of genetically related variants (haplotypes), one being predominant with frequencies of 32–57% and most of the others having a low frequency of 4–5%. Each predominant haplotypes was selected randomly to compare the nucleotide sequences. The predominant haplotype displayed a wide representative of sequence for others in a PMLVd isolate. The similarities and genetic distance between the predominant sequences from peach showing the same symptoms were higher than that with different symptoms: >98.8% and <1% between the predominant sequences with different symptomatic source, respectively. The results suggested that some correlations might exist between the genetic diversity and symptoms, but whether change in the bases are crucial factors in deciding the type of symptoms induced by the isolates of PLMVd has still to be determined by further investigation (Xu et al. 2008b).

7.5 Appendix

7.5.1 Detection of *Citrus Tristeza Virus* by Improved Direct Tissue-Blot Immunoassay (I-DTBIA) in Citrus Plants (Lin et al. 2000, 2006)

7.5.1.1 Preparation of Antibodies

- i) Use purified IgG as antibody sources for polyclonal antibody PCA1212 and MAb MCA 13; use undiluted cell culture fluid as source for MAb 17G11.
- ii) Use goat antimouse Ig (H + L) -alkaline phosphatase conjugate (GAM-AP) as secondary antibodies to react with CTV MAbs and goat antirabbit IgG alkaline phosphatase conjugate (GAR-AP) to react with CTV PCA (GAM-AP and GAR-AB) supplied by Southern Biotechnologies Assoc Inc., AL and Sigma Chemical Co, MO, USA.
- iii) Prepare prereaction solutions using PBS buffer containing 0.15 M sodium chloride, 0.015 M sodium phosphate, pH 7.0 or PBS-B buffer containing 1% fetal bovine serum (BS); prepare prereaction solutions for MAbs by directly adding GAM-AP into MAb17G11 cell culture fluid; maintain the concentrations of PCA1212 and MCA13 at $1.0 \mu\text{g mL}^{-1}$ and dilutions of GAM-AP were 1/1000 to 1/4000 and dilutions of GAR-AP at 1/5000 to 1/10000.

7.5.1.2 Improved DTBIA Procedure

- i) Cut sections (100–200 μm thick) using a new razor blade from fresh stems, petioles, or veins from healthy or infected plants; maintain 4–6 replicates for each sample; gently and evenly press the fresh-cut surface of the stems onto nitrocellulose membrane.
- ii) Incubate with prereaction solutions of CTV-specific antibodies ($1.0 \mu\text{g mL}^{-1}$) mixed with labeled secondary antibodies (1/1000 for GAM-AP, 1/5000 dilution for GAR-AP) for 20 min and rinse with PBST buffer (0.15 M sodium chloride, 0.015 M sodium phosphate, pH 7.0, 0.05% Tween-20) for 5 min.
- iii) Incubate the blots with 300–500 μL of a freshly prepared NBT-BCIP substrate mixture (66 μL of nitroblue tetrazolium at 0.3 mg mL^{-1} and 33 μL of 5-bromo-4-chloro-3-indolyl phosphate at 0.15 mg mL^{-1} in 10 mL of 0.1 M sodium carbonate buffer, pH 9.8) for 5–15 min; stop the reaction by removing the substrate solution and adding 500–1000 μL of water.
- iv) Record the results by observing under a light microscope at $\times 10$ to $\times 25$ magnification; positive reaction is indicated by development of purple color in blots.

7.5.2 Detection of Tomato Spotted Wilt Virus (TSWV) in *Ranunculus* Tubers by Tissue-Blot Immunoassay (Whitfield et al. 2003)

7.5.2.1 Preparation of Plant Tissue Samples

- i) Roll leaf samples into tight bundle; slice with a razor blade and gently blot the oozing sap onto 0.45 μm Nitro ME nitrocellulose membranes (Micron Separations, Inc., MA) for 3–5 s.
- ii) Incubate the rootlets sampled from dried tubers (*Ranunculus*) for 16–20 h in deionized (DI) water in 24-well microtitre plates at room temperature (RT); cut cross-sections of each rootlet using a razor blade and press the cut surface gently into nitrocellulose membrane.

7.5.2.2 Tissue-Blot Immunoassay (TBIA)

- i) Air-dry the blotted membranes at RT; place them in hybridization tubes and allow them to react with reagents as indicated in the following.
- ii) Carry out all incubations and wash steps at RT in a hybridization oven (Lab-Line, Barnstead, IA) at 16 rpm; expose membranes to reagents sequentially and pour in and out the reagents from the tubes without removing the membrane.
- iii) Block the membranes with 10 mL of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (0.14 M sodium chloride, 1.0 M potassium phosphate, 8.0 mM sodium phosphate and 2.5 mM potassium chloride at pH 7.5) for 1 h.
- iv) Treat the membranes with appropriate dilution of anti-NSs monoclonal antibody from a stock solution (MAb at 1 mg mL^{-1}) by diluting in 4 mL of antibody dilution buffer (PBS, 0.1% Empigen BBe (Calbiochem La Jolla, CA) and 0.1% BSA); incubate the antibody for 2 h; wash three times in PBS with 0.5% Tween (PBST) for 5 min/wash followed by incubation in 4 mL of a 1:2000 dilution (in antibody dilution buffer) of rabbit antimouse alkaline phosphatase conjugate (Biorad, Richmond, CA) for 1 h.
- v) Wash the membranes again in PBST (3 times, 5 min/wash); add alkaline phosphate substrate (Sigma Fast BCIP/NBT tablets, b5655); incubate the membranes for 5–15 min; rinse in water and examine after air drying at RT.
- vi) Observe for the development of purple color, indicating the positive reactions.

7.5.3 Detection of *Citrus Tristeza Virus* (CTV) by Hybridization Assays (Narváez et al. 2000)

7.5.3.1 Preparation of Nucleic Acid Extracts

- i) Pulverize young bark of CTV-inoculated and healthy sweet orange plants with liquid nitrogen; extract with 4 mL g⁻¹ tissue of 2 × STE (10 × STE = 0.5 M Tris-HCl, 1 M NaCl, 10 mM Na₂EDTA, pH 6.8), 1% SDS, 50 μL of a bentonite suspension (32 mg mL⁻¹) and 12 mL g⁻¹ tissue of STE-saturated phenol/chloroform (1:1 v/v) and centrifuge at 10,000 g for 15 min.
- ii) Collect the supernatant containing total nucleic acids; precipitate with cold ethanol and sodium acetate and resuspend in 50 μL distilled water (concentrated RNA).
- iii) Purify ds-RNA from the total nucleic acid extract by nonionic cellulose (CF-11, Whatman) column chromatography in the presence of 16% ethanol.
- iv) Denature aliquots of 2 μL of nucleic acid extract with an equal volume of formamide at 94°C for 3 min; chill on ice and spot the solutions to nylon membranes (Hybond, Amersham) or polyvinylidene difluoride (PVDF) (Immobilon-N, Milipore) and bake the membrane at 80°C for 2 h in a vacuum oven or irradiated with UV light (50 mJ) in cross-linking oven.
- v) Prepare tissue prints by cutting transversely tender shoots or leaf petioles, gently pressing the cut surface to the membrane and air dry.

7.5.3.2 Hybridization

- i) Prehybridize the membranes for 2 h in 6 × SSC (20 × SSC = 3 M NaCl, 0.3 sodium citrate, pH 7.0), 5 × Denhardt's solution (50 × Denhardt's = 1% Ficoll, 1% bovine serum albumin, 1% polyvinylpyrrolidone), 1% sodium dodecylsulfate (SDS) and 100 μg mL⁻¹ denatured Salmon sperm DNA at 60°C for nonradioactive detection.
- ii) Perform hybridization at 60°C overnight, adding 25 ng of the DIG-labeled probe; wash the membranes twice (15 min each) in 2 × SSC and 1% SDS at room temperature twice in 0.1 × SSC and 0.1% SDS at 60°C.
- iii) For differentiation of CTV isolates using tissue prints, hybridize the membranes as per the Labelling and Detection Kit (Boehringer) instructions at 60°C in 50% formamide; wash twice with maleate buffer plus 0.3% Tween-20, after incubation with anti-DIG antibody.
- iv) Detect positive hybridization with the chromogenic substrate 5-bromo-4 chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) or with the chemiluminescent substrates LPPD or CSPD (Boehringer Mannheim), as per manufacturer's instructions.

7.5.4 Detection of *Plum Pox Virus* (PPV) by Real-Time RT-PCR and Its Variants (Capote et al. 2009)

7.5.4.1 Preparation of Crude Extracts of Plant Tissues

- i) Collect samples from adult *Prunus* trees (dormant or swelling buds) in winter; grind in plastic bags containing a heavy net (Plant Print Diagnostics, Valencia) using a hammer in the presence of 1:20 PBS buffer, pH 7.2 supplemented with 2% polyvinylpyrrolidone and 0.2% sodium diethyl dithiocarbamate, and use the same extracts for all detection procedures.

- ii) Prepare serial dilutions (1:10 to 1:10¹¹ v/v) of the extract from PPV-infected and healthy peach seedling (GF305).
- iii) Use sections of leaf petioles and sectors of expanded leaves for GF305 peach seedlings to prepare imprints and squashes on membranes.

7.5.4.2 Real-Time RT-PCR and Dilution, Spot, Tissue Print, and Squash Variants

- i) For conventional RT-PCR, extract total RNA from 200 µL of crude extracts using RNeasy Plant MiniKit (Qiagen) or Ultraclean Plant RNA isolation kit (MoBio).
- ii) For real-time RT-PCR, use 5 µL of total RNA as template with plant crude extract; dilute 5 µL in 100 µL of conventional extraction buffer and use 5 µL of the diluted sample.
- iii) For spot procedure, load 5 µL of plant crude extract onto 0.45 µm positively charged nylon membrane (Roche) or on Whatman 3 MM paper filter.
- iv) For tissue print, gently press fresh sections of tender shoots or leaves on the membranes to make one or several prints; combine leaf petiole imprints from a PPV-infected GF305 peach seedling with overlapping petiole imprints of a healthy GF305 in the same site of the membrane in different ratios (infected:healthy) of 0:8, 1:7, 2:6, 3:5, 4:4, 5:3, 6:2, 7:1, and 8:0.
- v) Squash fresh tissues on the membrane with the bottom portion of the Eppendorf tube; insert sections of membranes with immobilized samples (spotted, tissue-printed, or squashed plant materials) into Eppendorf tubes or placed inside ELISA plate wells for large-scale assays and extract RNA targets with 0.5% Triton X-100 or glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA, pH 8.0).
- vi) Prepare reaction cocktail for SYBR Green Chemistry containing 1 × Power SYBR Green PCR Master Mix (Applied Biosystems), 10 units of RNase inhibitor (Applied Biosystems), 0.3 µM primer P₁ and 0.05 µM primer P₂.
- vii) Provide the RT-PCR conditions as follows: 48°C for 30 min, 95°C for 10 min and 45 cycles of amplification (95°C for 15 s, 60°C for 1 min); prepare a melting curve for temperatures between 60 and 95°C in all cases to verify the specificity of reactions and perform data acquisition and analysis using an ABI Prism 700 and StepOnePlus software packages (Applied Biosystems).

7.5.5 Detection of *Potato Spindle Tuber Viroid* by Reverse-Transcription Loop-Mediated Isothermal Amplification (LAMP) (Tsutsumi et al. 2010)

7.5.5.1 Extraction of Total Nucleic Acid from Potato Leaf, Tuber, and Tomato Leaf Tissues

- i) Extract total nucleic acid from infected plant tissue by sodium dodecylsulfate (SDS-) potassium acetate method; grind the samples (50 mg/sample) in 2 mL buffer solution (0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl, 1% 2-mercaptoethanol, 0.005 EDTA (pH 8.0), 1.25% SDS, 3.3% PVP and 1.25 M potassium acetate (40 v/w)) and stir thoroughly.
- ii) Centrifuge for 10 min at 15,000 g and separate the supernatant.
- iii) Add half volume of 2-propanol to the supernatant; mix and centrifuge for 10 min at 15,000 g.
- iv) Add equal volumes of 99.5% ethanol and 1/10 volume of 3 M sodium acetate to the supernatant and mix well; dissolve the precipitate containing total nucleic acid in 50 µL of 0.1 TE (pH 8.0) and use 1 µL of total nucleic acid as the template for the LAMP reaction.

7.5.5.2 RT-LAMP Reaction

- i) Perform RT-LAMP reaction with a LoopampRNA amplification reagent kit (Eiken Chemical co) in 25 μ L of the mixture containing 1 $\times$ reaction mix (40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM MgSO₄, 20 mM (NH₄)₂ SO₄, 0.2% Tween-20, 1.6 M Betain, 2.8 mM each dNTPs), 0.2 μ M each of F3 and B3 primers, 1.6 μ M each of FIP and BIP primers, 0.8 μ M each of LF and LB primers, 1 μ L Enzyme mix (AMV reverse transcriptase and *Bst* DNA polymerase) and 1 μ L of total nucleic acid.
- ii) Measure turbidity indicating target gene amplification by a real-time turbidity meter (LA-2003 Termecs Co) and carry out the reactions at 65°C for 80 min.
- iii) An increase in turbidity within 60 min indicates positive reaction.

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8

Biology and Infection Process of Viruses and Viroids

Viruses are ultramicroscopic and intracellular obligate pathogens, capable of infecting all living organisms from primitive prokaryotes to highly evolved human beings. Although infection of tulips resulting in tulip break disease was known of for a long time, it was not until the late nineteenth century that viruses were proven to be the causative agents of plant diseases. As viruses are obligate pathogens, requiring the presence of living cells for their existence, their presence in the plants and vectors involved in their transmission from infected plants to healthy plants has to be detected at the earliest possible time by employing sensitive, specific, rapid, and reliable techniques to identify the sources of inoculum. The management of plant virus diseases is largely limited to preventive methods by eliminating infected seeds and propagules and establishing virus-free seed stocks. If seeds and planting materials are not assessed for virus infection they may ruin crops within a short period of time due to the rapid increase in infection levels through subsequent generations. It is imperative to monitor the movement of seeds and planting materials, as globalization facilitates unrestricted exchange of seed materials between countries and regions in a country. Knowledge of various factors that influence virus infection and the development of disease in individual plants, and the spread of disease in individual plants and in plant populations, is required to develop short- and long-term strategies to restrict disease incidence and subsequent spread in the field. Furthermore, the biology of viruses has to be studied to understand their pathogenic potential (virulence), genetic variability, host range, virus–vector relationship, and their survival in the absence of primary host (crop) plant species in order to target the weak link in the disease cycle for the eradication of the virus disease(s).

8.1 Characteristics of Plant Viruses

Plant viruses have simple morphological characteristics with limited variations in the particle morphology that are useful for their reliable identification and differentiation. All viruses have a protein coat (covering) to protect viral genomic nucleic acid from inactivation. The viral nucleic acid may be of either ribonucleic acid (RNA), as in the majority of plant viruses, or deoxyribonucleic acid (DNA) molecules. The viral genome may be encapsidated either in one type of particle (monopartite) or in two (bipartite) or more types of particles (multipartite). There is no evidence for the production of any virus-specific enzyme present in the virus particle or in their capacity

for producing any enzyme of their own during the infection process (pathogenesis). They have to depend entirely on the synthetic machinery of the susceptible host plant for their replication, leading to the development of various types of external and internal symptoms depending on the virus–host interactions. The virus replication process is distinct from other cellular pathogens. Viruses represent particulate entities which are similar to cellular constituents and do not possess any organelle of cellular organisms. The viral components – coat protein (CP) and genomic nucleic acid – are synthesized separately in different sites in susceptible host cells. Viral genomic nucleic acids carry the required information for synthesizing viral components, taking command of the host's synthetic systems and directing the synthesis of the required amino acids and nucleotides for viral coat protein and genomic nucleic acid. When the viral coat protein and nucleic acid reach required concentrations, they are assembled to form infective mature virions (Narayanasamy 2011). The nature of viral genomic nucleic acid, particle morphology, presence of membranous envelope, immunological properties, nucleotide sequences of genomic nucleic acid and amino acid sequences of viral coat (capsid) protein, and the presence of virus-associated protein are the major characteristics used for the identification, classification, and differentiation of plant viruses and their strains (Chapter 7).

8.2 Biological Properties of Viruses

8.2.1 Types of Symptoms Induced by Viruses

The pathogenic potential (virulence) is the basic requirement of any organism to be considered as a pathogen. Plant viruses may infect one or more plant species which may produce visible external symptoms or may remain latent without inducing any recognizable symptoms of infection. The plant species that are asymptomatic can be potential reservoirs of virus inoculum which may be spread by the natural biological vectors or by other means. The symptoms induced by viruses may be macroscopic (externally visible) and/or microscopic (internal) symptoms characteristic of virus infection. The severity of symptoms may differ depending on the levels of susceptibility/resistance of the cultivars to the virus concerned. Likewise, the ability to allow the transmission of the virus(es) through seeds varies depending on the cultivars of the crops. The mechanism of virus transmission through seed was investigated by inoculating *Arabidopsis thaliana*, the model plant, with *Turnip yellow mosaic virus* (TYMV) and *Tobacco mosaic virus* (TMV). Serological and biological tests (grow-out) were conducted to identify the route by which the viruses could reach the seed and their locations in the seed. Both TYMV and TMV were detected in seed from infected plants. However, only TYMV was transmitted from infected seed to seedlings that grew from infected seed as revealed by the grow-out test and enzyme-linked immunosorbent assay (ELISA) test. The mere presence of the virus in the seed coat did not result in seedling infection. Embryo infection was shown to be necessary for seed transmission, which was inferred from the successful seed transmission of TYMV in *A. thaliana* (de Assis Filho and Sherwood 2000). Natural seed transmission of *High Plains virus* (HPV) to the seedlings grown from infected corn seeds was confirmed by the production of mosaic and broad chlorotic streaks in leaves similar to those on HPV-infected mature plants in the field (Forster et al. 2001).

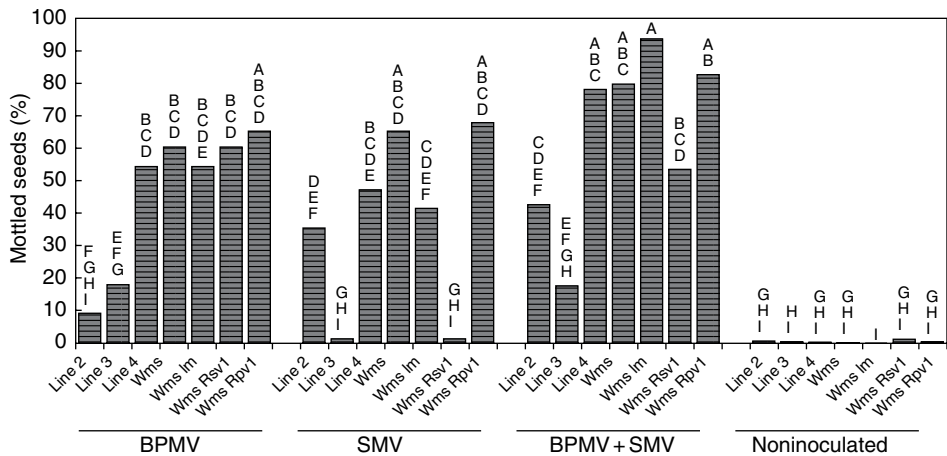


Figure 8.1 Influence of *Bean pod mottle virus* (BPMV), *Soybean mosaic virus* (SMV), and mixed infections on the percentage of seeds of soybean lines with mottling symptoms under field conditions in 2001. Mean percentages of mottled seeds (columns) followed by the same letter are not significantly different as per the Student's test ($P=0.05$). Source: Hobbs et al. (2003). Reproduced with permission of the American Phytopathological Society, MN, USA.

Plant viruses may cause systemic or localized infections. If the virus is able to move from the site of infection to other organs of plants, the virus is said to be systemic, inducing various kinds of discoloration of leaves such as mosaic, vein clearing and chlorosis, and deformation of affected leaves and other plant parts. Dwarfing of plants and smalling of leaves due to virus infection may also be observed. Seeds may also exhibit discoloration, reduction in seed size, and seed germination. Seed infection may result in seed coat mottling, which may pose problems for marketing. *Bean pod mottle virus* (BPMV) and *Soybean mosaic virus* (SMV) alone or in combination produced seed coat mottling to different intensities. A significant level of seed coat mottling was seen in a virus-isolate-soybean line combinations. Percentages of seed coat mottling were significantly higher in BPMV and SMV doubly infected soybean plants than in both BPMV and SMV singly infected plants for cultivars Williams in 2000 and Williams *Im* in 2001 (Fig. 8.1). SMV-resistant line 3 and cultivar Williams-*Rsv1* exhibited negligible percentages of seed coat mottling when challenged with SMV alone. The percentages of seed coat mottling observed in these two lines were therefore probably due to BPMV only. Seed coat mottling of seed from individual parent plants (+ or -), as determined by ELISA tests, were significantly correlated ($P=0.01$). SMV resistance in line 3 and Williams-*Rsv1* offered high levels of protection against SMV infection. The results showed that BPMV induced significant seed coat mottling which could adversely affect the marketability of bean seed (Hobbs et al. 2003). When the virus infection is localized in some plant species, countable chlorotic or necrotic lesions may be produced on inoculated leaves. In such plant species the virus is restricted to the lesions and it is not detectable in other plant parts. The number of local lesions formed on the inoculated leaves may reflect the concentration of the virus inoculum used for inoculation. As the local lesions are considered to be initiated by a single virus particle, the host plant species reacting with local lesions are used as diagnostic or indicator host for detecting and quantifying the virus titer in different systemic host

plants or samples. Furthermore, single lesion isolations are made to differentiate strains of a virus which may differ in some biological properties. Some of the plant species employed as diagnostic hosts are *Nicotiana glutinosa*, *Phaseolus vulgaris*, *Vigna unguiculata*, *Gomphrena globosa*, *Chenopodium amaranticolor*, and *C. quinoa*. Citrus cultivars/species used as diagnostic hosts such as Mexican lime, Dweet tanger, and *Citrus excelsa* have been useful to detect infection by *Citrus tristeza virus*, *Citrus vein enation mosaic virus*, and *Citrus psorosis virus* (Vidalakis et al. 2004; Narayanasamy 2011).

Tobacco mosaic virus (TMV), infecting tomato, is transmitted through tomato seed. The strain of TMV occurring in Sapopena-PR Brazil could not infect *Petunia hybrida*, described as a host for TMV and *Tomato mosaic virus* (ToMV). However, TMV induced severe mosaic symptoms in tomato cultivars Santa Clara and Avansus, while TMV-Sapopena caused only mild symptoms in cultivar Carmen. The TMV-Sapopena strain could be differentiated based on the differences in the type of symptoms induced on tomato cultivars and by its inability to infect *P. hybrida* (da Silva et al. 2008). The biological characterization of 10 isolates of *Cucumber mosaic virus* (CMV) associated with banana mosaic disease (BMD) in Taiwan were compared following inoculation of Cavendish banana plantlets using the cotton aphid (*Aphis gossypii*). The isolates were classified into four pathotypes based on the symptom types induced on banana, *Nicotiana glutinosa*, and cowpea (*Vigna unguiculata*) inoculated by sap inoculation. Three symptom types A, B, and C were distinguishable using *N. glutinosa* as a differential host plant. Two isolates infected systemically inducing mosaic symptoms on newly expanded leaves, while other isolates caused local lesions on inoculated leaves. Satellite RNA associated with CMV did not have any significant effect on symptom intensity induced in banana or in *N. glutinosa* (Chou et al. 2009).

Among the seedborne viruses, members of the genus *Potyvirus* form the largest group and induce characteristic inclusion bodies in the infected cells. The ultrastructural investigations revealed the presence of cylindrical or pinwheel inclusions in their respective host plants. The pinwheel inclusion has a central core from which rectangular or triangular curved plates radiate. In addition to pinwheel inclusions, they produce complex amorphous cytoplasmic inclusion bodies and secondary vacuolation of cytoplasm (Martelli and Russo 1984). *Artichoke yellow ringspot virus* (AYRSV), belonging to the genus *Nepovirus*, was found to be transmitted via onion seeds by up to 20%. *Nicotiana benthamiana*, the indicator plant species, was inoculated by sap inoculation. Cytopathological investigation showed that large cytoplasmic inclusions were produced in the mesophyll cells. These inclusions comprised voluminous accumulations of entangled membranous elements, more or less developed and specifically arranged particle aggregates and conspicuous globules of a possible lipid nature. A very characteristic and apparently specific cytopathologic structure occurred in many infected cells and in different stages of development and localization. This cytopathologic structure mainly contained complexes of two cisternae of rough endoplasmic reticulum (ER) in a parallel arrangement. In addition, a high proportion of infected cells showed the presence of virus particles. On the other hand, in the infected onion leaves extremely vacuolated cells with very low concentrations of cell constituents and AYRSV-induced cytological alterations were observed. AYRSV-On strain predominantly induced particle aggregates in the form of crystal-like arrays, either in particle monolayers enclosed between specially arranged ER cisternae or in double layers not enclosed between membrane elements (Maliogka et al. 2006).

8.2.2 Host Range of Viruses

Plant viruses may be able to infect a large number of plant species which may be other crops grown in the same farm or wild species growing in the vicinity of the cultivated fields. Viruses such as *Potato virus Y* (PVY) and *Alfalfa mosaic virus* (AMV) have wide host ranges including several crop plant species. AMV has been reported to naturally infect many herbaceous and woody plant hosts belonging to 150 species in 22 families. In addition, AMV could be experimentally transmitted to over 430 species in 51 dicotyledonous families (Jaspars and Bos 1980). *Potato virus Y* (PVY) belongs to genus *Potyvirus* to which more than 30 plant species are susceptible, including several crop plants such as potato, bean, peanut, pepper, soybean, lettuce, and watermelon (Narayanasamy 2001). On the other hand, some viruses have the ability to infect only a few wild plant species, in addition to the primary crop plant species. *Rice yellow mottle virus* (RYMV) infected wild rice species *Oryza barthii* and *O. longistaminata* and the presence of RYMV could be detected in the scales of these wild rice species by ELISA tests. RYMV also infected the other grass species *Dactyloctenium aegyptium*, *Eragrostis ciliaris*, *E. tenella*, and *E. tremula*. However, the virus could not be detected in the seeds of these grass species (Allarangaye et al. 2006).

Artichoke yellow ringspot virus (AYRSV), infecting onion crops, was inoculated onto 44 plant species belonging to eight families and 28 species were found to be infected by the virus. The host range of AYRSV included *Cucumis sativus*, *Phaseolus vulgaris*, *Vicia faba*, *Capsicum annuum*, *Nicotiana tabacum* cultivars Samsun, White Burley, and Xanthi which were systemically infected with the virus (Maliogka et al. 2006). *Indian peanut clump virus* (IPCV) infected peanut and was transmitted through seeds of finger millet (5.2%), foxtail millet (9.7%), and pearl millet (0.9%), but the virus did not infect sorghum. Transmission of IPCV in seeds of millets which are often grown in rotation with peanut is important because of the possibility of the infected millet plants being potential sources of inoculum that may be spread by the fungal vector *Polymyxa* sp. Millets in which the Hyderabad isolate of IPCV was seed-transmitted were also found to be the hosts of the vector *Polymyxa* sp. (Reddy et al. 1998).

8.2.3 Genetic Variability of Viruses

Biological, serological, and molecular properties of viruses have been used as the basis of differentiating strains of the viruses transmitted through seeds and propagules. *Plum pox virus* (PPV), causing plum pox or Sharka disease, infects stone fruit trees such as plum, peach, and apricot. Four groups of PPV, namely D, M, EA, and C, were differentiated based on the disease severity on natural hosts, geographical distribution, and latency period. The two most frequently observed groups – PPV-D in apricot and plum and PPV-M in peach – were investigated for their ability to be transmitted through seeds by testing the seeds obtained from infected plants and the seedlings developing from these seeds using immunocapture (IC-) RT-PCR assay. Although PPV was detected in seed coats and cotyledons, none of the seedlings showed symptoms or were PPV-positive in IC-RT-PCR assay, indicating lack of seed transmission of the virus (Pasquini et al. 2000). *Potato virus Y* (PVY) strains were differentiated based on the type of symptoms induced on potato cultivars and other plant species. The strain PVY^O causes mosaic symptoms on most potato cultivars. The induction of veinal necrosis in indicator plants (tobacco) forms the criterion for biological differentiation of PVY isolates. Necrotic ringspot symptoms in potato and tobacco are induced by the strain PVY^{NTN}.

Some isolates of PVY^{NTN} have been shown to be recombinants between PVY^O and PVY^N strains, where other strains might have arisen by point mutation. The potato cultivar Yukon Gold develops necrosis when inoculated with isolates of PVY^N (Chrzanowska 1994; Boonham et al. 2002; Nie and Singh 2002; Baldauf et al. 2006).

A one-step fluorescent RT-PCR assay on a single nucleotide polymorphism (SNP) was developed to examine the relationship between biological and molecular properties of PVY isolates. PVY isolates (42) could be assigned to their respective groups based on this approach. In addition, mixed infection of PVY^N and PVY^O strains could also be resolved, indicating the potential of the one-step fluorescent RT-PCR format for reliably classifying PVY isolates without the requirement of inoculating tobacco cultivar Xanthi plants in the greenhouse (Glais et al. 2002; Jacquet et al. 2005). *Potato virus Y* (PVY) strain PVY^{NTN} causes potato necrotic ringspot disease (PTNRD). The strain PVY^{NTN} was differentiated by applying strain-specific restriction endonucleases that cleave their respective PCR products. Single cleavages of PCR products derived from the 5' end of PVY^{NTN} genome by *Nco*I and that of the PVY^N by *Bgl*II were accomplished. The digests were then resolved by PAGE analysis. The strains of PVY were differentiated based on RFLP profiles. The components of mixed infections in potato by PVY strains could be identified and differentiated reliably and precisely (Rosner and Maslenin 1999; Lorenzen et al. 2006).

Variations in symptomatology, host range, and pathogenicity of strains/isolates of *Zucchini yellow mosaic virus* (ZYMV) have been indicated by Antignus et al. (1989) and Lecoq and Purcifull (1992). The reliability of differentiating isolates of ZYMV based on variation in biological characteristics was examined by inoculating different plant species and also sequencing of genomic portion of the C-terminal part of Nib and N-terminal part of the CP gene of ZYMV. All isolates (12) infected *Chenopodium amaranticolor* which reacted by producing necrotic local lesions, whereas *Gomphrena globosa* was infected only by four isolates. The inability of 8 of 12 isolates to infect *G. globosa* was confirmed by DAS-ELISA test which could not detect these isolates in inoculated *G. globosa* plants (Bananej et al. 2008). Strains of *Soybean mosaic virus* (SMV) were identified based on their pathogenicity on differential soybean cultivars. Use of differential cultivars was widely adopted since it was difficult to differentiate SMV strains by serological methods; most SMV strains were found to be serologically homogenous. A RT-PCR/RFLP procedure was therefore employed successfully for detection and identification of SMV strains. A primer pair amplifying a 1385 bp fragment of the cylindrical inclusion (CI) coding region was used for RT-PCR. The RFLP profiles of the RT-PCR products were compared after restriction digestion with restriction endonuclease enzymes *Rsa*I, *Eco*RI, or *Acc*I. Five strains, as well as the seedborne SMV isolates from local soybean cultivars grown in Korea, could be differentiated by RT-PCR/RFLP analysis (Kim et al. 2004).

Strains of *Citrus tristeza virus* (CTV) were distinguished based on the reactivity with monoclonal antibodies (MAbs), and were placed into two serogroups which were correlated with groups differentiated by sequencing of their coat protein (CP) genes (Shalitin et al. 1994). CTV strains inducing stem-pitting symptoms have to be differentiated from other strains, since the stem-pitting-inducing strains cause serious damages to citrus production. Different combinations of polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) were used in indirect DAS-ELISA format to identify stem-pitting-inducing strains so that the infected citrus trees could be eradicated to prevent the subsequent spread of these CTV strains (Nikolaeva et al. 1998). The decline-inducing

strains of CTV could be differentiated from non-decline-inducing strains by employing the RT-PCR assay. Two internal primers, one (CP4) specific for non-decline-inducing strains of CTV and two terminal primers HCP1 and HCP2 for the ends of the capsid protein gene, were employed. This RT-PCR protocol was able to detect and also differentiate decline-inducing and non-decline-inducing strains of CTV accurately, since the 320 bp fragments were only amplified from the decline-inducing strains of CTV (Huang et al. 2004). Genetic variations in two groups of CTV isolates (Spanish and Japanese sources) were investigated. Single-strand conformation polymorphism (SSCP) analysis of genes *p18* and *p20* was performed. The genetic diversity was estimated within and between isolates, and the genetic differentiation between populations was evaluated based on nucleotide sequences of representative haplotypes of each isolate and gene. In some isolates, within-isolate diversity was greater than diversity with other isolates because their populations contained distinctly related sequence variants. The genetic variation of different genes, repeated inoculation in the field, homologous RNA recombination between sequence variants, and the presence of different defective RNAs were found to be some of the factors that may contribute to biological variability of CTV isolates (Ayllón et al. 2006).

Isolates of *Citrus tristeza virus* (CTV) collected from different geographical locations were investigated for their differentiation based on the polymorphism in the coat protein (CP) gene by cleavase fragment length polymorphism (CFLP) and single-strand conformation polymorphism (SSCP) analyses. CTV sources were initially biologically characterized, followed by IC-RT-PCR and CFLP analysis targeted to CP gene. All CTV isolates (except one) formed different clusters which frequently shared some kind of geographical or biological relationship. SSCP analysis grouped the clones in a different way, however. The CP gene products of CTV isolates (12) obtained after RT-PCR amplification were analyzed by CFLP procedure. It was possible to group most of the isolates accurately by CFLP analysis according to the sequences of the variants constituting the isolates. The plant inoculated with a mixture of mild and severe isolates showed patterns that were more closely related to those of severe isolates, suggesting that severe isolates could multiply more rapidly and become more predominant in plants inoculated with the mixture of mild and severe isolates. The results indicated that CFLP procedure might be effective for identification and differentiation of CTV isolates more reliably and accurately (Marques et al. 2006).

Genetic diversity of isolates of *Citrus tristeza virus* (CTV) collected from 20 citrus growing countries was assessed using single-strand conformation polymorphism (SSCP) analysis. RT-PCR assay was employed for amplification of CP gene of these isolates followed by cloning and subjecting to SSCP analysis. Haplotypes that produced different patterns within each geographic region were sequenced and a database of 153 accessions of CTV was assembled. The existence of seven well-defined clusters with a coefficient of differentiation of 0.78 was revealed by phylogenetic analysis. An asymmetric PCR-ELISA typing (PET) assay was developed in the frame of this clustering pattern, using a set of eight hybridization probes. The results could be interpreted objectively through a visual basic application that could be compared to the rates of hydrolysis of the ELISA substrate of an assayed isolate to a matrix of rates of hydrolysis obtained from standard haplotypes. An additional notable factor is the possibility of automation as the ELISA test (Nolasco et al. 2009). Severe strains of CTV causing seedling yellows (SY) and/or stem-pitting symptom are potentially dangerous, being responsible for the destruction of citrus plants in several countries. The plants infected by the severe

isolates therefore have to be identified rapidly by employing sensitive and reliable diagnostic methods. Real-time RT-PCR assay was applied by employing a general primer set and three TaqMan locked nucleic acid (LNA) probes targeting sequences characteristic of severe, mild (non-SY, non-SP), and T-36-like isolates. It was possible to amplify expected DNA fragment from fresh or silica-desiccated CTV-infected samples and all isolates except one, which reacted with one or more probes. RT-PCR assays with homologous and heterologous transcript RNA mixes demonstrated that each probe reacted only with its cognate sequence which was detected even at ratios below 2.5%. In a test with 56 CTV isolates from different countries analyzed by real-time RT-PCR format, mild isolates reacted only with the mild probe. In contrast, severe SP and SY isolates reacted with severe SP or the T36-like probes, respectively. The real-time RT-PCR protocol could be effective in differentiating the severe strains of CTV from the mild strains, facilitating the development of required systems for the eradication of citrus plants infected by severe strains of CTV (Ruiz-Ruiz et al. 2009).

Monoclonal antibodies (MAbs) generated against coat protein (CP) of *Grapevine leafroll-associated virus 1* (GLRaV-1) exhibited variations in their levels of specificity of reactions with different isolates of GLRaV-1, as indicated by the results of DAS-ELISA and Western blot analysis. Two MAbs IC4 and IB7 reacted positively with 25 and 32 isolates of GLRaV-1 obtained from infected grapevine plants, but not with GLRaV-3 coat proteins. The MAb IG10 reacted with both GLRaV-1 and GLRaV-3 coat proteins, indicating that these two viruses had some antigenic determinants (epitopes) in common (Seddass et al. 2000). The MAb specific to *Grapevine virus D* (GVD) could react differentially with GVD when tested by ELISA test (Boscia et al. 2001). In order to determine the variability of CP gene of *Citrus psorosis virus* (CPsV), isolates from 50 field sources were analyzed by immunoassay. By employing 23 MAbs and 9 serogroups, at least 10 different epitopes on CPsV coat protein were recognized. The reaction patterns of field sources were determined using triple-antibody sandwich (TAS-) ELISA format. The field sources were grouped into more patterns named A to I. Pattern A was the most frequent, accounting for 39.6% of the field sources. Different *Citrus* species and cultivars growing in the same plot frequently showed distinct reaction patterns. By using TAS-ELISA to analyze 40 isolates from different countries with 24 MAbs, 14 reaction patterns and at least 17 different epitopes of CPsV coat protein were identified (Alioto et al. 2003).

Several strains of *Plum pox virus* (PPV) infecting plum trees have been identified. Serotypes of PPV designated PPV-D and PPV-M could be differentiated by employing specific MAbs in DAS-ELISA tests (Candresse et al. 1998). In a later investigation, two more serotypes C and EL Amar were identified in addition to the serotypes D and M (Myrta et al. 1998). Triple-antigen sandwich (TAS-) ELISA format with MAbs specific to PPV-M and PPV-D serotypes was employed for assessing the reactivity of 43 isolates collected from orchards in Romania. All isolates reacted positively to at least one of the two MAbs, as well as to PPV-D- and/or PPV-M-specific primers in PCR assays. Using TAS-ELISA test 21 isolates were identified as PPV-D (48.8%), 17 isolates as PPV-M (39.6%), and 5 isolates as a mixture of D and M strains (11.6%). The results were corroborated by those of immunocapture (IC-) RT-PCR format (Zagarai et al. 2008).

The need to assess the extent of genetic diversity of *Plum pox virus* (PPV) was emphasized because of the destructive nature of Sharka or plum pox disease infecting *Prunus* spp. all over the world. The serologically distinct strains PPV-D and PPV-M could be differentiated by *RsaI* polymorphism in RFLP analysis. The unique 243bp DNA

fragment was amplified by P1 and P2 primers located at the C-terminus of PPV CP gene or by direct IC-RT-PCR typing using PD- and PM-specific oligonucleotides (Olmos et al. 1997). RT-PCR was performed using primers capable of amplifying the 243 bp fragment in the C-terminus of the CP coding region. Restriction fragment length polymorphism (RFLP) analysis applied to the amplified 243 bp fragment showed that restriction sites of *AluI* and *RsaI* were present in the samples extracted from plum and apricot. An amplified 836 bp cDNA fragment derived from the P3-6 K coding regions of both isolates had restriction profiles typical of PPV-D strain. Nucleotide identities of 99–100% were observed for the 243 bp fragments of the Kazakhstan isolates when compared to the corresponding regions of strain D, and the similarity of identity with strain M was 94–95%. Nucleotide sequence analysis of the entire CP coding region of the plum and apricot isolates resulted in the identification of a unique deletion of six nucleotides in the N-terminal region of the plum isolate. Several nucleotide substitutions in the CP coding region were commonly seen in plum and apricot isolates and they seemed to be unique to the Kazakhstan isolates (Spiegel et al. 2004).

Pepino mosaic virus (PepMV) induced two types of yellowing symptoms, namely interveinal leaf yellowing and yellow mosaic, in glasshouse tomatoes. After several weeks, the interveinal yellowing symptoms gradually disappeared and leaves in the apical portions became green (green heads) again, with yellow mosaic patterns on the leaves as intermediate stage. The sequencing of multiple isolates causing interveinal leaf yellowing identified two point mutations, occurring in positions 155 and 166 of coat protein (CP), as unique to the yellowing pathotype. Site-directed mutagenesis of infectious clones confirmed that both CP mutations are determinants of the interveinal leaf yellowing symptoms. Sequencing of CP clones from plants or plant parts with yellow mosaic symptoms resulted in a mixture of wild-type and mutated sequences, whereas sequencing of CP clones from the green heads of recovered plants resulted in only wild-type sequences. Yellow mosaic symptoms could be reproduced by inoculation of an artificial 1:1 mixture of RNA transcripts from wild-type and mutated infectious clones. The results showed that the ratio of mutated versus wild-type sequences could determine the nature and severity of symptom development. The gradual recovery of the plants, which coincided with disappearance of the yellowing mutations, suggested that selection pressure might act to the advantage of the wild-type virus. Experiments with wild-type and mutated infectious clones showed that reverse mutation events from mutants to wild-type occurred and the wild-type virus did not have a replicative advantage over the mutant (Hasiów-Jaroszewska et al. 2013).

8.2.4 Modes of Transmission of Viruses

Plant viruses, being obligate pathogens, cannot exist in a free state except in the case of a few viruses. In contrast, most of the fungal or bacterial pathogens are dispersed freely as spores, sexual spores, or other structures which may be resistant to adverse conditions. Further, like fungal or bacterial pathogens, plant viruses do not produce any enzymes or toxins that may aid in the penetration of or ingestion through unwounded plant surfaces. Plant viruses therefore cannot infect unwounded plant surfaces and have to be introduced and deposited inside the susceptible cells of the host plants. Transmissibility of plant viruses via pollen, seed, and propagules has been discussed already (Chapter 7). Another important characteristic of viruses is their natural dissemination through vectors such as insects, mites, nematodes, or fungi. In a few

cases such as *Tobacco mosaic virus* (TMV) and *Potato virus X* (PVX), transmission through leaf contact may be possible under field or glasshouse conditions. Potato viruses are known to be transmitted through cutting knives and other handling equipments. Transmission of viruses may be accomplished under in vitro conditions, by mechanical inoculation, or sap inoculation. Grafting and budding operations and other nursery practices have been shown to be responsible for significant spread of viruses infecting fruit crops. Transmission of some viruses through parasitic dodder (*Cuscuta* spp.) and natural root grafts may also occur.

Plant viruses are transmitted by different types of vectors with varying efficiency. The nature of relationship between the virus and the vectors is determined by the virus concerned. Three kinds of major virus–vector relationships have been recognized. The viruses may be grouped as nonpersistent (styletborne), semipersistent (circulative), and persistent (propagative) viruses based on the nature of their relationship with the vectors. Nonpersistent viruses are acquired by their vectors after a short acquisition feeding period (a few minutes) on the infected source plants, and can be transmitted to healthy plants immediately after acquisition of the virus from the infected plants. There is no demonstrable latent (incubation) period in the vectors. The vectors of nonpersistent viruses lose their infectivity after moulting. These viruses are also known as styletborne viruses. The semipersistent viruses are acquired by their vectors after several hours of acquisition access period on the infected source plants. A latent period of few hours or days may be required after the acquisition access period on the infected source plants before the vectors become infective and are able to transmit the viruses to healthy plants. These viruses are called semipersistent or circulative viruses. The vectors remain infective for a long time (several days) and even after moulting. The third type of viruses is designated persistent or propagative viruses. These viruses are acquired by their vectors after a long feeding period (several days) on infected source plants and can be transmitted after a long latent period (several days or weeks) to healthy plants. Some of the persistent viruses are able to multiply in their vectors and may be transmitted to the next generation through eggs. This type of transmission is known as transovarial transmission or congenital transmission. Viruses transmitted via seeds have predominantly different aphid species as vectors. Some of the aphid-transmitted viruses such as *Alfalfa mosaic virus*, *Cucumber mosaic virus*, *Pea enation mosaic virus*, *Barley stripe mosaic virus*, *Potato virus Y*, and *Lettuce mosaic virus* are also transmitted through seeds. Viruses infecting citrus, stone fruits, strawberry, and banana are transmitted by different species of aphids and these viruses are also transmitted through propagules. The vector efficiency differs with species of vectors, virus, and the environmental conditions that influence vector population and movement. Nematodes and fungi function as vectors of members of the genera *Nepovirus* and *Furovirus*, respectively. The nature of virus–vector relationship and vector specificity may be useful for putative identification of plant viruses to some extent. However, the identity of the virus concerned has to be conclusively established using immunoassays and/or nucleic acid-based techniques.

8.2.5 Cross-Protection of Viruses

The phenomenon of cross-protection of plants by mild strain against severe strain of the same virus or related virus was demonstrated in tobacco against *Tobacco mosaic virus* (TMV) (McKinney 1929) and *Potato virus X* (PVX) (Salaman 1933). No additional symptom due to inoculation with severe strain was observed in plants already

inoculated with the mild strain. Cross-protection is one of the biological properties of viruses used to establish relationships among viruses and their strains. The degree of cross-protection, as a measure of relatedness of strains, may be determined quantitatively by inoculating a systemically invading strain and a local lesion-forming strain on an appropriate host plant species. For example, relationships between TMV strains could be determined by inoculating *Nicotiana sylvestris* with the type strain causing systemic infection; when these plants were inoculated with aucuba mosaic strain of TMV, the number of local lesions formed by the challenging strain was significantly reduced (Kunkel 1934). The phenomenon of cross-protection by mild strains against severe strains of viruses has been exploited for protecting plants as an effective disease management strategy. Cross-protection between virus strains as a practical disease management strategy was assessed.

CM95, an attenuated isolate of *Cucumber mosaic virus* (CMV), was evaluated for its ability to protect plants against severe isolates of CMV under field conditions. RT-PCR-RFLP procedure was used to rapidly detect and differentiate CMV-95 from severe isolates. Preimmunized plants were challenge-inoculated at 14 days after inoculation with CMV-95 isolate. The appearance of symptoms on newly formed upper leaves was monitored at 30 days after inoculation. RT-PCR-RFLP technique rapidly revealed the presence or absence of the severe isolates in the preimmunized plants much earlier than assessment of cross-protection based on symptoms expression which required 30 days after inoculation (Nakazono-Nagaoka et al. 2005).

The degree of protection varied with *Citrus tristeza virus* (CTV) isolate and climatic conditions at a given location. Preimmunization with mild CTV isolate (PB61) protected Marsch grapefruit trees against stem-pitting strain for 35 years. However, a gradual increase in CTV severity occurred in the preimmunized trees used as budwood sources, commencing 17 years after planting. Nevertheless, the preimmunized trees remained vigorous and productive with only 6 of 64 trees showing mild trunk-pitting. Breakdown in mild strain protection was seen more significantly in subpropagations from trees with mixed infections (Broadbent et al. 1995). Mild strains of CTV have been shown to have the potential to protect grapefruit plants against severe strains. Grapefruit cultivar Ruby Red plants on sour orange rootstock were graft-inoculated with three mild isolates of CTV whose ability to protect the plants was determined at 16 years after inoculation with mild isolates. The mild isolate DD102 protected the grapefruit plants most effectively against severe strains of CTV (Powell et al. 1999). Kagzi lime plants inoculated with mild strain showed a significantly reduced incidence of decline due to severe strain of CTV. The presence/absence of severe strain of CTV was checked by employing enzyme-linked immunosorbent assay (ELISA) tests (Chakraborty and Ahlawat 2001).

The efficacy of preimmunization against superinfection was influenced by the inoculation method, the challenge isolate involved, and the host and its physiological status. The protective capacity of a mild strain (PB61) of *Citrus tristeza virus* (CTV) to protect Marsh grapefruit seedlings against superinfection with the severe grapefruit stem-pitting (OSP) isolates (PB155 or PB235) was assessed. Symptom development in the test plants was monitored by applying isolate-specific RFLP analysis of amplicons generated by RT-PCR and multiplex RT-PCR assays. Only 1 of 15 Symons sweet orange (SSwO) seedlings preimmunized with PB61 (mild strain) and challenged with PB235 by aphid inoculation was superinfected by 90 days postinoculation, compared to 14 of 15 days in non-preimmunized control plants. Preimmunization with PB61 did not ameliorate the

expression of OSP symptoms once superinfection with OSP-inducing isolates was initiated, and the mild strain could not prevent movement of challenge virus. Preinoculation with a severe OSP isolate (PB155) did not delay superinfection by PB61 when introduced via grafting. Preimmunization with PB61 isolate protected plants more effectively against OSP isolate PB235 than against OSP PB155. The higher level of protection provided against PB235 isolate might be due to its closer nucleotide sequence homology to PB 61 (97.6%) than to the PB155 isolate (91.2%). The more effective protective capacity of PB61 against the isolate PB235 might be a direct function of the sequence homology between them. A model of RNA-mediated defense was suggested whereby infection with the preimmunizing isolate might trigger the host to produce a ds-RNA-specific nuclease which targets the viral RNA for degradation to low levels. This might result in the appearance of small nucleotide fragments such as those observed during posttranscriptional gene silencing (PTGS) process. Once PTGS is established other transcripts homologous, or nearly so, to the silenced gene are also subsequently degraded if they infect the plant. The mechanism of development of cross-protection appeared to be through operation of the PTGS in citrus plants inoculated with the challenged strain (mild strain) (Zhou et al. 2002).

Changes in the populations of sequence variants of *Citrus tristeza virus* (CTV) isolates from different geographic origins and pathogenicity properties were followed using single-strand conformation polymorphism (SSCP) analysis of cDNA of the genes *p18*, *p13*, *p20*, and *p23*. RT-PCR with primers specific for selected genes yielded DNA fragments of the expected sizes: 425, 438, 520, and 698 nucleotides for genes *p18*, *p13*, *p20*, and *p23*, respectively. The mild isolates of CTV used in this investigation generally yielded a SSCP profile with two DNA bands, indicating that their viral populations might be more complex. The set of SSCP profiles of the four genomes allowed identification of individual isolates, but no profile characteristic of a geographic location or a biogroup could be recognized. The SSCP profiles of the mild isolate T425 for genes *p18* or *p20* differed from the corresponding profiles of the severe isolates T388 or T305 and provided a convenient system to discriminate between the predominant sequence variants of these isolates in mixed infection (Fig. 8.2). Sweet orange plants singly inoculated

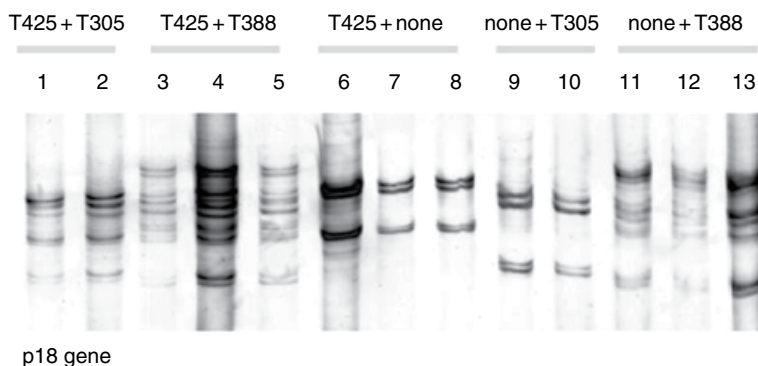


Figure 8.2 Differentiation of *Citrus tristeza virus* (CTV) isolates in individual and mixed infections of sweet orange plants based on SSCP profiles characteristic of *p18* gene. SSCP profiles of mild isolate T425, severe isolates T388 or T305, or a mixture of T425 and T388 or T425 and T305 in the extracts of inoculated plants determined at 3 years after challenge inoculation with T388 or T305 isolates. Source: Sambade et al. (2002). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

with a mild or with a severe isolate yielded the SSCP profile characteristic of each isolate, whereas the SSCP profile of plants successively inoculated with both mild and severe isolates was a composite of two individual profiles. In these plants, the DNA bands characteristic of the mild isolate T425 were often less intense than those characteristic of T388 or T30 (severe isolates challenge inoculated). The SSCP profile of plants singly inoculated remained constant, but the profile of doubly inoculated plants varied with time. Plants in which the SSCP profile of the severe isolates became predominant showed stem-pitting symptom. On the other hand, the plants in which the predominant profile corresponded to the mild isolate remained asymptomatic. In cross-protection experiments, the virulent RNA population usually out-competed and reached a higher titer than the pre-existing mild RNA population. The shift in population could be efficiently monitored (during 3–6 months after challenge inoculation) by periodical SSCP analysis of gene *p20*. The observation that DNA bands characteristic of T425, T11, or T32 were always present in the SSCP profile of the doubly inoculated plants indicated that the population of the mildly pathogenic isolates were overgrown by populations of the severely pathogenic isolates, but they were not totally displaced. Periodical SSCP analysis of the budwood sources could be a simple and reliable procedure to check the authenticity of the mild protecting isolate and to detect a potentially dangerous change in its RNA population before bud propagation to produce new cross-protected plants (Sambade et al. 2002).

Citrus psorosis disease has been presumed to be due to *Citrus psorosis virus* (CPsV), the type member of the genus *Ophiovirus* (Milne et al. 2000). CPsV was generally detected by biological assay on sensitive sweet orange seedlings (*Citrus sinensis*) grown in a temperature-controlled (18–26 °C) greenhouse. Later, specific diagnosis of psorosis was performed by cross-protection tests or immunoassays. Direct tissue-blot immunoassay (TBIA) and enzyme-linked immunosorbent assay (ELISA) were employed for the detection of CPsV in field-grown *Citrus* spp. Field samples of different varieties of sweet orange, grapefruit, lemon, clementine, Satsuma, and sour orange (*Citrus aurantium*) showing psorosis symptoms from different citrus growing areas in Spain and Italy were tested. For biological indexing of field sources, two seedlings each of sweet orange and *C. excelsa* were graft-inoculated with bark pieces from each candidate tree. Pineapple sweet orange plants were challenge-inoculated with the isolate PB108 and evaluated for cross-protection at 3 and 6 months later. A cross-protection test was performed for the diagnosis of CPsV. Sweet orange seedlings preinoculated with candidate psorosis isolate A were challenge-inoculated with psorosis B. Plants already inoculated with psorosis A did not develop leaf pustules and twig blisters characteristic of psorosis B. Healthy sweet orange seedlings inoculated with psorosis B exhibited characteristic symptom within 6 months, whereas plants already infected with psorosis A were protected and did not exhibit the symptoms of psorosis B. Cross-protection against psorosis B was observed in 41 of 47 citrus plants, as indicated by the absence of symptoms induced by psorosis B.

As this procedure was found to be slow and expensive in terms of trained personnel and large greenhouse space with temperature-controlled facilities, detection of CPsV by ELISA, RT-PCR, molecular hybridization, and ISEM techniques was compared with the results of cross-protection tests. CPsV was detected by the four techniques in plants inoculated with all isolates of psorosis A and B and two of the ringspot type isolates (RS-108 and RS-ALM). The presence of biologically characterized CPsV isolates was detected by ELISA, RT-PCR, dot-blot hybridization, or ISEM, and the results were

correlated with psorosis diagnosis by biological indexing and cross-protection tests. The results suggested that CPsV was the causal agent of psorosis, as defined by the cross-protection test against psorosis B and that rapid diagnosis of CPsV disease could be accomplished by employing one or more of the rapid tests employed in these investigations (Martin et al. 2002, 2004).

8.3 Infection Process of Plant Viruses

8.3.1 Virus Replication

Plant viruses have structural features distinctly different from those of cellular bacterial pathogens and multicellular fungal pathogens. Plant viruses constitute a group of obligate intracellular and molecular pathogens resembling the host cellular constituents rather than the cells. As the requirements of viral replication are different, their strategies for successful infection and development of symptoms (pathogenesis) are also different and unique. As no known physiological functions and enzymatic activities of viruses are known, the viruses depend on the wounds created by mechanical damage on the plant surface or feeding injuries made by the vectors of viruses. The viruses have to be deposited directly into the susceptible host cells because of their inability to enter through the intact host cells. Viral infection of susceptible hosts is a complex process. Four principal stages in the process of infection by viruses have to be completed for successful infection of the natural host plant species: (i) entry into plant cells; (ii) replication in the primarily infected cells; (iii) cell-to-cell movement through plasmodesmata (PD); and (iv) long-distance movement through vascular system. As the animal cells lack a cell wall, animal viruses are able to enter the cells through a receptor-mediated endocytosis. In contrast, plant cells have cell walls forming a formidable barrier to the entry of the viruses and there is no receptor-mediated mechanism operating for effecting initial transfer of the plant virus into a plant cell. The cell-wall barrier has to be breached and the virus particle needs to be placed inside the cell through wounds or injuries by feeding vectors.

Soon after the entry of the virus particle into the susceptible plant cell, uncoating of viral coat protein is initiated and the viral genome is released into the cell cytoplasm. The infecting viral genome is translated directly if it is + sense RNA. Single-stranded (ss) RNA or mRNAs are formed and translated to produce early products such as virus replicase and perhaps other virus-specific proteins. The viral replicase or replicase-associated protein(s) required for the synthesis of subgenomic mRNAs are formed. The new viral RNA genomes are synthesized by the viral replicase or replication-associated proteins. Proteins needed relatively late in the viral replication cycle, such as coat protein (CP) and movement protein (MP) essential for cell-to-cell movement of the viruses, are synthesized. When they reach sufficient concentrations, coat protein subunits and viral genomes are assembled together for the production of new progeny virions, usually in the cytoplasm. The mature virions move from the initially infected cell to adjacent cells. If the virus has the capacity to move to other cells, different plant organs are infected in systemic infection. If the cell-to-cell movement is restricted by the host defense system the virus is localized, leading to local infection and giving rise to a relatively small zone of infected tissue which may be recognized as chlorotic or necrotic local lesions on the inoculated leaves. The principal stages in virus replication may vary depending on the nature of host–virus combinations.

For the successful infection of plants, viruses need to overcome a series of pre-existing physical and chemical barriers in plants. If the virus succeeds in overcoming this first line of defense, it has to face the nonspecific defensive reactions with which the plant responds to some molecular patterns that are common to different pathogens. Plants are able to recognize virulence factors of viruses and create a new, more specific resistance layer that is induced only when faced with viruses expressing the virulence factor(s). A virus can cause productive infection only in plants that are unable to develop specific defense responses to its virulence factors (Jones and Dangl 2006). Viral RNA induces specific plant defense responses in which a large number of plant proteins participate. Dicer-type ds-RNases, ss-RNases belonging to the Argonaute-type protein family, assemble in RNA-induced silencing complex (RISC), RNA polymerases, and RNA helicases (Dunoyer and Voinnete 2005). This antiviral response, which is one of the manifestations of a complex set of cellular proteases known as RNA silencing, is apparently universal and the virus not only needs to escape the defenses that are host-produced obstacles but must also successfully accomplish all stages in its life cycle. The initiation of this cycle depends on the nature of the genetic material of the virus concerned. The induction of plant defense mechanisms, their suppression by counteracting viral strategies, and the cooperation of host factors required for virus replication and movement may confer a pathological condition upon successful virus infection (Pallás and García 2011).

RNA silencing is a potent surveillance system targeting parasitic RNA in a highly sequence-specific manner, manifesting as posttranscriptional gene silencing (PTGS) in plants. The RNA silencing pathway in plants constitutes a formidable defense against viral pathogens. Most plant viruses have adopted counter-defensive strategies to overcome the host silencing pathway. RNA silencing has two distinct stages: initiation and maintenance. At the initiation stage, the host cells sense the presence of ds-RNA as abnormal and employ a ds-RNA-specific RNase called Dicer or Dicer-like (DCL) to digest the ds-RNA into small RNA species of 21–24 nucleotides known as small interfering RNAs (siRNAs). The viral RNA may also be converted into a ds-RNA target by one of the RNA-dependent RNA polymerases (RdRps) encoded by the plant host (Yu et al. 2003; Molnar et al. 2005). The siRNA produced by the action of DCLs is then recruited into a RNA-induced silencing complex (RISC) where it mediates the sequence-specific digestion of homologous RNA by RISC. At the maintenance stage, silencing of homologous RNA persists in the absence of the ds-RNA trigger. This is accomplished through a siRNA amplification process in which host RdRp synthesizes new ds-RNA, using siRNA as a primer and the homologous cellular RNA as template (Vaistij et al. 2002). Local induction generating sequence-specific signals that spread systemically throughout the plant is the unique characteristic of RNA silencing in plants (Voinnet 2005).

Plant viruses encode suppressors of RNA silencing to specifically counteract the RNA-silencing-based defense mechanisms to ensure successful systemic infection of the host plant. Virus-encoded silencing suppressors have been investigated in some host–virus interactions. In potyviruses, the protein P1/HC-Pro has been shown to be multifunctional in that it influenced aphid transmission, polyprotein processing, genome amplification, and long-distance virus movement (Kasschau et al. 1997). The P1/HC-Pro was also able to act as a broad range pathogenicity enhancer causing increased viral RNA accumulation and dramatically more severe viral symptoms in many unrelated viral functions (Pruss et al. 1997). P1/HC-Pro has been shown to strongly suppress RNA

silencing most likely acting on a maintenance step affecting the assembly and/or targeting of RISC complex (Chapman et al. 2004). The role of P1/HC-Pro in genome amplification and long-distance movement was found to be highly correlated with its silencing suppressor activity. Mutant viruses that lacked suppressor activity were able to initiate the replication process but unable to sustain robust amplification; consequently, long-distance movement was significantly reduced. The results indicated that suppression of the RNA silencing-based on host defense was crucial for vigorous viral RNA replication followed by efficient systemic spread of the virus (Kasschau and Carrington 2001). The 2b protein of cucumoviruses was demonstrated to have silencing suppressor activity, as the P1/HC-Pro of potyviruses. This protein enhanced long-distance movement of *Cucumber mosaic virus* (CMV) in a host-dependent manner. CMV 2b mutants invaded tobacco systemically, but not cucumber plants (Ding et al. 1995). Systemic infection of plants containing a silenced GFP transgene by either CMV or a PVX vector expressing 2b protein restored GFP expression in the leaves emerging after infection, but not in leaves where GFP silencing had already established. The results suggested that CMV 2b might function to prevent the systemic spread of RNA silencing signals (Brigneti et al. 1998; Guo and Ding 2002).

8.3.2 Movement of Plant Viruses

Plant viruses replicate in initially infected cells, attaining the required concentration for infecting neighboring cells and sustaining their subsequent pathogenic activities. The progeny virus particles have to move out of the initially infested cells to infect adjacent cells or plant tissues away from the initially infected. Two phases of virus movement within the host plant, namely cell-to-cell or short- and long-distance, have been distinguished. When a virus is unable to move to neighboring cells after replication in the initially infected cells, the infection is referred to as subliminal infection of the plant species. Such plants show a high form of resistance (extreme sensitivity) restricting the virus movement, resulting in necrosis of infected cells and subsequent inactivation of virus particles which cannot survive in the absence of live cells (Cooper and Jones 1983). Viruses differ in their ability to become systemic or confined to local lesions depending on the nature and level of resistance/susceptibility of plant species.

8.3.2.1 Cell-to-Cell Movement

Plant viruses utilize host plant proteins generally required for host-specific activities during the life cycles commencing from deproteinization of virus particles to intracellular, local, and systemic movement, leading to development of various symptoms induced by different viruses. The movements from one cell to another adjacent cell via plasmodesmata (PD) are modified to a lesser or greater extent and to other distant plant organs, including flowers and seeds, through the vascular system. Plasmodesmata function as cytoplasmic bridges and establish membrane (endoplasmic reticulum (ER) and plasma membrane) as well as cytoplasmic continuity between adjacent plant cells. The plasmodesmata form an important route for communication between plant cells, regulating cell-to-cell communication. As such, PDs are too small to allow free passage of mature virus particles or viral genomes which are far larger than the size exclusion limit (SEL) of plasmodesmata. The viruses first move from replication sites to PD at the cell periphery and then traverse the intracellular channels to enter the neighboring cells (Nelson and Citovsky 2005).

In the case of many plant viruses, cell-to-cell transport is mediated by specific virus-encoded factors known as movement proteins (MPs). The function of MPs may be augmented by other viral proteins. The involvement of MPs in the modification of the size exclusion limit (SEL) of plasmodesmata (PD) has been shown by the investigation using *Tobacco mosaic virus* (Wolf et al. 1989). Many viral MPs may be delivered to PD via endoplasmic reticulum (ER), whereas actin/myosin filaments may regulate the flow of proteins in the ER membrane (Boevink and Oparka 2005). Many viruses form replication centers enriched in endoplasmic reticulum (ER) and many viral proteins are translocated to ER. The role of ER translocation and plasmodesmatal targeting was explored using calreticulin, a cellular protein that localizes to PD. The N-terminal signal peptide critically influenced the ability of calreticulin to accumulate within plasmodesmata (Chen et al. 2005). Coat protein (CP) has a role in virus movement, in addition to providing protection to the viral genome against inactivation. The requirements of *Cucumber mosaic virus* (CMV) for cell-to-cell movement seemed to be different. Both MP and CP were found to be necessary for the cell-to-cell movement. CMV variants lacking CP could not move from infected cell to adjacent cells (Canto et al. 1997). Virion assembly was not found to be a prerequisite for CMV movement, since assembly-defective CMV variants efficiently spread and induced local lesions (Kaplan et al. 1998).

During infection of plants by tobamoviruses such as *Tomato mosaic virus* (TMoV), viral replication complexes containing a viral 126 kDa replicase component (126 K) are generated temporarily in association with a viral movement protein (MP) that is indispensable for viral intracellular movement. The *Tobamovirus* 126 K protein has been shown to play an important role not only in virus replication, but also in intercellular transport of viral RNAs. Transient expression of the 126 K protein was shown to result in the formation of cytoplasmic bodies with shapes similar to those of the replication complexes. Roles of TMoV MP in the formation, localization, and dynamics of cytoplasmic bodies of 126 K protein were investigated by using confocal microscopy for visualizing fluorescently tagged 126 K and MP that were transiently and simultaneously expressed in *Nicotiana benthamiana*. The 126 K protein was evenly distributed in the cytoplasm with occasional formation of small 126 k bodies, when MP was not expressed. These small 126 K bodies moved in the cytoplasmic stream and along the endoplasmic reticulum (ER) network. In contrast, the coexpression of MP markedly promoted the formation of larger MP-associated 126 K bodies which were generally static, but moved occasionally in the cytoplasmic stream. Further, small mobile 126 K bodies were captured at the terminal part of the MP-derived filamentous structures, resulting in the formation of 126 K-MP complexes and their translocation to another cellular site (Sasaki et al. 2014).

Barley stripe mosaic virus (BSMV), a seedborne virus in the seeds of barley, has RNA B genome which contains a series of overlapping open reading frame (ORF) known as triple gene block (TGB). The three most abundant proteins, β b, β c, and β d, have essential roles in infectivity. The involvement of each of these proteins in cell-to-cell movement of BSMV in planta was studied by engineering fusions of the green fluorescent protein designated γ b. Microscopic examination of inoculated and systemically infected barley and oat leaves revealed high levels of fluorescence that moved rapidly through the compact striate vascular tissue without infecting epidermal cells. In contrast, a radial pattern of fluorescence spread through a large number of epidermal and mesophyll cells before entry into the reticulate vascular tissue of the dicotyledonous plants *Nicotiana benthamiana* and *Chenopodium amaranticolor*. Mutational analysis indicated that the

β b, β c, and β d proteins were essential for cell-to-cell movement in local lesion and systemic hosts, whereas the β d' protein was dispensable. The results demonstrated that the three major triple gene block-encoded proteins could act in concert to mediate cell-to-cell movement of *Barley stripe mosaic virus* (Lawrence and Jackson 2001).

The cell-to-cell movement of several groups of viruses is mediated by proteins translated from a set of three overlapping ORFs known as triple gene block (TGB). Two classes of triple gene blocks have been distinguished: class 1 TGB1 includes hordeiviruses having a 42–63 kDa protein and class 2 TGB2 contains potexviruses and carlaviruses with TGB1 encoding a product of 25 kDa (Solovyev et al. 1996). The transport component ribonucleoprotein (RNPs) or virions involved in the cell-to-cell movement of *Potato virus X* (PVX) genome include TGBp1 and CP. Both proteins are able to move through plasmodesmata (Batten et al. 2003; Howard et al. 2004). The PVX TGBp1 is able to modify plasmodesmata (PD) and move between cells. In rod-shaped viruses, TGBp2 and TGBp3 move to PD and act in concert to transport TGBp1 to and through PD (Schepetilnikov et al. 2005). In virus-infected plants, RNA silencing involves a mobile silencing signal that has the ability for cell-to-cell movement and also systemic movement through plants. The silencing signal may be linked to the defense activity if it moves through the plant either with or ahead of an invading virus. Many plant viral genomes encode suppressors of RNA silencing that are also effectors of long-distance virus movement through phloem. The protein P19 from *Cymbidium ringspot virus* is a suppressor of silencing not required for virus replication in isolated cells, but it is necessary for invasion of leaves systemically (Havelda et al. 2003).

The two groups of viruses containing TGB have the major difference in their requirement of coat protein (CP) for Group 2 and dispensability of CP for Group 1 viruses for cell-to-cell movement. Studies on subcellular localization of TGB proteins of *Potato mop-top virus* (PMTV) show that, when expressed as N-terminal fusions to GFP from a TMV-based vector, both the PMTV GFP-TGB2 and GFP-TGB3 fusions were associated with cellular endomembranes, particularly the ER network and membranes surrounding the nucleus. The GFP-TGB3 labeled opposing pairs of fluorescent spots across neighboring cell walls that suggested localization at or near plasmodesmata were present (Cowan et al. 2002). The TGB1 protein interaction with RNA and its transport as a part of the RNP complex out of the cell and long distance throughout the plant appears to be possible. The fluorophore-labeled TGB2 and TGB3 proteins of PMTV showed an early association with ER and colocalized in mobile granules that used the ER-actin network for intracellular movement. Both proteins were able to increase size exclusion limit (SEL) of plasmodesmata. TGB3 accumulated at PD in the absence of TGB2. Both fusion proteins were incorporated into vesicular structures during expression cycle. TGB2 was associated with its structures independently, while TGB3 required TGB2 for its incorporation into vesicles. Furthermore, in addition to localization to ER and motile granules, mRFP-TGB3 was incorporated into vesicles when expressed in PMTV-infected epidermal cells, indicating recruitment by virus-expressed TGB2. The experiments involving labeling of the TGB fusion of protein-containing vesicles with FM4-64 indicated that the endocytic pathway was involved in viral intracellular movement (Haupt et al. 2005).

Members of *Potyvirus* group cause the most numerous and devastating diseases among plant viruses, negatively impacting production of agricultural, horticultural, and floricultural crops. The viral genome has about 10,000 nucleotides, encoding a polyprotein that is processed by three proteinases to synthesize 10 mature viral proteins.

The proteinases are P1, HC-Pro, and NIa and mature proteins are P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb, and CP. All viral proteins are involved at one or other stages of the viral cycle. Three essential HC-Pro regions responsible for different functions have been identified. The N-terminal part controls virus transmission by aphids, virulence (symptom severity), genome amplification, and virus accumulation. The central region controls both long-distance movement and replication maintenance functions. The C-terminal region of HC-Pro is a cysteine-type proteinase and has a role in virus cell-to-cell movement. HC-Pro has also been shown to be a pathogenicity determinant (Revers et al. 1999; Redondo et al. 2001; Syller 2003). The coordinate activity of HC-Pro with capsid protein (CP) for transmission of potyviruses by aphid vectors and virus movement appears to indicate a direct interaction between these proteins. By using HC-Pro-specific monoclonal antibodies in a two-site triple-antibody sandwich (TAS-) ELISA format, an interaction between HC-Pro and CP of *Lettuce mosaic virus* (LMV) was demonstrated in extracts of LMV-infected leaves, as well as for two other potyviruses *Plum pox virus* (PPV) and *Potato virus Y* (PVY) (Roudet-Tavert et al. 2002). The cell-to-cell movement of two potyviruses *Bean common mosaic necrosis virus* (BCMNV) and *Lettuce mosaic virus* (LMV) was demonstrated to be dependent on HC-Pro. The interaction of this protein with viral CP was revealed by microinjection techniques using *Escherichia coli*-expressed proteins. Both proteins trafficked from cell to cell induced an increase of plasmodesmatal SEL and facilitated cell-to-cell movement of viral RNA, as a mutation in the C-terminal part of HC-Pro appears to have a role in short-distance movement of potyviruses (Rojas et al. 1997).

In addition to the cell-to-cell movement, the involvement of HC-Pro in different steps of the viral cycle such as aphid transmission, replication, systemic movement and as a suppressor of posttranscriptional gene silencing (PTGS) has also been indicated by various investigations (Plisson et al. 2003). Increasing the size exclusion limit (SEL) of the plasmodesmata (PD) is a critical factor affecting cell-to-cell movement and subsequent long-distance movement of viruses. The viral movement protein (MP) is involved in the process of enhancement of SEL of PD which is known as gating (Wolf et al. 1989). The movement complexes, comprising many proteins, function in a coordinated manner with different roles for the protein components of the complex. Gating appears to be a basic requirement for cell-to-cell movement of non-tube-forming viruses. Actin is involved in depolymerizing the actin, leading to an increase in SEL. In contrast, deposition of callose in response to defense and wound responses may lead to closure of plasmodesmata (Roberts and Oparka 2003). The interaction between PVX TGB2 and proteins that interact with β -1,3-glucanase, a callose-degrading enzyme, has been suggested to be a strategy of PVX employed to gate PD to accelerate callose degradation (Fridborg et al. 2003). The interaction of *Tobacco mosaic virus* (TMV) MP with pectin methyl esterase (PME) may regulate the activity of PME, loosening the cell wall around PD and allowing the PD to open more easily. Alternatively the MP may recruit additional PME to PD to assist gating rather than relying on PME for targeting (Chen et al. 2005).

8.3.2.2 Long-Distance Movement of Viruses

Plant viruses have to move from the initial site of infection or inoculations and travel long distances to infect other plant organs such as petiole, stem, roots, flowers, and seeds. The majority of plant viruses depend on the phloem for long-distance movement, whereas a few viruses such as *Lettuce necrotic yellows virus* (LYNV) and *Rice yellow mottle virus* (RYMV) can move through xylem (Hull 2002). Different mechanisms may

operate singly or in combination depending on the virus concerned. A host defense system silencing signal spreads between cells and also long distances from the lower to upper leaves. The movement of viruses to other plant organs depends on an RNA-dependent RNA polymerase (RDR), whereas the signal for cell-to-cell movement does not depend on RDR (Himber et al. 2003). Systemic infection by plant viruses entails cell-to-cell movement into, through, and out of various cell types of the vascular system, in addition to long-distance transport through sieve elements. A barrier formed as constitutive or postinfectious responses may block the systemic spread of the virus at different stages.

The long-distance movement of *Potato leafroll virus* (PLRV) was investigated using two potato clones showing resistance to virus multiplication and accumulation. These clones were graft- or aphid-inoculated with PLRV and grown in the greenhouse along with a moderately susceptible cultivar for comparison. A high concentration of aphid-borne PLRV inoculum was used to ensure strong inoculum potential. Clones M62759 and PS1706 were highly resistant and susceptible to PLRV, respectively. Both clones expressed a high level of resistance to virus multiplication when primary or secondary infection was assayed by ELISA test. In addition, PLRV could only be detected in a few or none of the progeny plants of clone M62579, therefore indicating strong inhibition of virus transport to tubers. Translocation of PLRV from aphid-inoculated shoots to uninoculated shoots sprouted from the same tubers was monitored. No specific mechanism appeared to impair PLRV movement through tubers of the resistant genotypes. The results indicated that three valuable components of resistance to PLRV seemed to be closely linked in the genotype, a combination occurring rather rarely in potato clones (Syller 2003). Reduction in the rate of cell-to-cell movement in inoculated leaves resulted in inhibition of systemic infection of zucchini squash (*Cucurbita pepo*) by M strain of *Cucumber mosaic virus* (M-CMV) (Shintaku and Palukaitis 1990). However, coinfection of zucchini plants with M-CMV and *Zucchini yellow mosaic virus* strain A (ZYMV-A) facilitated M-CMV to move systemically, as revealed by tissue-print hybridization analysis. Gel analysis of RT-PCR products obtained from nucleic acids extracted from the upper leaves of plants doubly inoculated with M-CMV and ZYMV showed that M-CMV CP gene-specific production was absent from extracts of plant inoculated with M-CMV alone. On the other hand, the expected size band for M-CMV CP was detected in plants doubly inoculated with M-CMV and ZYMV. ZYMV-encoded HC-Pro or other proteins of ZYMV involved in the systemic movement of M-CMV into uninoculated leaves away from the site of inoculation (Choi et al. 2002).

Transcriptional gene silencing (TGS) is recognized by the absence of transcription from silenced gene and occurs where homology exists between promoter sequences of the affected genes. In contrast, PTGS genes contain a homologous sequence within the coding region. Although PTGS genes are actively transcribed, only low levels of steady-state mRNA can be detected. PTGS is a homology-dependent RNA degradation process that may target RNA exclusively in the cytoplasm of host cells. Plant viruses replicating in the cytoplasm can function as both initiator and target of PTGS. Many plant viruses encode protein that can suppress PTGS as a counter-defense strategy. HC-Pro encoded by potyviruses may be able to “switch off” gene silencing in plants wherever it is expressed. The 2b proteins encoded by *Cucumber mosaic virus* (CMV) do not have any effect in tissues where PTGS is established, but are able to prevent initiation of gene silencing in newly emerging tissues (Brigneti et al. 1998; Li et al. 1999). The intracellular distribution of CMV 2b protein (Cmv2b) was investigated by fusing it with GFP to

understand the mechanism(s) of counter-defensive suppression effect of Cmv2b. This protein localized to the nuclei of tobacco suspension cells and whole plants via an arginine-rich nuclear localization signal. The nuclear targeting of 2b protein was essential for the efficient suppression of PTGS, indicating that PTGS might be blocked in the nucleus. The PTGS suppressor activity was not sufficient for virulence determination by Cmv2b protein, although it is important (Lucy et al. 2000).

The tripartite genome of *Cucumber mosaic virus* (CMV) encodes replicase function in RNA1 and RNA2, with RNA3 encoding MP and CP proteins. For local cell-to-cell movement and also for systemic movement in squash plants, both MP and CP are required (Canto et al. 1997; Kaplan et al. 1997). The systemic movement of CMV in cucumber plants was investigated by analyzing the structure that is translocated and its putative interactions with phloem components in the phloem exudates (PE) samples. The movement of CMV as intact virus particles was indicated by the rate zonal centrifugation and electron microscopy observations of PE from CMV-infected plants. The virus particles interacted with a PE protein p48 which was found to be a cucumber homolog of phloem protein 1 (PP1) from pumpkin. The PP1 from pumpkin has the PD gating property and ability to translocate in sieve tubes. Interaction between encapsidated CMV particles and p48 protein seemed to make the viral RNA more resistant to RNase. The results indicated that the interaction with p48 might modify CMV particle structure and suggested that the virus particles might interact with cucumber homolog of PP1 during translocation in sieve tubes of cucumber (Requena et al. 2006). Long-distance movement is required for the virus to become systemic. Mutations within HC-Pro coding region of *Wheat streak mosaic virus* (WSMV) were introduced by misincorporation during PCR; nonsynonymous substitutions, including that of consistent reversion to wild-type, lead to attenuation of systemic infection. Mutants bearing GUS reporter gene were evaluated for their ability to establish primary infection foci. HC-Proteinase activity was observed in mutants that could not establish foci of infection. The results indicated that a complete HC-Pro deletion mutant could infect plants systemically and that certain substitutions in this dispensable gene probably prevented systemic infection by WSMV via interference (Stenger et al. 2006).

Cucumber mosaic virus (CMV) 2b protein has dual functions as an RNA silencing suppressor protein and also inducer of disease symptoms. A hybrid virus, FRad352b-CMV (renamed as CMV-FRad2b-Pro), generated by replacement of the 2b gene of the strain Fny-CMV with that from Rad35-CMV, displayed markedly lower pathogenicity than Fny-CMV on *Nicotiana* species. However, the replacement of proline with leucine at position 55 of the 2b protein of CMV-FRad2b-Pro (protein Rad2b-Pro) created a virus (CMV-FRad2b-leu) that induced severe symptoms. Experiments on *Arabidopsis thaliana* showed that Rad2b-Pro was a weaker RNA silencing suppressor than the protein Rad2b-Leu. This was confirmed in *Nicotiana benthamiana* using agroinfiltration assays showing that, compared with either Rad2b-Leu or the Fny2b protein, Rad2b-Pro was ineffective in inhibiting local or systemic silencing of expression of a green fluorescent protein (GFP) reporter gene (*gfp*). Transgenic expression of Rad2b-Leu but not of Rad2b-Pro in *Arabidopsis* induced symptom-like phenotypes and rescued the accumulation of the 2b-deletion mutant Fny-CMV Δ 2b. Bimolecular fluorescent complementation indicated that in planta Rad2b-Leu self-interacted, but not Rad2b-Pro. Self-interaction was crucial to the ability of the 2b protein to suppress silencing and induce a symptom-like phenotype and was dependent on the properties of the residue at position 55 of the 2b protein of *Cucumber mosaic virus* (Xu et al. 2013).

8.3.3 Colonization of Plant Tissues by Viruses

Plant viruses systemically infect different kinds of plant organs such as leaf petiole, stems, roots, floral parts, and seeds. They colonize these different plant tissues which may support their replication to varying degrees and consequently exhibit different abnormalities. In some plant species, the viruses multiply to a fairly high titer without inconveniencing the host and producing any visible symptoms. These latent infections may become potential sources of virus inoculum that may be spread by the natural biological vectors. The presence of viruses in various plant tissues may be detected by employing light and electron microscopy, immunoassays, and nucleic acid-based molecular techniques.

Citrus tristeza virus (CTV) was detected by applying the in situ immunoassay (ISIA) procedure. Sections of petioles, leaf veins, and stems of CTV-infected and healthy plants were fixed with 70% ethanol and incubated with specific polyclonal antibodies (PAb) 1212 or monoclonal antibodies (MAbs) MCA13 or 17G11. The antibodies bound to plant cells were labeled with enzyme-conjugated species-specific secondary antibodies and exposed to a substrate mixture containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Development of a purple color, indicating the presence of CTV antigen, could be visualized by observing under the light microscope. The presence of purple color was only observed in the phloem tissues of CTV-infected plants, but not in comparable tissues of healthy plants. Locations of CTV antigens could be observed directly and accurately by in situ immunoassay (ISIA) method in both fresh tissues as well as in samples stored in plastic bags at 4°C or frozen for 4 weeks. The ISIA procedure provided a unique advantage of determining the three-dimensional distribution of CTV within the host. In addition, the ISIA test may also have applications for cross-protection and pathogen-derived resistance (PDR) investigations (Lin et al. 2000). The transcriptional responses of citrus to *Citrus tristeza virus* (CTV) were investigated. Genes (430) differentially regulated by severe CTV isolates in lime plant were identified by applying suppression subtractive hybridization (SSH) procedure. The differential expression levels of 25 expressed sequence tags (ESTs) in CTV-N21 infected lime were validated by qRT-PCR. The largest set of those genes were involved in primary metabolism (26.0%) and then in protein metabolism (15.1%). The results revealed that a large set of genes (51 ESTs) were commonly upregulated by two severe strains CTV-N21 and CTV-T305 in lime. The quantitative analysis for temporal and spatial transcriptional levels of those genes fluctuated during CTV infection and differed in different citrus leaves (Yang et al. 2013).

Tomato spotted wilt virus (TSWV) infects *Ranunculus asiaticus*, an ornamental crop grown for tuber production, potted plants, and cut flowers. Distribution of TSWV in tuber and root tissues of *R. asiaticus* was monitored by employing tissue-blot immunoassay. The cut surfaces of the desired tissues were gently blotted on nitrocellulose membranes and the tissue-blot immunoassay (TBIA) was performed using the monoclonal antibody generated against NSs of TSWV. The positive reaction was indicated by the formation of a purple precipitate. Presence and distribution of TSWV in *R. asiaticus* was monitored. TSWV was not uniformly distributed in *Ranunculus* tubers and the percentages of infected rootlets per tuber varied. The virus was detected in more than 50% of the rootlets per tuber for the majority of infected tubers assayed. When one or more roots had detectable TSWV titer, the central stem portion always tested positive. Further, TSWV could be detected in the foliage produced from the tubers in which

TSWV was detected earlier by TBIA technique. The results indicated that TSWV could be replicated in different organs of *R. asiaticus* plants, reaching sufficient titers detectable by TIBA technique (Whitefield et al. 2003). The presence of TSWV in different parts of potato tubers was monitored using enzyme-linked immunosorbent assay (ELISA). Rates of TSWV transmission from infected foliage to potato tubers and from infected tubers to progeny plants were assessed. The results indicated that cultivars of potato had a significant effect on TSWV transmission from plant to tuber and from tuber to plant. As the virus was unevenly distributed in the tubers, composite samples including different parts of potato tubers might provide reliable detection of TSWV (Bulajić et al. 2014).

Cytopathology of developing anthers and pollen mother cells from young flowers of lettuce plants infected by *Lettuce mosaic virus* (LMV) was studied. Ultrastructural investigations revealed that within infected pollen mother cells, structures reminiscent of bundles and partly formed pinwheel inclusions were discernible. Immunogold labeling showed that particles of LMV were associated with these structures. Neither large aggregates of LMV particles nor complete pinwheel inclusions were observed in pollen mother cells. The infection of the pollen mother cells was consistently correlated with infection of the adjacent tapetal cell layer. The pollen mother cells of two other LMV-infected plants were not infected, indicating that LMV infection was not successfully achieved in all pollen mother cells of LMV-infected plants. Early invasion of the floral meristem appeared to be necessary for virus infection of mature pollen grains (Hunter and Bowyer 1997). Cytopathological effects of colonization of onion tissues by *Artichoke yellow ringspot virus* were studied by electron microscopy and decoration techniques. Large cytoplasmic inclusions were observed in mesophyll cells of infected plants, but not in healthy tissues. The cytoplasmic inclusions comprised voluminous accumulations of entangled membranous elements, more or less developed and specifically arranged particle aggregates, and conspicuous globules of a supposed lipid nature. The characteristic cytopathologic structures principally consisted of complexes of two cisternae of rough endoplasmic reticulum (ER) in a parallel arrangement. A high proportion of infected cells contained AYRSV particles. Antiserum against AYRSV did not react with the onion isolate in immunosorbent electron microscopy (ISEM) and decoration tests (Maliogka et al. 2006). An antiserum specific to *Fig latent virus 1* (FLV-1) was used for visualization of the virus by ISEM procedure in seedlings of different ages and geographical origin. All seedlings irrespective of the age and origin were found to be heavily infected with FLV-1. Virus particles were readily decorated by the FLV-1-specific antiserum. The sections stained with lead citrate showed well-preserved cytological organization. Major organelles appeared to be normal, except for the chloroplasts which were swollen, misshapen, and exhibited a somewhat disarranged membrane system. Bundles of filamentous virus particles were frequently visualized in the cytoplasm of cells of parenchyma in asymptomatic seedlings, indicating the usefulness of electron microscopy to study the anatomical changes induced by FLV-1 even before the appearance of symptoms of the disease (Castellano et al. 2009).

Grapevine leafroll-associated virus 3 (GLRaV-3), a major grapevine pathogen, causes considerable production losses. A single-chain fragment variable (scFv) antibody (scFvLR3) directed against the coat protein (CP) of GLRaV-3 was expressed in *Escherichia coli* and used in immunosorbent electron microscopy (ISEM) examination for the presence of GLRaV-3 in grapevine tissue extracts. The antibody was fused to the light chain constant domain of human immunoglobulin to create the bivalent reagent C_L-LR3.

The bivalent reagent bound strongly to GLRaV-3 and the protein either captured or decorated GLRaV-3 in electron microscopy assays. The results of ISEM corroborated those of enzyme-linked immunosorbent assay (ELISA) tests (Cogotzi et al. 2009). The route of infection of cucumber by *Cucumber green mottle mosaic virus* (CGMMV) was investigated by using scanning electron microscopy (SEM). At anthesis, pollen was collected from the flowers of the infected cucumber plants and they were used to pollinate noninoculated plants. The presence of CGMMV in pollen from infected plants was detected by SEM observations. Transmission of CGMMV to the seeds and subsequently to the seedlings growing from these seeds was revealed by RT-PCR analysis (Liu et al. 2014).

The genomic viral RNA/DNA and/or the coat protein (CP) of *Carnation mottle virus*, *Carnation latent virus*, and *Carnation etched ring virus* were localized in the carnation shoot tip to determine whether any of them were capable of infecting the shoot apical meristem. The results showed that all viruses were excluded not only in single-infected but also in double-infected plants. However, these viruses showed a different infection pattern inside the shoot-tip, with different cell types being their main targets. For mixed infections, a spatial separation pattern within carnation shoot-tip meristem between the viruses in mixed infection was observed, strongly suggesting the existence of an exclusion mechanism in the shoot-tip tissue of carnation (Gonsalvez-Bernal et al. 2006).

The mechanism of seed transmission of *Pepper mild mottle virus* (PMMoV) in pepper (*Capsicum annuum*) was investigated. The virus immunolocalized in pepper seeds was visualized using fluorescence microscopy technique. Two distinct patterns of virus distribution were observed. The presence of PMMoV in the epidermis and parenchyma was seen but not in the endosperm or embryo, whereas in other seeds the virus was restricted to the surface of the epidermis and parenchyma (Genda et al. 2005). In a later study, the infection cycle of PMMoV was studied through vertical transmission of the virus. The location of PMMoV within seeds as they developed on inoculated seedlings of pepper was followed by detecting the viral coat protein using immunofluorescence microscopy procedure. Seedlings were inoculated with PMMoV when the flower buds on the first and second branching nodes were in bloom. The presence of PMMoV was inferred by the fluorescence around immature seeds and placentas in the ovaries on the fourth branching node at 20 days postanthesis (20 dpa), which corresponded to 39 days postinoculation (39 dpi). The area with fluorescence progressively expanded from the placenta into the integument and the parenchyma and finally reached the tip of immature seeds by 34 dpa. The embryo or endosperm beyond the endothelium did not fluoresce during the experiment (until 102 dpi). For visualizing the viral routes of invasion from seeds into developing seedlings, PMMoV-infected *C. annuum* seeds that were heterologous for the L³ tobamovirus-resistance were grown in soil at 30 °C. The cotyledons developed virus-induced necrosis (Genda et al. 2011).

Prunus necrotic ringspot virus (PNRSV), belonging to the genus *Ilarvirus*, infects many stone fruit trees including cherry, sour cherry, almond, peach, apricot, and plum. Infected pollen has a key role in both seed and plant-to-plant transmission of the members of *Ilarvirus* (Milne and Walter 2003). The route of infection and the pattern of distribution of PNRSV in apricot pollen were investigated. By employing dot-blot hybridization technique, PNRSV was detected inside as well as on the outside of infected apricot pollen grains. On the pollen surfaces, PNRSV was probably attached to the exine. The flower buds at different stages of development were tested to determine the route by which PNRSV invaded pollen grains. In the earliest stage in which flower buds

with anthers contained pollen mother cells (PMCs), PNRSV was detected in all flower bud tissues including sepals, petals, and anthers. Different degrees of infection of anthers were revealed by in situ hybridization assay. Viral RNA was localized only in the vascular tissue in some, whereas other anthers showed the complex invasion of PNRSV with PMCs also being infected. The virus could be detected in all tissues of flower bud, including anthers containing unicellular pollens. PNRSV infection persisted until flower maturation, when the virus was detected in the whole flower. In addition, in infected pollen grains in the early stages of maturation, PNRSV infection was uniform with cytoplasm particularly being highly invaded. PNRSV was not detected in the nucleus of any of the pollen grains examined. In the bicellular pollen grains, PNRSV was detected in the cytoplasm of the vegetative cell and the virus was sometimes localized around and inside the generative cell. The presence of viral RNA inside the generative cell was confirmed by in situ hybridization at electron microscopy level, as the gold particles corresponding to PNRSV were localized to the cytoplasm of the generative cell. In the germinated infected pollen grains, viral RNA was seen to be evenly distributed inside pollen grains as well as in the pollen tube with a higher concentration at the tip or growth zone of germinating pollen tube, but no signal was detected in comparable healthy pollen grains. The results indicated that the route of infection of PNRSV commenced from another version by first infecting the vascular system of the anther filament. The virus could then move from the vascular system to infect the anther connective tissue, using the high number of cytoplasmic connection bridges characteristic of this tissue. PNRSV invaded the tapetum cells and then pollen mother cells (PMCs). Apparently PMCs might be infected prior to the callose deposition as the callose layer might separate the PMCs from one another and also from the anther tissue. Pollen tubes, once germinated, could reach the apricot embryo sacs. Infected pollen could therefore play an important role in the vertical transmission of PNRSV in apricot (Amari et al. 2007).

In situ hybridization analyses of *Prunus necrotic ringspot virus* (PNRSV) distribution in apricot reproductive organs at different developmental stages were carried out. PNRSV was detected in the early stages in both the ovule and ovary. The virus was present in the ovules and nucellus in the megaspore stage. PNRSV was localized in the later developmental stages, in the funiculus as well as in the corresponding ovule, and the distribution of the virus was uneven in the ovary at this stage. Later, PNRSV invaded all parts of the ovule including inner and outer integuments surrounding the nucleus which enclosed the embryo sac. At this stage, two different infection patterns could be differentiated. Infection of the whole ovule occurred in some floral buds, whereas the nucellus was free of PNRSV except for the cells close to and surrounding the embryo sac. However, the presence of PNRSV was always observed in the nucellar cells located around the embryo sac, even in the more advanced developmental stage, where viral RNAs were rarely detected inside the embryo sac; when present, they were found in the fused polar cells.

Invasion of the apricot embryo by PNRSV in the early developmental stages was confirmed by dot-blot hybridization procedure. PNRSV was detected in embryos in immature seeds at globular stage. PNRSV RNA was detected in embryos in the globular developmental stage, following hand-pollination of virus-free flowers with infected pollen grains. Viral infection of seed integuments, nucellus, and testa in immature seeds could be detected using in situ hybridization assay. Infection of the testa mainly affected areas in and around its vascular tissue. Tissue-printing hybridization (TPH) from fruits obtained from pistils of hand-pollinated PNRSV-infected pollen revealed the presence of viral RNA in the periphery of fruits, whereas crosses between infected pistils and

infected pollen resulted in total invasion of entire fruits, indicating the vertical transmission of PNRSV from gametes to apricot seedlings (Amari et al. 2009).

Cocoa swollen shoot virus (CSSV), belonging to the genus *Badnavirus*, causes the devastating disease in cocoa. As cocoa is propagated largely by seeds, transmission of CSSV via seed is considered a critical factor impacting adversely cocoa production; the route of virus invasion of seed was therefore investigated. Seed embryo infection by CSSV could occur by two routes, either directly from infected gametes at fertilization or by direct invasion of immature embryo from virus-infected testa tissues (Maule and Wang 1996). All pollen grains isolated from the anthers of cocoa trees (T2 and T4) were found to contain the virus, as indicated by PCR assay/agarose electrophoresis (AE) and PCR/capillary electrophoresis (CE). Cocoa seeds from infected T2 and T4 plants and healthy plants were separated into component parts: testa, cotyledon, and embryo and tested for the presence of CSSV. The component parts of seeds of infected trees showed varying levels of CSSV infection by PCR/agarose electrophoresis, but PCR/capillary electrophoresis revealed 100% infection of seed components by CSSV. The seedlings raised from infected seeds obtained from CSSV-infected trees showed virus infection at a high frequency as revealed by PCR/CE techniques, indicating the potential danger of using seed lots without checking the levels of seed infection by CSSV (Qainoo et al. 2008). *Apple latent spherical virus* (ALSV) was detected in apple flower buds by in situ hybridization assay. ALSV was present inside almost all pollen grains and in all ovary and ovule tissue, including the embryo sac and inner integument. Although low level of seed embryo infection was detected, ALSV was not transmitted to the seedlings (Nakamura et al. 2011).

Pea enation mosaic virus (PEMV) disease is due to two obligately symbiotic viral genomes: PEMV-1, the sole member of the genus *Enamovirus* in the family Luteoviridae; and PEMV-2, a member of the genus *Umbravirus*. A satellite RNA (RNA3) is associated with PEMV-2. The PEMV-1 genomic RNA contributes to encapsidating CP gene and aphid transmissibility, while the PEMV-2 genome is responsible for mechanical transmissibility and systemic viral movement in the plant (Demler and de Zoeten 1989; de Zoeten and Skaf 2001). Accumulation and relative concentrations of PEMV-1 and PEMV-2 viral genomes in leaf and seed tissues were estimated using a quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The PEMV-1 and PEMV-2 genomes accumulated in all tissues, with large relative differences in mean normalized accumulation (MNA) between tissues. The PEMV genomes accumulated to high levels in stipules, whole pea, and seed coat, but to much lower levels in pea embryo tissues. On an average, PEMV-1 and PEMV-2 genome RNAs accumulated to c. 1240 and 13,000 times higher levels in stipule than in embryo, respectively. Accumulation of PEMV genomic RNA in developing seed tissues from PEMV-infected plants was investigated. PEMV-1 and PEMV-2 genomic RNAs accumulated to high amounts in all pod wall and seed coat samples. The genomic contents accumulated to similar levels in seed coats and pod walls. PEMV-1 and PEMV-2 genomes could often be detected in embryo tissues, but at much lower levels. PEMV-1 was not detected in embryo axis in three plant or pod samples or from one cotyledon and embryo axis sample. PEMV-2 was not detected in one cotyledon and two embryo axis samples. In all samples in which PEMV-1 was detected, concentrations of PEMV-1 were greater in the cotyledons than in the embryo axis. No evidence was obtained for seed transmission of PEMV, since none of the >50,000 plants grown from seeds derived from PEMV-infected source plants exhibited symptoms of pea enation mosaic disease (Timmerman-Vaughan et al. 2009).

The connection between the ability of the plant viruses to reprogram host gene expression and the pathogenicity of viruses has been investigated. The interference of viruses with cellular transport process is potentially the effective form of altering the physiology of susceptible plants. Virus multiplication and movement are necessary for the disease symptoms to develop. The rate and extent to which these processes occur may be the primary determinants of symptom development. The infective viral cycle in a susceptible host mostly begins through epidermal cells or through roots, as a result of either mechanical damage or feeding injuries caused by vectors of viruses. When the viral genome replication cycles have been completed, the progeny viruses move from infected cells and reach the vascular system to invade the distal plant parts. Movement proteins (MP) can act by forming ribonucleoprotein complexes with the viral genomes or tubular structures that hold the virions and allow them to cross plasmodesmata (Lucas 2006; Sanchez-Navarro et al. 2006). The most common way to restrict the viral invasion is through blocking its cell-to-cell movement function which may directly influence the development of disease symptoms. Studies conducted with natural or artificial mutants of the corresponding MPs or with pseudorecombinant viruses have contributed to the understanding of the role of virus movement in pathogenesis. The symptomatic variant was correlated with a mutation in MP which corresponded to the thermosensitive Ls-1 mutant of *Tobacco mosaic virus* (Nishiguchi et al. 1978). This mutant could not become systemic at high temperatures. The Ls-1 mutant had a proline-to-serine change in the MP gene (Ohno et al. 1983). An association between less symptomatic phenotypes and lesser accumulation of the MP from TMV was later established (Arie-Johnson et al. 1995).

The severity of symptoms is not always correlated with virus titer, indicating that disease development may be due to specific interaction between virus and host components. Plant virus MPs have been shown to interact with a range of host proteins to facilitate movement of the virus in the susceptible host plant. Host proteins with which the viral MPs interact may be localized in the nucleus, cytoplasm, and endoplasmic reticulum, microtubules, and plasma membrane. Most of these interactions may be essential for the intra- and intercellular movement of viruses and these interactions have significant effects on the symptom expression. TGBp2, *Potato virus X* MP, interacted with cytoplasmic protein Tip-1 which in turn interacted with β -1,3-glucanase (Fridborg et al. 2003). This enzyme is known to participate in the regulation of callose levels which have an important role in the regulation of the size exclusion limit (SEL) of the plasmodesmata (Iglesias and Meins 2000). Plants overexpressing the MP proteins have been used to shed light on the involvement of MPs in viral pathogenesis. As transgenically expressed MPs preferentially located to the plasmodesmata, the appearance of symptoms of viral infection such as abnormal accumulation of sugars, reduced photosynthesis, chlorosis, and dwarfism was attributed to overexpression of MPs in plants, causing functional interference of these proteins with cytoplasmic communication channels. Plants expressing TMV MP showed alterations in the carbohydrate distribution originating in mesophyll tissues while the primary site of action of the MPs was restricted to the phloem, as in MP17 from *Potato leafroll virus* (PLRV) which resided in the phloem tissue (Balachandran et al. 1995).

There are many determinants of viral pathogenicity. Protein P3 from potyviruses is an inducer of the responses mediated by several dominant resistance genes (Hajimorad et al. 2006; Chowda-Reddy et al. 2011). Protein P3 along with the small 6K1 peptide was bound to *Soybean mosaic virus* polyprotein and they conditioned the symptomatology

and host range of the virus (Desbiez et al. 2003; Salvador et al. 2008a). Replacement of the P1-coding sequence of *Plum pox virus* (PPV) by that of another potyvirus *Tobacco vein mottle virus* (TVMV) had no effect on the virus infectivity in plants that were hosts of both PPV and TVMV, but inhibited infection on the PPV host *Prunus persica* which was not susceptible to TVMV (Salvador et al. 2008b).

Sweet potato plants are commonly infected by *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV), resulting in a complex called sweet potato virus disease (SPVD) complex. Aphids and white flies are involved in the transmission of SPFMV and SPCSV, respectively, and the transmission of these two viruses occurs independently in a sequential manner under natural conditions. The effect of sequential infection on symptom development and individual virus titers was assessed by inoculating the plants infected by one virus with the other virus. Symptoms were significantly more severe in plants infected with SPCSV followed by SPFMV, compared with plants infected first with SPFMV followed by SPCSV. Virus titer were not significantly different for SPCSV, but SPFMV titers in plants infected with SPCSV followed by SPFMV, were significantly higher than that of all other combinations. The sequence of infection of sweet potato plants had significant influence on disease severity and SPFMV reached higher titers in plants already infected with SPCSV (McGregor et al. 2009).

8.4 Characteristics of Viroids

Viroids are obligate plant pathogens comprising small unencapsidated, covalently closed, circular, and nonencoding single-stranded RNA molecules (246–401 nucleotides). They share some structural similarities with certain satellite RNAs. However, satellite RNAs depend on the helper viruses for their replication. Viroids do not code for any protein and therefore remain unassociated with any structural protein(s) that are present, as in the case of plant viruses. Viroids may be considered as RNA parasites that have shed off all the protein-synthesizing capacity and made efficient use of the host's synthetic machinery for their replication (Diener 1999; Tabler and Tsagris 2004; Flores et al. 2005a). Based on the structural characteristics and site of replication in the host cells, the viroids are classified into two families: Pospiviroidae and Avsunviroidae (Chapter 7). *Potato spindle tuber viroid* (PSTVd), the first viroid to be discovered and characterized, has a serial arrangement of 26 double-stranded segments interrupted by bulge loops of varying sizes. The extended secondary structure is folded into a more globular tertiary conformation, as the single-stranded loops do not interact. The viroids possess both stability and flexibility. The native rod-like structure of viroids is converted through thermal denaturation into hairpin-containing circle in a highly cooperative fashion (Diener 1979; Henco et al. 1979). The International Committee on Taxonomy of Viruses (ICTV) admitted 29 distinct viroid species and six were listed as tentative viroid species (Flores et al. 2005b).

Fruit crops are infected by several viroids. Six viroids, *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd), *Apple fruit crinkle viroid* (AFCVd), *Pear blister canker viroid* (PNCVd), *Hop stunt viroid* (HSVd), and *Peach latent mosaic viroid* (PLMVd) have been reported to infect naturally pome (apple, pear, and quince) and stone (peach, plum, and almond) fruit trees (Hadidi et al. 2003). Likewise, *Citrus* spp. are naturally infected by *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus viroid IV*

(CVd-IV, renamed *Citrus bark cracking viroid* (CBCVd)), *Citrus bent leaf viroid* (CBLVd), and *Citrus viroid III* (CVd-III, renamed *Citrus dwarfing viroid* (CDVd)). In addition, the incidence of two viroids – *Citrus viroid original source* (CVd-OS) and *Citrus viroid V* (CVd-V) – has also been observed (Ito et al. 2001; Serra et al. 2008). These viroids usually occur as complex mixtures coinfecting the same plant and producing overlapping symptoms. Precise, sensitive, and reliable technique(s) may be required to resolve the components of the viroid complex and to differentiate the viroids involved.

8.4.1 Biological Properties of Viroids

8.4.1.1 Types of Symptoms

Viroids cause symptoms that may be similar to those induced by viruses. However, some of the viroids such as *Potato spindle tuber viroid* (SPTVd) have a characteristic effect on tubers, which become abnormally elongated assuming a spindle or cylindrical shape with prominent eyes (Diener 1979). Likewise, symptoms caused by *Apple scar skin viroid* (ASSVd) are confined to apple fruits and symptoms on fruits may differ depending on the apple cultivar (Koganezawa et al. 2003). When graft-inoculated onto sensitive selection of Etrog citron, citrus viroids induce distinguishable symptoms; however, this required 3–9 months, good greenhouse facilities, and experienced personnel (Roistacher 1991). *Peach latent mosaic viroid* (PLMVd) causes irregularly shaped, discolored, and deformed fruits with cracked structures and enlarged pits. Some strains also induce clear-cut foliar leaf discoloration or yellowing (Malfitano et al. 2003). When the environmental conditions existing in the field were not favorable for symptom expression, the forced cutting method was followed. This procedure involved forcing cuttings taken directly from citrus orchards to sprout in a growth chamber at temperatures around 30°C. This treatment appeared to promote viroid replication. This process facilitated detection of viroids by other diagnostic methods. Forcing citrus cuttings was effective for detection of citrus strain of *Hop stunt viroid* (HSVd-cit), *Citrus exocortis viroid* (CEVd), and *Citrus viroid III* (CVd-III), even when no positive amplification by PCR was obtained from field-grown plants (Guardo et al. 2005).

8.4.1.2 Diagnostic Hosts and Host Range

The majority of the viroids cause symptoms similar to those induced by other viroids and viruses infecting the same host plant species, resulting in confusion in establishing the identity of the viroid(s) with certainty. Diagnostic or assay hosts which react by producing characteristic and distinguishable symptoms more rapidly than the natural host plant species (crops) are therefore employed. This test, known as biological indexing, usually requires a long time and a large glasshouse space. *Potato spindle tuber viroid* (PSTVd) produced necrotic local lesions in *Scopolia sinensis* following mechanical inoculation (Singh 1971). Likewise, cucumber (*Cucumis sativus*) could be inoculated to detect *Hop stunt viroid* (HSVd) in greenhouses maintained at 30°C. The cucumber bioassay was found to be useful even when the viroid was at a low concentration (1:200). The practical utility of cucumber bioassay was demonstrated by testing samples from field-grown HSVd-infected plants (Sasaki and Shikata 1977; Sasaki et al. 1981).

The use of *Gynura aurantiaca* plants as a bioindicator for effective detection and identification of *Citrus exocortis viroid* (CEVd) was demonstrated. *G. aurantiaca* plants were inoculated with total nucleic acid preparations from grapevine plants and the inoculated plants were topped to promote the production of new shoots.

The development of symptoms of CEVd infection was monitored for 30 days after inoculation. An infectious RNA isolated from *G. aurantiaca* was subjected to electrophoresis and this RNA had the same electrophoretic mobility of CEVd, establishing the identity of the viroid infecting the grapevine (Flores et al. 1985). Arizona 861-S1 Etrog citron plants were graft-inoculated with 112 citrus samples collected during a survey. The bioindexing procedure to detect and identify *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), and *Citrus viroid III* (CV-III) was effective in 104 of 112 citrus samples tested (Malfitano et al. 2005). The distribution of CEVd and HSVd in the orchards of Central Italy was monitored by applying one-tube RT-PCR assay. Green bark and leaves from 21 varieties belonging to 9 citrus species, and 1 hybrid which showed no specific symptoms of viroid infection, were tested by one-step RT-PCR assay. All *Citrus* species except citron, limeta, and kumquat were infected by CEVd to a different extent. Sweet orange (4/4), mandarin (3/4), satsuma (2/2), clementine (2/3), and lemon (3/3) were infected by CEVd in addition to the hybrid lemon x citron. On the other hand, infection by HSVd was detected in mandarin (2/4), clementine (1/3), and kumquat. The predominance of CEVd was revealed by the one-tube RT-PCR analysis. CEVd infection in 16 citrus varieties and HSVd infection in 4 citrus varieties could be detected, although the infected plants did not exhibit any specific symptom of viroid infection (Ragozzino et al. 2005).

Citrus viroid V (CVD-V) was initially identified after transmission to *Atlantia citrioides* which was immune to CEVd, CBLVd, CDVd, HSVd, and *Citrus bark cracking viroid* (CBCVd), but allowed replication of CVD-V (Barbosa et al. 2005). CVD-V induced mild but typical symptoms and, in coinfection CVD-V, interacted synergistically with either *Citrus bent leaf viroid* (CBLVd) or *Citrus dwarfing viroid* (CDVd) producing enhanced leaf symptoms and highly severe dwarfing of infected plants (Serra et al. 2008). Grapevine viroids, *Grapevine yellow speckle viroid* 1 and 2 (GYSVd-1 and -2), *Hop stunt viroid* (HSVd), *Citrus exocortis viroid* (CEVd), and *Potato spindle tuber viroid* (PSTVd) were detected and differentiated by using cucumber (*Cucumis sativus*) cultivar Alpha, tomato (*Lycopersicon esculentum*) cultivar Castle Rock, and *Gynura aurantiaca* as bioindicators (Nasr-Eldin et al. 2012). Australian grapevine viroid (AGVd), a member of the family Pospiviroidae, was inoculated by agroinfiltration of dimer constructs of AGVd into the leaves of cucumber, tomato, squash (*Cucurbita pepo*), purple passion (*Gynura aurantiaca*), tobacco cultivar Turkish tobacco, *Nicotiana glutinosa*, and pot marigold (*Calendula officinalis*). All plant species inoculated with AGVd either showed visible symptoms or the viroid could be detected in the infected plants that were asymptomatic, with the exception of tobacco. Tobacco was not infected by AGVd, which also could not be detected by PCR assay (Zaki-Aghl et al. 2013).

Viroid infection of sweet pepper (*Capsicum annuum*) in the glasshouse-grown pepper was observed. Based on the biological and molecular properties, the pepper viroid was named as the new species *Pepper chat fruit viroid* (PCFVd) in the genus *Pospiviroid*. Seven solanaceous plant species were mechanically inoculated with PCFVd. Tomato cultivar Money-maker and potato cultivar Nicola were infected by PCFVd; leaf distortion and tuber deformation, in addition to the formation of necrotic lesions on leaves, were observed in the infected tomato and potato, respectively (Verhoeven et al. 2009). *Potato spindle tuber viroid* established appears to have the widest host range, infecting several plant species including potato. Economically important crops infected by PSTVd include tomato, avocado, pepino, and sweet potato, in addition to wild *Solanum* spp. Infection by PSTVd of ornamental species may go undetected since the infected

plants mostly remain asymptomatic; such plants may become potential sources of viroid inoculum (Lenarčič et al. 2012). *Solanum jasminoides* plants were found to be symptomless hosts of PSTVd (Anonymous 2006). Infection of *Brugmansia suaveolens*, *B. cordata*, and *B. variegata* was reported from Netherlands (Verhoeven et al. 2007). *Solanum jasminoides* and *S. rantonnetii* were found to be susceptible to PSTVd under natural conditions. The presence of PSTVd was detected by tissue-printing hybridization technique in these plant species (Di Serio 2007). The experimental host range includes a wide range of plant species from other families such as sweet potato and avocado. Infection of avocado remained latent, but when coinfecting by *Avocado sun blotch viroid* (ABVd) symptoms due to PSTVd were expressed (Querici et al. 1995).

The host range of *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) was determined. Various plant species and cultivars (51) were inoculated with circular and linear forms of CSVd purified from infected chrysanthemum plants. CSVd was pathogenic to *Gynura aurantiaca* and other plants belonging to the families Solanaceae and Asteraceae, and these plants remained symptomless. Under natural conditions, *Petunia hybrida*, *Ageratum* sp., *Dahlia* sp., *Senecio cineraria*, and *Argyranthemum frutescens* were found to be infected. The host range of CChMVd was restricted to two species of *Chrysanthemum* (Cho et al. 2013).

Viroids affecting pome and stone fruit trees have been reported to occur naturally in all countries where these crops are grown. *Apple scar skin viroid* (ASSVd) and *Apple dimple fruit viroid* (ADFVd) infect both apple and pear, while *Apple fruit crinkle viroid* (AFCVd) can infect apple, hop, and Japanese persimmon. Incidence of *Pear blister canker viroid* (PBCVd) on pear, quince (*Cydonia oblonga*), nashi (*Pyrus serotina*), and wild pear has been recorded. *Hop stunt viroid* (HSVd) has been reported to infect several herbaceous and woody plants including hops, almond, apricot, peach, pear, plum, grapevine, pomegranate, citrus, and cucumber (Cañizares et al. 1999; Lolic et al. 2007; Sano and Matsura 2004). *Tomato apical stunt viroid* (TASVd) was found to infect several plant species under natural conditions such as the ornamental species, *Brugmansia* sp., *Cestrum* sp., *Lycianthes rantonnetii*, *Solanum jasminoides*, *S. pseudocapsicum*, and *Streptosolen jamesonii*. The viroid caused serious production losses in tomatoes and possibly also in potatoes (Anonymous 2011).

8.4.1.3 Modes of Viroid Transmission

Viroids may be transmitted from infected plants via seeds, pollen, and propagules. Transmission by vectors is yet to be demonstrated conclusively. Mechanical transmission of viroids to several plant species through contaminated cutting knives and other handling equipment has also been reported.

Conventional seed testing methods, such as the growing-out test, may be adopted for estimating the extent of transmission through seeds under greenhouse conditions. Development of symptoms characteristic of the viroid infection may be observed in the seedlings growing from the specific number of seeds and the percentages of infected seeds are determined. Transmission of *Potato spindle tuber viroid* (PSTVd) was observed through true seeds and pollen of potato (Fernow et al. 1970) and through seeds of tomato (Bensen and Singh 1964). Transmission of *Grapevine yellow speckle viroid 1* (GYSVd-1) and *Hop stunt viroid* (HSVd) through seeds of eight grapevine cultivars was demonstrated by using a combination of dot-blot hybridization, Northern hybridization, and RT-PCR procedures (Wah and Symons 1997, 1999). The role of pollen in the seed transmission of *Peach latent mosaic viroid* (PLMVd) was investigated.

Pollen from nine peach trees of three cultivars infected with an Italian isolate of PLMVd as donors was used to pollinate healthy peach plants grown in insect-proof greenhouse and maintained for 6 years. PLMVd was detected in the pollen used for pollination using RT-PCR and spot-blot hybridization techniques. Five of 18 plants pollinated with pollen from infected trees were positive for PLMVd. Seeds from PLMVd-infected trees were also found to be infected. However, the seedlings from the PLMVd-positive seeds were apparently viroid-free. The results showed that PLMVd was transmitted through pollen but not through seeds of infected peach plants, indicating the possibility of pollen from peach plants being potential sources of PLMVd inoculum (Barba et al. 2007).

The route of *Hop latent viroid* (HLVd) transmission from hop plant through pollen to seed and then to seedlings was investigated during different stages of pollen and seed development. HLVd was eliminated during maturation and germination of hop pollen. HLVd degeneration in hop pollen was found to be a selective process that efficiently eliminated HLVd during pollen maturation and germination. The RT-PCR was applied to detect HLVd in pollen from infected plant. HLVd was detected by RT-PCR only in very low titer in mature pollen after anthesis and no viroid could be detected in vitro in germinating pollen. The results suggested complete degradation of circular and linear forms of HLVd. Elimination of HLVd from pollen was considered to be due to activities of pollen nuclease and specific RNases (Matoušek et al. 2008). In contrast, transmission of *Hop stunt viroid* (HSVd) through pollen and subsequently to seeds and seedlings was indicated by a later investigation. In order to verify the pollen transmission of HSVd, pollen from infected plum trees was tested by collecting pollen grains from closed flowers, using RT-PCR assay. The presence of HSVd isolate in the parental pollen donor plants, in the pollen, and in the seedling grown from seeds from plants pollinated with pollen infected by HSVd was revealed by RT-PCR assays. The dissemination of HSVd through pollen-seed-seedlings emphasized the need to check the plants, seeds, and pollen for freedom from HSVd in breeding programs (Luigi et al. 2010).

Potato spindle tuber viroid (PSTVd) can be easily transmitted by mechanical inoculation. Contaminated cutting knives used for tuber propagation led to high percentages of PSTVd transmission. Further, PSTVd transmission to healthy tubers occurred to an extent of 80–100% when tractor wheels became contaminated with infectious sap due to bruising of infected plants. Similar levels of high transmission (100%) of PSTVd were observed when cultivating and hilling equipment was used in the field where large plants were present (Bonde and Merriam 1951; Manzer and Merriam 1961). As the viroids can be readily transmitted by sap inoculation, the involvement of biological vectors appears to be less limited. The aphid species *Myzus persicae*, the most efficient vector of several plant viruses, was reported to transmit *Tomato planta macho viroid* (TPMVd) from the wild host *Physalis foetens* to tomato plants under glasshouse conditions. *Macrosiphum euphorbiae* was able to transmit *Potato spindle tuber viroid* (PSTVd), whereas *M. persicae* or *Aulacorthum solani* could not transmit PSTVd. *M. persicae* was shown to transmit PSTVd from plants doubly infected with PSTVd and *Potato leafroll virus* (PLRV) (Salazar et al. 1995). The successful transmission of PSTVd by *M. persicae* could be explained by the finding that PSTVd was heterologously encapsidated within particles of PLRV for which *M. persicae* was the natural biological vector. This phenomenon might have important implications for the epidemiology and spread of PSTVd under field conditions (Querci et al. 1997). *Tomato apical stunt viroid* (TASVd) was found to be transmitted by bumble bees in greenhouse tomato crops (Antignus et al. 2007).

8.5 Infection Process of Viroids

Viroids pathogenic to plants are classified into two families, Pospiviroidae and Avsunviroidae, with type members *Potato spindle tuber viroid* (PSTVd) and *Avocado sun blotch viroid* (ASBVd), respectively. The members of Pospiviroidae replicated in the nucleus and generally lack ribozyme activities. They have highly conserved regions in their rod-shaped secondary structure. Five broad structural domains have been identified in the secondary structures of viroids belonging to Pospiviroidae which include the left-terminal pathogenicity and central, variable, and right-terminal domains. On the other hand, members of Avsunviroidae possess highly branched secondary structures. They replicate in the chloroplasts and all have ribozyme activities (Keese and Symons 1985; Flores et al. 2005a). All viroids are readily transmitted by grafting and mechanical inoculation to healthy plants. After inoculation, development of systemic infection by viroids depends on the successful completion of the following processes: (i) import of viroid molecules into a subcellular organelle chloroplast or nucleus of the cell for Avsunviroidae or Pospiviroidae respectively; (ii) replication inside the organelle; (iii) export out of the organelle after replication; (iv) trafficking into neighboring cells; (v) entry into the phloem; (vi) long-distance trafficking within the phloem to upper leaves (away from inoculated leaf) and roots; and (vii) exit from the phloem to invade nonvascular cells and repeat the replication and trafficking cycle. As the viroids do not possess any proteins, as for viruses, the viroid RNA has to interact with host-encoded enzymes that may have the capacity to degrade RNAs (Ding 2009).

After entry into the susceptible cells that eventually become infected, viroids recruit host proteins for intracellular, cell-to-cell, and long-distance trafficking. *Potato spindle tuber viroid* (PSTVd) is the best characterized among plant-infecting viroids. PSTVd is localized in the nucleus where it is considered to replicate through the activities of the DNA-dependent RNA polymerase II via the rolling circle mechanism (Branch and Robertson 1984; Schindler and Muhlbach 1992). The tomato VirP1 protein was identified based on its property of interacting with the PSTVd replicating in the host cell nucleus (Martinez de Alba 2000). VirP1 cDNA clones were isolated from an expression library of tomato leaves by applying an RNA ligand screening procedure (Sagesser et al. 1999) using radio-labeled PSTVd RNA as a ligand. In addition to PSTVd RNA, VirP1 bound in vitro to *Hop stunt viroid* (HSVd). The amino acid sequence of VirP1 does not contain any known RNA-binding motifs; however, a bromo domain and two nuclear localization signals were identified (Martinez de Alba 2000). The partial self-complementarity that occurs along the 359 bases of PSTVd genome results in the formation of a secondary structure of an interrupted double helix with a branch at the left terminus (Gast et al. 1996).

Potato spindle tuber viroid (PSTVd) or *Hop stunt viroid* (HSVd), belonging to Pospiviroidae, have been investigated more frequently. A specific and saturable receptor mediates the cytoskeleton-independent import of PSTVd plus strands into the nucleus, where processing to the final circular forms presumably occurs. PSTVd seems to have a specific motif for transport through plasmodesmata; when injected into mesophyll cells, PSTVd moved from cell to cell through plasmodesmata (Ding et al. 1997). Two PSTVd strains were found to enter the phloem for long-distance transport but were unable to exit the phloem, suggesting regulated cell-to-cell trafficking (Zhu et al. 2002). Mutational studies on two PSTVd strains, PSTVd^{NT} and PSTVd^{NB} which differed by five nucleotides, were used to identify a bipartite motif required in young tobacco leaves for

trafficking from bundle sheath to mesophyll, but not required for trafficking in the reverse direction. This motif was not required for trafficking through the same cellular boundary in mature leaves, indicating developmental control of RNA trafficking (Qi et al. 2004). Members of the family *Avsunviroidae* might also follow the same route as members of *Pospiviroidae*. Investigations of long-distance movement of PSTVd and HSVd indicated that they were translocated through the phloem, forming complexes with phloem proteins, and this mode of trafficking might be sustained by viroid replication and governed by viroid RNA motifs and plant development. This motif was not required for trafficking in the reverse direction however, but was dependent on leaf developmental stages (Qi et al. 2004).

Potato spindle tuber viroid (PSTVd) was considered to move from the photoassimilate-source leaf into sink organs in a pattern consistent with phloem transport. This view was supported by in situ localization of PSTVd in the phloem. PSTVd failed to invade the shoot apical meristem (SAM), suggesting either inhibited trafficking into this tissue or inhibited replication once the viroid reached this tissue. However, selective transport of PSTVd RNA into sepals, but not into the other floral organs of *Nicotiana benthamiana* was observed (Zhu et al. 2001). PSTVd was transported following mechanical inoculation on the leaves of *N. benthamiana* to sepals, but not to other floral organs (petals, stamens, and ovary) of developing flowers. Although PSTVd could replicate in all floral organs, the absence of PSTVd in petals, ovary, and stamens might be attributed to restricted PSTVd movement into these organs and not to suppression of replication. The results indicated that PSTVd RNA movement within sieve tubes does not simply follow mass flow from source to sink organs, but is transported into selective sink organs. In addition, two PSTVd mutants could enter phloem to spread systemically, but could not exit the phloem in leaves of tobacco. It seems that the viroids have most likely evolved structural motifs that may mimic endogenous plant RNA motifs, so that they may be recognized by cellular factors involved in RNA trafficking (Zhu et al. 2002). Seed-transmissibility of viroids depends on the embryonic infection by the viroid concerned. In order to identify the major pattern of seed transmission of viroids, in situ hybridization was employed to histochemically analyze the distribution of *Potato spindle tuber viroid* (PSTVd) in each developmental stage of petunia from flowering to mature seed stages. PSTVd was present in the reproductive tissues of infected female $\times$ infected male and infected female $\times$ healthy male, but not of healthy female $\times$ infected male before embryogenesis. After pollination, PSTVd could be detected in the developed embryo and endosperm in all three crosses. The results indicated that PSTV was indirectly delivered to the embryo through ovule or pollen during the development of reproductive tissues before embryogenesis, but not directly through maternal tissues as cell-to-cell movement during embryogenesis (Matsushita and Tsuda 2014).

Movement of *Potato spindle tuber viroid* (PSTVd) in sections of infected tomato plants was monitored using in situ hybridization assay. Viroid signals were observed in the vascular tissues as well as in other cells in the subapical region but not in the shoot apical meristem (SAM), suggesting that the surveillance system that restricted the entry of most RNA viruses into the SAM also limited the PSTVd access to this plant compartment (Forster et al. 2002; Qi and Ding 2003). In contrast, *Peach latent mosaic viroid* (PLMVd), belonging to the family *Avsunviroidae*, was able to invade the SAM of infected peach tree (Zhu et al. 2001, 2002). Some of the PLMVd variants (PL-C40) contained an insertion of 12-13-nt folding into a hairpin capped by a U-rich loop that was responsible

for a variegated-albino phenotype known as peach calico (PC) (Malfitano et al. 2003; Rodio et al. 2006). Northern blot and in situ hybridization assays showed that PLMVd replicated in the albino leaf sectors and it could bypass the RNA surveillance system, resulting in the invasion of SAM and changes in protoplastids (Rodio et al. 2006). These results indicated a demarcating difference in the movement patterns of the two representative members, PSTVd and PLMVd, of the two viroid families. PLMVd was found to be well distributed in shoots, leaves, bark, roots, and fruits. Young developing fruits appeared to be the best source of PLMVd (Delgado et al. 2005).

Lacking the capacity to encode any protein, the genome of a viroid expresses biological functions directly. Understanding the nature of infections of a viroid requires identification of structural elements that may interact with host factors or serve as reaction substrates necessary in establishing infection. Functional genomic and proteomic analyses may provide vital inputs for determining the roles of structural motifs in the viroid genome necessary for systemic infection. A number of viroid-interacting proteins of potential functional significance have been identified. These are the chloroplast protein PARBP33 interacting with *Avocado sun blotch viroid* (ASBVd), Virp1 interacting with PSTVd, and phloem proteins interacting with several viroids (Darós and Flores 2002; Kalantidis et al. 2007). VirP1, a protein from tomato, was found to be an excellent host for PSTVd and was isolated by its capacity for its specific binding to PSTVd RNA (Maniataki et al. 2003).

Six proteins that interacted with *Peach latent mosaic viroid* (PMLVd) were identified using northwestern hybridization with peach leaf extracts partially purified by affinity column chromatography followed by mass spectrometry. These include elongation factor 1- α (eEF1A), aminomethyl transferase, putative chaperone, dynamin, 1,5-ribosomol protein and β -1,3-glucanase (JP Perreault, personal communication, see Ding 2009). The molecular mechanisms involved in the induction of effects (symptoms) characteristic of viroid infection remain unclear. Viroids may modify host gene expression interfering with normal developmental pathways. The mature RNA or its replicative intermediates are likely to be the primary pathogenic effectors interacting with host protein or RNA (Diener 2001).

Cellular changes associated with viroid disease symptoms caused by specific viroid pathogenicity determinants in both the nuclear and chloroplastic viroids have been investigated. A U257A substitution in *Potato spindle tuber viroid* (PSTVd) was recovered as a spontaneous mutation in transgenic tobacco plant expressing the viroid cDNA (Zhu et al. 2002). Incorporation of this nucleotide substitution into several PSTVd strains converted them into lethal strains that caused severe stunting and premature death of infected plants (Qi and Ding 2003). The peach calico symptoms, characterized by extreme chlorosis of infected tissues, was shown to be associated with the insertion of an extra 12-13-nt sequence which folded into a hairpin to the left terminal loop of *Peach latent mosaic viroid* (PLMVd). Such an insertion occurred sporadically de novo and could be acquired or lost during infection (Malfitano et al. 2003; Rodio et al. 2006).

It is hypothesized that simple competition for the transcription and/or subcellular, cell-to-cell, and long-distance movement of endogenous plant molecules may result in several physiological activities that contribute to the development of disease symptoms. However, the accumulation level of a viroid appears to have a minimal effect on the symptom severity. Viroid diseases may therefore be considered as a result of altered host gene expression, including gene transcription and posttranscriptional modification of gene products, caused by specific interaction between noncoding viroid RNA

and the host plant (Ding 2009). Small RNA-mediating gene silencing has been shown to be an antiviral mechanism operating in plants and animals. RNA silencing involves several classes of 21-24-nt small RNAs as key mediators. These small RNAs include microRNAs (miRNAs) and short interfering RNAs (siRNAs), produced by cleavage of structured or ds-precursor RNAs, respectively. RNase III dicer or dicer-like proteins and their associated proteins carry out the cleavage, yielding duplex miRNAs or siRNAs. One strand of the duplex is incorporated into the RNA-induced silencing complex (RISC) to guide degradation or translation inhibition of a target RNA or into an RNA-induced initiator of transcriptional silencing complex to guide DNA methylation and chromatin modifications (Jones-Rhodes et al. 2006; Chapman and Carrington 2007). The sequences of small RNAs from *Potato spindle tuber viroid* (Itaya et al. 2007; Machida et al. 2007), *Citrus exocortis viroid* (Martin et al. 2007) and *Peach latent mosaic viroid* were determined (Landry et al. 2004; St-Pierre et al. 2008).

Tomato plants infected with *Potato spindle tuber viroid* (PSTVd) showed severe leaf curling and vein clearing symptoms. The severity of disease symptoms decreased at later stages of infection however, and almost healthy-looking leaves appeared on the upper portion of tomato plants. PSTVd titers attained the peak levels in leaves with severe symptoms and declined in upper leaves recovering from severe symptoms. PSTVd-specific short interfering RNAs (siRNAs), characteristic of RNA silencing, accumulated in all leaves in which PSTVd reached a detectable level. The results suggested that recovery from a severe phase of disease was associated with RNA silencing via sequence-specific degradation of PSTVd RNA genome (Sano and Matsuura 2004). Tomato plants expressing hairpin RNA derived from PSTVd developed symptoms similar to those of PSTVd infection, suggesting that viroids may cause disease symptoms by directing RNA silencing against physiologically important host genes. The results indicated that viroid RNAs were significantly resistant to RNA silencing-mediated degradation (Wang et al. 2004). Small interfering RNAs (siRNA) resulting from the host RNA silencing, the defensive response triggered by double- or quasi-double-stranded RNAs, are considered to induce viroid disease symptoms. Viroid pathogenicity derived from viroid RNA sequences (21-24-nt) during infection may guide RNA silencing of host genes and subsequent induction of disease.

A positive correlation was observed between levels of small RNAs and symptom severity due to *Potato spindle tuber viroid* (PSTVd) and *Avocado sun blotch viroid* (ASBVd). In addition, development of symptoms was correlated with the production of small RNAs in tomato transgenic lines expressing nonreplicating hairpin PSTVd RNAs (Markarian et al. 2004; Matoušek et al. 2007; Wang et al. 2004). RNA-directed RNA polymerase 6 (RDR6) involved in the biogenesis of small RNA and many aspects of RNA silencing was required for symptom production by *Hop stunt viroid* (HSVd) in *Nicotiana benthamiana* (Chapman and Carrington 2001; Gómez et al. 2008).

Peach latent mosaic viroid (PLMVd) could induce an RNA silencing response in their hosts. By loading the second key RNase of the RNA silencing pathway, forming part of the RNA-induced silencing complex (RISC), the viroid-specific siRNAs may be able to guide the complex against their cognate genomic RNAs. Further, some of the viroid-specific siRNAs could by chance guide RISC against host messenger RNAs. This may downregulate the expression of specific host genes and eventually result in the production of disease symptoms. A link between certain macroscopic symptoms such as the albino-phenotype induced by variants of PLMVd and the underlying cytopathic and molecular alterations such as malformed protoplastids and impairment of chloroplast

formation was inferred (Flores et al. 2006). Posttranscriptional gene silencing (PTGS), considered to have an important role in antiviral defense, is triggered by the presence of ds-RNAs that are cleaved by an RNase known as Dicer. Following the cleavage, ss-RNAs (21-25-nt in length) designated small interfering RNAs (siRNAs) are formed. The siRNAs are incorporated into a multisubunit protein complex, the RNA-induced silencing complex, and act as a guide to direct this RNA degradation machinery to its target RNAs. The members of the two families of viroids have been shown to induce PTGS, based on the detection of siRNA, representing different regions of the viroid genome. Incubation of *Peach latent mosaic viroid* (PLMVd) transcripts of either plus or minus polarity with DCL enzyme(s) resulted in the detection of siRNA. Only these regions including the long p11 hairpin resulted in the formation of siRNA. The hairpin known to be implicated in the replication of PLMVd has the ability to trigger the Dicer enzyme(s). It appears unlikely that the P11 hairpin evolved specifically to be a Dicer substrate, since the viroid can evade PTGS-mediated degradation by replicating in the chloroplasts (Landry and Perreault 2005). The viroids can interact with cell factors which redirect them to replicate and traffic throughout the plant, while avoiding the RNA silencing machinery they trigger in their hosts.

Distinctly different symptoms are induced by viroid strains which are unencapsidated and lack the ability to code any protein, but have subtle difference in nucleotide sequences. A comprehensive analysis of differential gene expression patterns of tomato plants at various stages of infection by a mild and severe strain of *Potato spindle tuber viroid* (PSTVd) was carried out. Macroarrays containing 1156 clones from the subtracted cDNA library to monitor their expression patterns throughout the infection process was constructed. Alterations in the tomato gene expression following infection by PSTVd and *Tobacco mosaic virus* (TMV) were compared. The PSTVd strains altered expression of both common and unique tomato genes. The genes encode products involved in defense/stress response, cell-wall structure, chloroplast function, proven metabolism, and other diverse functions. The expression of some, but not all, of those genes altered by PSTVd was also altered by TMV infection. Viroids have the potential to trigger complex host responses, in spite of their simple structure (Itaya et al. 2007).

Coinfection of *Citrus* spp. with *Citrus tristeza virus* (CTV) and viroids occurs frequently. An assay to determine the effects of interactions between *Citrus dwarfing viroid* (CDVd) and CTV was developed. CTV encodes three RNA silencing suppressors (RSSs): p23, p20, and p25. Transgenic Mexican lines expressing each RSS were employed in this investigation. The assays were performed by comparing single and double infections of CDVd and CTV in Etrog citron (*C. medica*) (viroid bioindicator). Interactions between the pathogens were host-dependent. In the Mexican lime, but not in Etrog citron, CTV enhanced CDVd accumulation, while concomitantly CDVd limited the accumulation of CTV. The reduced CTV titer in the presence of CDVd suggested that these pathogens compete for a common biological factor. However, the higher CDVd titer in the coinfecting plants indicated that CTV might provide a beneficial effect for viroid accumulation. The potential role of RSS proteins in the accumulation of viroid RNAs (generated from ds-RNAs) in plants coinfecting with CTV and CDVd was studied. In Etrog citron CDVd-sRNAs were detected at similar concentration, regardless of whether plants were singly infected with CDVd or coinfecting with CTV and CDVd. The results indicated that, as with the accumulation of genomic CDVd, CTV did not have any effect on the accumulation of CDVd-sRNAs.

In Mexican lime, in which the accumulation of circular and linear CDVd forms was enhanced in the presence of CTV, CDVd-sRNAs were only detected in the doubly infected plants. A consistent positive correlation between the concentrations of viroid genomic RNA and sRNAs was therefore observed. Transgenic Mexican lines expressing p23 displayed an enhanced CDVd titer similar to that of nontransformed controls coinfecting with CTV. Transgenic lines expressing p20 had a viroid titer similar to that of nontransformed controls singly infected with CDVd. Transgenic lines expressing p25 had a CDVd titer slightly higher than that of singly infected nontransformed controls. Sequencing of RT-PCR amplicons from CDVd-infected plants showed that the CDVd used as inoculum remained stable in all treatments. The results showed that interaction between CDVd and CTV affected accumulation of viroid, but not symptom expression. On the other hand, CDVd infection caused a slight, but statistically significant, reduction of CTV titer (Serra et al. 2014).

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Volume 2

Epidemiology and Management of Crop Diseases

9

Epidemiology of Seed- and Propagule-Borne Diseases

Epidemiology is one of the different aspects concerned with host–pathogen interactions, resulting in the incidence and spread of crop diseases and consequent losses in quantity and quality. The host–pathogen interactions are markedly influenced by the environment and, as a result, epidemiology also deals with the effects of biotic and abiotic environments existing in a geographic location. The term “epidemic” was originally coined for diseases affecting human beings (epi = upon; demos = people). In plant pathology, the term epidemiology refers to the production and spread of pathogen inoculum and the factors that influence both pathogen and the host plant. Interaction between a susceptible host plant species/cultivar and a virulent pathogen species/strain in a favorable environment results in the development of disease induced by the pathogen under investigation (Fry 1982). Variability in the pathogenic potential of isolates/strains of microbial pathogens and levels of resistance of host plant genotypes are important factors that determine the outcome of host–pathogen interactions, namely the incidence or absence of disease. The study of pathogen ecology helps to understand the influence of various factors that affect the buildup of pathogen populations. Disease incidence depends on the availability of susceptible host plant species or cultivar, virulent strains/isolates of pathogen, and environment favorable for the development and spread of the pathogen to wider areas. Each of these parameters are influenced by the duration (length of time) for which it is available. The term “disease triangle” applied previously has therefore been modified to “disease tetrahedron” to include the effect of duration of time of each of the three components – host, pathogen, and environment – on disease development.

A thorough understanding of epidemiology of seedborne diseases may provide a basis to evaluate the need to suppress disease development and spread and to develop effective and sustainable disease management systems. Molecular epidemiology aims to shed light on those processes that may result in a successful state of disease in a host plant, primarily to enable identification of a weak link in the biotic and molecular interactions or processes that may be capitalized upon to achieve desired level of disease reduction. When a pathogen species or its variants appear at a location, the identity of the pathogen has to be established precisely and rapidly. It is also essential to quantify the pathogen population at various stages of disease progress. Furthermore, the effects of various ecological factors on the pathogen population buildup have to be assessed. Information about the population genetics of pathogens has been useful for the advancement of epidemiological investigations (Narayanasamy 2008, 2011).

A basic need in epidemiology is the availability of a reliable diagnostic system and information on the pathogen genomics. The need to precisely and rapidly determine the pathogen population in a range of environmental settings has been emphasized for constructing models of risk of invasion by the target pathogen. Moreover, the detection of pathogens in asymptomatic plants, seeds, and propagules assumes great importance in eliminating the primary sources of infection that may be otherwise unwittingly introduced to the location(s) where the pathogen may be either absent or of minor importance. Molecular diagnostic techniques have been applied for effective assessment of host resistance/susceptibility or pathogen presence and virulence when infection is still at very low levels, providing a distinct advantage in developing effective strategies for containing the spread of the disease in the field (von Bodman et al. 2003).

9.1 Epidemiology of Fungal Diseases

Seeds and propagules infected by fungal pathogens constitute important primary sources of infection. The seedlings emerging from the infected seeds or propagules provide the inoculum for secondary spread of the disease. Seed transmission of fungal pathogens to the young seedlings or older plants may depend on the host–pathogen combinations. Rate of seed infection to seed transmission or plant infection and subsequent establishment in the field and further spread of inoculum depends on the pathogenic potential (virulence) of the fungal species/strains and levels of host plant resistance, in addition to existence of favorable environmental conditions. Seedborne pathogens have a reverse mode of transmission from infected roots, stems, and leaves to earheads/spikelets, resulting in different rates of seed infection in the earhead/seed-bearing organs of the host plant. The propagules obtained from infected mother plants may or may not exhibit symptoms of infection. Asymptomatic propagules may become potential sources of pathogen inoculum, as the plants growing from infected seeds and propagules form foci of infection from which secondary spread of the disease may occur depending on the availability of favorable environmental conditions, supporting the production of spores and other infection structures that may be spread by the wind and rain.

9.1.1 Dynamics of Host–Fungal-Pathogen Interactions

Epidemiology encompasses several subprocesses. Infection cycle, infection chain, or cycle of pathogenesis, the major subprocess, is the duration required for one dispersal unit (or infecting unit) to grow and produce the next generation of dispersal units. Some fungal pathogens, such as *Fusarium graminearum*, produce only one infection cycle during a crop season and such pathogens are known as monocyclic pathogens. On the other hand, pathogens such as *Alternaria* spp. and *Colletotrichum* spp., with many infection cycles during a single crop season, are referred to as polycyclic pathogens. The number of infection cycles produced during a crop season influences disease dynamics markedly. Another type of pathogen, infecting perennial crops, increases in population from year to year. The increase in pathogen populations and disease development therefore has to be considered over several seasons. These pathogens are called polyetic pathogens. As the susceptible plants are available for several seasons, even small increases in pathogen population in one season may result in severe epidemics.

The severity (intensity) of the disease varies with the nature of the pathogen, the virulence level (pathogenic potential) of the pathogen species, the levels of resistance/susceptibility of the crop cultivar, and environmental conditions. Further, the amount of disease is related to the pathogen population size. Often, the disease is analyzed more easily by visual observations; the scale of the disease is then quantified rather than the pathogen population, since it is easier to determine the disease severity using a standard scale than to estimate the population of the pathogen at a specified time. Yield reduction is attributed to the extent of disease occurrence in a location. The amount of diseased tissue is expressed as a proportion of total plant tissue and it is plotted over time. The shapes of disease progress curves may vary with location and time, since disease development is influenced by factors that may differ with location and time. The disease progress curve for disease induced by monocyclic pathogen usually resembles a saturation curve. On the other hand, the disease progress curve for disease induced by a polycyclic pathogen is likely to be sigmoid. The dynamics of polyetic pathogen-induced disease (represented by the proportion of infected plants over several seasons) lead to an exponential curve. Disease progress curves are useful for gathering information on disease dynamics. The influence of management strategies applied for the disease has to be assessed for forecasting disease incidence and subsequent spread.

Severe epidemics may occur, irrespective of the type of pathogen, if susceptible cultivars and favorable environmental conditions are available for long periods. Disease assessments are made to quantify the effects of diseases caused by the pathogens. Quantification of disease is often made, rather than the measurement of pathogen population, since the amount of plant tissues destroyed by the pathogen is directly related to the magnitude of crop losses. Further quantification of pathogen population requires time-consuming conventional methods or expensive modern molecular techniques and expertise. The contribution of the pathogen factors, host plant factors, and environmental factors to the buildup of epidemics of pathogen diseases are discussed in the following sections.

9.1.1.1 Pathogen Factors

Fungal pathogens exist in the form of strains, races, varieties, or biotypes that differ in their virulence (pathogenic potential), resulting in varying levels of disease severity and consequently in the degree of crop losses under a given set of environmental conditions. Using a set of differential host plant species or cultivars, formae speciales, varieties, strains, races, or biotypes have been recognized within a morphologic species of fungal pathogen. Different strains of *Fusarium graminearum*, *Ustilago tritici*, *Magnaporthe grisea* (= *Pyricularia oryzae*) and other fungal pathogens have been differentiated using different techniques. Strains of *Fusarium* spp. differ in their ability to produce the mycotoxins which cause ailments in human beings and animals following consumption of contaminated feed. These mycotoxins also function as virulence factor complementing other factors that enhance the virulence of the fungal pathogens. Mycotoxigenic species are distributed in the genera *Fusarium*, *Alternaria*, *Aspergillus*, and *Penicillium* and infect plants in the field, processed plant products after harvest, and products in storage. *Fusarium graminearum*, *F. culmorum*, *F. cerealis*, *F. avenaceum*, *F. sporotrichoides*, and *F. poae* produce deoxynivalenol, nivalenol, fusarenone, zearalenone, and moniliformin in wheat, other small grains affected by head blight or scab, and maize (corn) affected by red ear disease. Further, *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*, inducing maize ear rot, produce fumonisins, beauvericin, fusaproliferin,

and moniliformin. Toxigenic *Alternaria alternata* strains, causing tomato black mold, black point of small cereals, and black mold of vegetables, produce tenuazonic acid and alternariols. *Aspergillus flavus* strains infect peanut, pea, and sesame, producing aflatoxins in seeds. Toxigenic strains of *Penicillium expansum* infecting apples and pear produce patulin in fresh and stored fruits. These mycotoxins reduce the quality of the produce significantly, rendering them unfit for consumption by humans and animals because of their carcinogenic and teratogenic properties (Logrieco et al. 2003; Narayanasamy 2005).

The coexistence of genetically divergent populations of *Fusarium graminearum*, causative agent of Fusarium head blight (FHB) disease of wheat, was observed in the upper Midwest and Gulf Coast regions of the USA. High molecular diversity was recorded within 12 individual populations of *F. graminearum* in Germany and with restricted diversity between the populations (Gale et al. 2011; Talas et al. 2011). Variations in two traits – the aggressiveness of isolates from individual field populations in terms of FHB infection and deoxynivalenol (DON) concentration in the host – within three populations of *F. graminearum* sensu stricto (s.s.) were estimated. Each population consisted of 30 single-spore isolates of a collection from four countries (Syria, Romania, Hungary, and Canada). All isolates were spray-inoculated onto a moderately resistant spring wheat cultivar at field locations over two years. The genetic proportions of phenotypic variance within population were significant for both traits. The environmental interaction was more important for mean FHB severity. Both traits were significantly ($P < 0.05$) correlated within and across populations. Within-field variation for traits of parasitic ability and selection was high. The high diversity within individual field populations as revealed by phenotypic traits and molecular markers was well reflected in the high evolutionary potential of *F. graminearum* s.s. The pathogen ability to adapt to environmental factors, including resistant cultivars, largely depended on the genetic variation within natural populations governed by the evolutionary forces mutation, recombination system, selection, genetic drift, and gene flow (Talas et al. 2011).

Although pathogenic specialization within *Fusarium graminearum* was not clearly established, difference in aggressiveness among isolates of *F. graminearum* could be recognized. High levels of variations in vegetative compatibility groups (VCGs) among *F. graminearum* isolates collected in the Midwestern USA were observed (Parry et al. 1995a; Bowden and Leslie 1996). In contrast, a low amount of genetic diversity among isolates of *F. graminearum* present in Canada was reported by Ouellet and Seifert (1993). *Fusarium avenaceum*, one of the components of Fusarium head blight (FHB) disease complex, showed wide variations in virulence of the isolates (91) on wheat. They were grouped into 15 phenotypes and 10 VCGs which formed 7 ITS rDNA phylogenetic clades and 3 EF-1 α PCR-DGEE groups. Variations in virulence within *F. avenaceum* isolates ranged from high virulence to avirulence to wheat. Virulent isolates, in general, had a wider host range and geographic distribution, while avirulent isolates exhibited greater heat tolerance, color plasticity, and growth rate compared to virulent isolates. Pathogen dynamics and outbreaks of disease might be influenced by the abundance ratio of virulent/avirulent isolates related to climate characteristics of each agroecosystem. The characterization of *F. avenaceum* sensu lato isolates obtained from western and eastern ecozones of Canada provided baseline information on the variation existing in FHB-inducing *Fusarium* species (Abdellatif et al. 2010).

Dissemination of fungal pathogens over short or long distances is one of the important factors for their survival and spread of the disease caused by them. *Fusarium graminearum* (teleomorph *Gibberella zeae*), the principal causal agent of Fusarium

head blight (FHB) disease of wheat and other cereals, is seedborne and can survive in the corn (maize) residue left in the field. Overwintered cereal debris is a potential reservoir of *Gibberella zeae* inoculum. Ascospores released from the crop debris become airborne and play a predominant role in the infection of spikes of wheat and barley at anthesis. The movement of *G. zeae* from a local source of inoculum in infested cereal debris was studied for the management of wheat FHB disease. Well-characterized clones of *G. zeae* were released and recaptured in five wheat fields in 2007 and 2008. Amplified fragment length polymorphism (AFLP) procedure was employed to track and identify unambiguously the released clones in the heterogeneous populations of the pathogen recovered from infected wheat spike collected at 0, 3, 6, and ≥ 24 m from small-area sources of infested corn residues. Isolates of *G. zeae* recovered at different distances from the center of source areas shared 65%, 19%, 13%, and 5% of the genotypes of clones respectively. The incidence of spike infection attributable to the released clones averaged 15%, 2%, 1%, and $<1\%$ at these distances. The percentages of spikes infected with the released clones varied significantly across sampling distances. The percentage of spikes infected with the released clones did not differ significantly between plots containing 3-acetyldeoxynivalenol (3-ADON) genotypes. The results suggested that FHB field experiments including a cereal debris variable have to include debris-free borders and interplot of at least 3 m (preferably 6 m) to avoid significant interplot interference from spores originating from within-field debris (Keller et al. 2010).

The relationship between *Gibberella zeae* inoculum dose and the resulting disease incidence levels in wheat was investigated under controlled condition for conidia alone and conidia and ascospores. The Fusarium head blight (FHB) disease metric (incidence or severity) was determined and this parameter increased sharply in proportion to spore contents until a maximum was reached where additional inoculum did not substantially increase the disease severity (Stack and McMullen 1985). Both kinds of pathogen propagules, conidia and ascospores, had similar pathogenic potential for infecting wheat and initiating FHB disease. The role of inoculum concentration (pathogen population) in the development of FHB disease and accumulation of deoxynivalenol (DON) was investigated to assess the effectiveness of risk advisory systems. Greenhouse-raised spring wheat plants were inoculated at flowering with suspensions that varied in *Gibberella zeae* macroconidia concentration. The spikes were covered for 72 h to enhance the infection percentage and the plants were maintained under ambient conditions for 15 days. Disease severity and DON concentrations were determined. DON concentration was assessed in grain-only and whole-spike samples for each treatment. Regression analysis was employed to evaluate the mathematical relationship between inoculum dose, disease metric (severity score), and DON contents. Both disease incidence and severity were found to increase sharply in relation to inoculum concentration until an asymptote (lack of symptom expression) was reached, where no further effects were observed with addition of inoculum. In contrast, DON accumulation in the ranges of inoculum concentration 0 to 25,000 conidia tested showed a linear function where the concentration of conidia continued to increase with additional inoculum. DON accumulation increased at a greater rate in whole-spike tissue. Functions developed from the greenhouse studies for disease severity and DON contents were not particularly effective at predicting events in the field. Disease incidence was overestimated at lower inoculum concentration, while disease severity and DON were underestimated. However, the DON accumulation in the grain was positively correlated to inoculum concentration under both greenhouse and field conditions. The combination of the models developed

in this investigation showed the potential to increase the accuracy of the existing risk advisory systems for predicting disease severity, and the risk of economically important DON concentrations (Stein et al. 2009).

Determination of sources of primary or initial inoculum for epidemics is an integral step toward understanding and managing plant diseases. Primary inoculum may be derived from dormant structures of a pathogen such as sclerotia or sexual spores or from actively growing pathogens from other plant populations. Primary inoculum may pre-exist in the field or may be introduced once or multiple times, and it may continue to arrive and cause primary infections in a plant population even after secondary cycles have commenced for polycyclic diseases. Barley net blotch caused by *Drechslera teres* became one of the most important diseases with increasing use of no-tillage agriculture and monocropping of susceptible cultivars. The pathogen is seedborne and the inoculum potential in infected seeds is a critical factor determining the extent of disease incidence and consequent yield losses. The symptomatic transmission efficiency (STE) of *D. teres* was calculated using the formula:

$$\text{STE}(\%) = \text{PS}(\%) / \text{S}(\%) \times 100$$

where PS is incidence of *D. teres* in plumules and S is incidence of *D. teres* in seeds. Seed infection of *D. teres* was determined using four replicates of 100 seeds each plated on the agar selective medium. The disease incidence in plots sown with fungicide-treated and untreated seeds was compared. The maximum *D. teres* symptomatic transmission efficiency (MSTE) registered from seed to plumule was 18.8% with mean values of 14.5%. In the absence of other sources of inoculum of *D. teres*, plumule infection after seedling emergence indicated that the infected seeds formed the primary sources of inoculum, resulting in higher levels of plumule infection in control treatment compared with fungicide treatment which reduced the seed infection percentage. The alarm threshold for net blotch disease control was established as 60% for leaf incidence. The results indicated that seedborne inoculum of *D. teres*, if unchecked, was a potential risk to barley crop (Carmona et al. 2008).

The influence of varying inoculum potential of *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*), incitant of ear rot of maize, was investigated on the development of disease development in maize seedlings/plants in early stages under controlled conditions. Osmo-priming procedure was followed to obtain seeds with different levels of inoculum. The nongerminated seeds of the same seed lot exhibiting typical fructifications of *F. verticillioides* were grouped into five categories according to a grading system (0, 1–25, 26–50, 51–75, and 76–100) that was established based on the visible area of seeds covered by the pathogen propagules. The seeds of different categories were sown on soil and plants were grown under controlled conditions (25°C and 16 h light/day) for 25 days. Correlation variables, namely plant population, speed of seedling emergence, and height/weight of emerged plants were negatively correlated with increase in inoculum density of the pathogen initially present in the maize seed. The regression equations for all variables had a high correlation coefficient (>0.85), except plant height which had a lower correlation coefficient (0.77). The most drastic effect of *F. verticillioides* was noted on the root system of emerged seedlings, the adverse effect increasing with increasing inoculum density on the maize seeds planted in the soil under the greenhouse conditions. The results revealed the risk of using maize seeds

with high levels of inoculum density, which might result in drastic reduction in the quality and quantity of grain yield (Machado et al. 2013).

Fusarium verticillioides colonizes maize kernels and contaminates the grains with mycotoxins. The migration of *F. verticillioides* isolate transformed with foreign genes from inoculated to noninoculated maize ears was tracked to assess the extent of population flow of the pathogen among maize plants under field conditions. Maize ears were inoculated with *F. verticillioides* PATg strain carrying two foreign genes, one coding for hygromycin resistance (hyg^r) and the other for β -glucuronidase (GUS), through either the shuck or silk channel at one of two stages of silk development, green or brown. The average expression of hyg^r was 66% and of GUS was 96% for mycelia emerging from kernels of ears inoculated with *F. verticillioides*. Mycelia with hygromycin resistance appeared in cultures from noninoculated and water-inoculated ears at low rate (3%). However, none of the mycelia stained positive for GUS in kernels originating from noninoculated ears and only 7% of those from water-inoculated ears. The results indicated that dissemination of *F. verticillioides* PATg strain was minimal from plant to plant under field conditions during 2001–2003 in Georgia, USA. Only a few ears inoculated with water through the silk during the green silk stage of development yielded the transformed strain. The results suggested that mechanical injury during inoculation might have provided an entry site for infection by *F. verticillioides* PATg strain (Yates and Sparks 2008).

The sources of primary (or initial) inoculum required for buildup of epidemics have to be determined to develop integrated disease management systems. Primary inoculum may be derived from dormant or survival structures such as sclerotia, sexual spores of pathogens, or from actively growing pathogens from other plant populations or debris. Primary inoculum may pre-exist in the field or may be introduced once or several times during a crop season. It may continue to arrive and cause primary infections in a susceptible plant population, even after secondary cycles have begun for polycyclic pathogens. Assessment of the relative contributions of sources of primary inoculum may be of vital importance for effective management of the target disease(s). Infected seed may be a major source of primary inoculum for foliar epidemics of *Phaeosphaeria nodorum* (anamorph *Stagonospora nodorum*) as seed infection in commercial wheat seed lots is common; the mean seed infection incidence is 23% with a range of 1–71% (Shah and Bergstrom 1993). *P. nodorum* was shown to be efficiently transmitted over a broad range of soil temperatures from infected seed to aerial portions of seedlings, where the pathogen formed pycnidiospores that could be dispersed by splashing rain. In addition to revealing significant correlations between seed infection and disease severity, the genotypes of *P. nodorum* were tracked from inoculated seed to foliar epidemics and then to the next generation of wheat seed in the field. The results of these investigations provided direct evidence for the infected seed being the primary source of inoculum for *P. nodorum* (Shah et al. 1995; Shah and Bergstrom 1999). Seed infection in wheat by *P. nodorum* was observed in wheat seeds from New York, the extent of seed transmission being 70% or greater on the coleoptiles which might be exposed above soil line. Transmission also occurred to the first leaves at frequencies of 2–37% and possibly also to other leaves. The results indicated the possibility of infected wheat seeds being a primary source of inoculum of *P. nodorum* (Shah and Bergstrom 2000).

Phaeospora nodorum can infect wheat, rotational crops, and graminaceous weeds which may be potential sources of primary inoculum. In-field and out-of field wheat debris and infected wheat seeds are also important primary sources of inoculum for

P. nodorum. The relative contributions of seed-transmitted inoculum to foliar populations of *P. nodorum* in winter wheat were investigated. By infecting seed with genetically marked isolates of *P. nodorum*, releasing them by planting in the field, allowing natural seed-to-seedling transmission, and then recapturing them by sampling over the growing season, the relative contribution of seedborne isolates of the pathogen was assessed. Recapture of the released marked isolates of *P. nodorum* was performed by isolating them from leaves with necrotic lesions at spring tillering and flowering stages and mature grains from spikes showing glume blotch. The isolates from these samples were genotyped by applying amplified fragment length polymorphism (AFLPs) procedure and differentiated as released or nonreleased to infer the sources of inoculum. Both infected seed and other sources of the pathogen contributed significant primary inocula to populations recovered from leaves and harvested grains. Seed-transmitted genotypes accounted for 57% of all isolates recovered from inoculated plots with a range of 15–90% of the populations of *P. nodorum* collected over the season in individual, inoculated plots at four locations. Plants in the noninoculated control plots also yielded 95% or more of nonreleased isolates recovered from these plots. The results suggested reduction of seedborne inoculum of *P. nodorum* alone might not result in desired levels of control because of the availability of additional sources of inoculum of the pathogen. The release–recapture experimental approach provided valuable insights into the relative importance of seed-transmitted and other primary inocula in foliar epidemics of *P. nodorum* (Bennett et al. 2007).

Atmospheric motion systems are essential for the transport of many fungal pathogens. The movement of fungal pathogens in the atmosphere is characterized by the process of spore liberation, drift, and deposition on plant surfaces. The temporal patterns of deposition of viable spores of *Gibberella zeae* (anamorph *Fusarium graminearum*), causing wheat Fusarium head blight (FHB) disease, over rotational wheat fields were studied. Petri dishes containing the selective medium were placed at 30 cm above wheat canopies to collect viable, airborne spores of *G. zeae*. Over 40,000 spores were collected over a period of 73 days (06:00–20:00) or night (20:00–06:00) sampling periods in 3 years (2002, 2004, and 2005). The vast majority of spores (82–94%) was collected during night. Viable spores were deposited in all but one sampling period, spanning spike emergence in New York State. Seven major deposition events (>50 colonies/plate) occurred all at night and three of these coincided with rainfall. The results suggested that spore deposition might be separated from spore release in both time and space, and the cumulative exposure of wheat spikes to airborne spores of *G. zeae* and the deposition of these spores mainly at night are important factors to be considered while prediction models are developed (Schmalle III et al. 2006).

Chickpea Ascochyta blight disease is caused *Ascochyta rabiei* (teleomorph *Didymella rabiei*). The pathogen produces pseudothecia and pycnidia on overwintered chickpea debris. Timing of primary inoculum release from overwintered chickpea debris was investigated in the field, using potted susceptible chickpea plants as trap plants and debris heavily infested with *D. rabiei* spread over an area of 1 m² in the early fall season. Primary inoculum was trapped in spring by placing trap plants (27) adjacent to the overwintered debris for 2–3 days, followed by incubation in the greenhouse under conditions to promote infection. Disease lesions were counted 2 weeks later. Primary inoculum release began in mid-March, with the majority of lesions associated with rainfall during May in 2004 and 2005. Release continued through the beginning of June when the number of lesions on trap plants reduced dramatically,

despite the continuation of rainfall. Primary inoculum release correlated well with pseudothecial maturity and ascospore release in the laboratory. Regression analyses revealed the closest relationship of rainfall with primary inoculum release from overwintered debris, facilitating the attempts for the control of the disease to be directed to the critical period of inoculum release (Chilvers et al. 2007). *Didymella pinodes*, causing Ascochyta blight disease of pea, infects pea crop through ascospores released at the time of sowing. The relationship between the number of ascospores of *D. pinodes* at sowing and disease severity at crop maturity was investigated. Field pea stubble infested with *D. pinodes* from one site was exposed to ambient conditions at two sites, repeated in 3 years. Stubble samples were retrieved every 2 weeks, moistened and placed in the wind tunnel, and up to 2500 ascospores $\text{g}^{-1} \text{h}^{-1}$ were released. Secondary inoculum was monitored using seedling field peas as trap plants in canopies, arising from three sowing dates, and external to field pea canopies reached its peak in the early sown crops. A model was developed to calculate the effective number of ascospores using predictions from G1 blackspot manager, distance from infested stubbles, and winter rainfall. Maximum disease intensity was predicted based on the calculated number of effective ascospores, soil-borne inoculums, and spring rainfall over two seasons. Predictions were validated in the third season with data from field trials and commercial crops. A threshold amount of ascospores of *D. pinodes*, $294 \text{ g}^{-1} \text{ stubble h}^{-1}$ was determined, above which it did not increase. Below this threshold, there was a linear relationship between ascospore number and maximum disease intensity (Davidson et al. 2013).

Potato late blight disease caused by *Phytophthora infestans* is a highly destructive disease of historical importance, being primarily responsible for the infamous Irish potato famine. *P. infestans* infects potato and tomato accounting for heavy losses, especially in glasshouse crops. *P. infestans* is a polycyclic pathogen capable of producing several asexual cycles in one crop season and the asexual sporangia are well adapted for aerial dispersal. The sporangia may be disseminated over long distances (40–60 km) depending on the environmental conditions (Fry and Goodwin 1997; Aylor et al. 2001). The role of aerial dispersion of sporangia has been recognized as fundamental to the development of late blight disease since, without dispersal of infectious units, epidemics fail to progress. The temporal and spatial dynamics of disease epidemics have to be understood for development of effective strategies to restrict the incidence and spread of crop diseases. Several decision support systems (DSS) have been developed in different countries for application of fungicides at a particular time when a genuine risk was perceived to exist. These systems for predicting the risk of infection were considered to be effective strategies for management of late blight disease by making better use of fungicides than calendar-based fungicide treatments (Aylor et al. 2011).

Management of potato late blight disease caused by *Phytophthora infestans* has been found to be ineffective because of the difficulty in determining when, where, and how abundant airborne inoculum that may lead to epidemics would be present in a geographic location. It is possible that decision support system (DSS) may overestimate the risk when very little or no airborne inoculum is available. As *P. infestans* can produce multiple cycles of infection and inoculum production, the new infections may increase proportionally to both initial amount of inoculum present and amount of new inoculum produced in situ or carried by wind from external sources. The value of airborne sporangia concentration (ASC) as a monitoring tool for late blight disease inoculum has not been duly recognized. Further, information on the spatial distribution of airborne

sporangia of *P. infestans* over several seasons is also lacking. The usefulness of real-time monitoring of *P. infestans* airborne inoculum as a complement to DSS was assessed during 2010–2012 potato production seasons at two locations in New Brunswick, Canada. Airborne sporangia concentrations (ASCs) were monitored using 16 rotating-arm spore samplers placed at 3 m above ground. In both areas, the highest mean ASC in all samplers was detected between the end of July and end of August. In general, no correlation could be established between ASCs and the weather conditions such as temperature, duration of the period of high humidity, and rainfall, except for a weak significant correlation between the ASCs and a high-humidity period during 2010–2012. A gap between the magnitude of the risk estimation by DSS and the degree of ASC was discernible. The results suggested that the two DSS underestimated the late blight risk in 2010 and overestimated it in 2012. DSS estimated the potential for the disease to occur, but it could not measure the real inoculum level. The results suggested the spore sampling network devices coupled with DSS might be useful for targeting the optimal time to apply a disease control strategy (application of fungicides). The farmers in New Brunswick used the spore-sampling network as a functional strategy to control late blight epidemics. This investigation showed that area under the inoculum progress curve (AUIPC) could be used to assess disease progression and as a monitoring tool for potato late blight disease, facilitating optimization of fungicide use (Fall et al. 2015).

Severe outbreaks of late blight disease caused by *Phytophthora infestans* on potato and tomato have been observed since 2008 in the states of Andhra Pradesh, Karnataka, and Tamil Nadu in India. The phenotypic and genotypic characteristics of 63 potato isolates and 94 tomato isolates of *P. infestans* were compared with reference isolates based on the mating type, in vitro metalaxyl sensitivity, mitochondrial DNA haplotype, DNA fingerprint patterns (using the RG57 multilocus DNA probe), simple sequence repeat (SSR) markers, and aggressiveness (pathogenic potential) on potato and tomato leaves. All isolates collected from potato and tomato in different fields in South India belonged to A2 mating type, mitochondrial DNA haplotype Ia, and they were resistant to metalaxyl and highly aggressive (virulent) on both tomato and potato. This clonal lineage showed a characteristic RG57 pattern and SSR loci which matched the multilocus genotype (MLG) 13_A2 lineage present in European countries. Severe disease occurrence was observed during August–December when the average temperature ranged over 20.8–22.5°C. The results showed that the 13_A2 lineage causing severe late blight outbreaks in South Indian states replaced the US-1 and other genotypes of *P. infestans* that occurred earlier. As all isolates showed resistance to metalaxyl, alternative strategies have to be worked out for effective management of late blight disease infecting two important vegetable crops (Chowdappa et al. 2015).

Plasmopara halstedii, causal agent of sunflower downy mildew disease, is a registered quarantine pathogen on sunflower seeds in European Union. Many races (35) of *P. halstedii* have been recognized. New pathotypes are constantly emerging in different countries and can overcome resistance of sunflower cultivars available (Gulya 2007). In addition, the appearance of fungicide-resistant strains has become a great concern for growers, as the fungicide treatment was found to be less effective in reducing the disease incidence/severity. The prevalence of metalaxyl-resistant *P. halstedii* races was reported to exceed 50% of the total number of races in France (Moinard et al. 2006). This obligate pathogen resides in a quiescent state in sunflower seeds and the disease could be spread from areas of crop production by international seed trade. Hence, an

efficient optimized duplex real-time PCR technique was used to achieve the detection of fungicide-resistant strain of *P. halstedii* at the level of <1 infected seed per 1000 seeds. Such diagnostic techniques might be useful to prevent the introduction and spread of potentially new genotypes or fungicide-tolerant strains (Ioos et al. 2012).

9.1.1.2 Host Factors

The development of epidemics of fungal diseases may be dependent on host factors such as plant structure, plant age at the time of infection, genetic resistance of cultivars, host range, and susceptibility/resistance levels of rotational crops. The effect of timing on when the plant is most susceptible for successful infection of barley by *Fusarium graminearum*, causing Fusarium head blight (FHB) disease, was investigated. Wheat is highly susceptible to FHB disease during anthesis, possibly because extended anther extrusion is initially infected. Wheat is generally chasmogamous (open flowering type), whereas barley has both chasmogamous and cleistogamous (closed flowering) cultivars. The florets in cleistogamous cultivars do not open and anthers are not extruded at anthesis. The effect of infection timing on FHB disease incidence and mycotoxin accumulation in barley cultivars with two different flowering types were studied under greenhouse conditions. Cleistogamous cultivars were resistant at anthesis but susceptible at 10 days after anthesis, whereas chasmogamous cultivars were susceptible at anthesis. Regarding accumulation of mycotoxin deoxynivalenol (DON) and nivalenol (NIV) in mature grains, cleistogamous cultivars accumulated more mycotoxins (DON and NIV) when inoculated at 10 or 20 days after anthesis than at anthesis. On the other hand, chasmogamous cultivars accumulated greater concentrations of DON and NIV when inoculated at anthesis. The results showed that the most crucial time for *F. graminearum* infection and mycotoxin accumulation in barley differed with cultivar (Yoshida et al. 2007).

The relationship between incidence of Fusarium head blight (FHB) disease of wheat and number of spikelets infected on an ear (incidence of spikelet infection) was studied during three seasons and in four countries in order to derive a simple relationship for predicting disease at the spikelet level using ear infection. A robust relationship between incidence of ear infection and average number of infected spikelets per ear was obtained, assuming a fixed variance–mean relationship and a negative binomial distribution for the number of infected spikelets. A relationship between incidences of spikelet and ear infection was also obtained based on the complementary log-log or logit transformation of ear and incidence of spikelet infection. These models were found to be consistent over years and counties and it might be useful for arriving at meaningful disease management decisions involving the fungicidal applications (Xu et al. 2004).

The impact of seed infection by *Mycosphaerella pinodes*, causal agent of *Mycosphaerella* blight disease of field pea, on the development of epidemic proportions was investigated. Seed lots of susceptible and partially resistant field pea cultivars with different levels of seed infection by *M. pinodes* were produced at a single site, providing uniform handling and storage conditions. Field trials were conducted in a location where external inoculum levels were very high and another site with little external inoculum. For each cultivar, seed lots with high seed infection (26–47%), intermediate seed infection (6–15%) and low seed infection (0–2%) were planted. Seedling establishment was higher in plots planted with low seed infection than in other categories of seed infection. However, consistent differences in four of eight locations of trials were observed.

The results indicated that seed infection of field pea with *M. pinodes* did not substantially contribute to aboveground symptoms and very high levels of seed infection could reduce seedling establishment and plant stand, resulting in low yield levels (Gossen et al. 2010).

Seedborne fungal pathogens such as *Fusarium graminearum*, incitant of Fusarium head blight (FHB) of wheat and other cereals, may have a wide host range encompassing both monocotyledonous and dicotyledonous crop hosts as well as weed plant species. These sources of inoculum can contribute to development of epidemics and may supply inoculum to sustain many cycles of infection by such pathogens (Burlakoti et al. 2007). Pathogens such as *Ustilaginoidea virens*, causing rice false smut disease, have a limited host range and the availability of inoculum from other plant hosts is restricted (Lu et al. 2009). The levels of susceptibility of 20 graminaceous plant species to two sorghum ergot pathogens *Claviceps sorghi* and *C. africana* were assessed by inoculating them with a conidial suspension of 10^6 conidia mL⁻¹. Five species, namely *Sorghum arundinaceum*, *S. halepense*, *S. versicolor*, *S. virgatum*, and *Pennisetum glaucum*, were found to be susceptible to both pathogens. A marked variation in size of macroconidia of both *C. sorghi* and *C. africana* from different host plant species was observed. The results indicated that *C. sorghi* and *C. africana* had similar host ranges and the alternative hosts might be able to provide the inoculum from out-of-field locations (Muthusubramanian et al. 2005).

Host preferences of *Microdochium majus* and *M. nivale* infecting cereals have been investigated. *M. majus* occurred more frequently on the stem bases and ears of wheat compared to *M. nivale* which infected rye and turf grasses more frequently. *M. majus* had a weak/slight selective advantage over *M. nivale* in wheat, whereas *M. nivale* showed strong selective advantage over *M. majus* in oats and rye (Parry et al. 1995b; Simpson et al. 2000). Temperature-dependent differential host preference between *M. majus* and *M. nivale* was revealed under controlled environmental conditions (Simpson et al. 2004). *Fusarium graminearum* (Fg), causing Fusarium head blight (also known as scab disease), is primarily a pathogen of cereals but it could infect noncereal crops such as potato and sugar beet in the USA. Isolates (21) of *F. graminearum* collected from potato, sugar beet, and wheat inoculated on wheat earheads produced nine mycotoxins as revealed by gas chromatography and mass spectrometry. All isolates contained the *Tri5* gene coding trichodiene synthase involved in the biosynthesis of the mycotoxins. They were virulent to wheat cultivar Grandin. Isolates of *F. graminearum* differed significantly in virulence (expressed as disease severity), Fg DNA content, and mycotoxin accumulation. Potato isolates showed greater variability in producing different mycotoxins than sugar beet and wheat isolates. The results showed that *F. graminearum*, causing potato dry rot and beet decay, could be potential sources of inoculum for FHB epidemics in wheat (Burlakoti et al. 2007).

Infested spinach seed was suggested to introduce *Verticillium dahliae* into lettuce fields. Evidence for linking spinach seedborne inoculum of *V. dahliae* to wilt epidemics in lettuce was provided by experiments conducted in commercial spinach fields in four coastal CA counties in the USA. *V. isaachi* was the predominant species isolated from spinach seed, followed by *V. dahliae* and, less frequently, *V. klebahnii*, irrespective of the county. The frequency of recovery of *Verticillium* spp. was unrelated to the *Verticillium* wilt incidence in lettuce, but was related to the area under spinach production in individual counties. *Verticillium* wilt disease incidence on lettuce was determined in microplots. The disease developed on lettuce when *Verticillium*-infested spinach seeds

were planted in the same soil twice or three times. The identity of the pathogen recovered was confirmed to be *V. dahliae* using PCR assays. In addition, green fluorescent protein (GFP-) tagged mutant strain of *V. dahliae* was employed to demonstrate transmission of *V. dahliae* from infested spinach seed to lettuce roots in a greenhouse study, after two cycles incorporating infected residue into the soil. The results provided direct evidence for the transmission of *V. dahliae* from infested spinach seed to lettuce plants (Short et al. 2015).

The influence of time of infection of wheat by *Fusarium graminearum* on reduction of grain weight and mycotoxin accumulation was investigated. Earheads of Norm wheat were spray-inoculated with a macroconidial suspension of a deoxynivalenol (DON-) producing isolate of *F. graminearum* at each of six reproductive stages from flowering to hard dough. Inoculated plants were incubated in a mist chamber for 48 h and then placed in the greenhouse until maturity. Norm wheat was susceptible at all stages inoculated, but the greatest grain weight reduction and DON accumulation in grains occurred in plants inoculated from post-flowering to late milk stages. However, a high level of kernel infection and marked accumulation of DON were observed in plants inoculated at the late hard dough stage, although reduction in grain weight was not in proportion to infection levels. Commercially significant levels of DON in plump, high-yielding wheat could result from infections well after flowering stages depending on the availability of favorable environmental conditions (Del Ponte et al. 2007).

The incidence of Fusarium head blight (FHB) disease of wheat caused by *Fusarium* spp. may be influenced by the structural characteristics of spikelet organs at the time of anthesis. Wheat cultivars differ distinctly in anther extrusion. The characteristic of anther retention (anther held within the spikelets) and trapped anthers (partially extruded and trapped between the lemma and palea of wheat spikelet) was assessed. A higher proportion of anthers was weakly correlated with increased susceptibility to FHB disease (Graham and Browne 2009). In a later investigation, the relationships between the degree of anther extrusion (AE) and FHB damage were determined using double haploid lines (DHLs) derived from F1 plants from crosses between closed-flowering and opened-flowering cultivars. Various degrees of AE were seen in the DHLs, and FHB severity was the lowest in closed-flowering DHLs. The DHLs with partially extruded anthers showed significantly more severe FHB symptoms than those with closed flowering phenotypes. FHB severity was significantly correlated with Fusarium-damaged kernels (FDKs) and deoxynivalenol (DON) concentrations in the grains. Partially extruded anthers were considered to be potential sources of FHB infection (Kubo et al. 2013).

The ergot disease incidence and severity in male-sterile sorghum lines as a function of distance from pollen source and its impact on seed production and quality were determined. *Claviceps africana* causes significant losses in yield. Sorghum male-sterile lines E15A × E15B and the hybrid A155 × R8509 were planted in seed production fields with a female to male row ratio of 8:4, 0.76 m row width, and 0.1 m spacing between plants. Disease incidence, ergot severity, and seed yield were determined in female rows 1, 3, 5, and 8 and in male rows 1 and 3. Regression analyses between distance from pollen emission source and disease incidence, disease severity, and seed yield were carried out for each field. The results indicated that yield reduction, ergot incidence, and disease severity were lower in female rows of the line than in those of the hybrid seed production field. As the distance increased from pollen source to the female row, the ergot incidence increased from 42 to 67% in the furthest row (3.04 m) and from 19 to 22% for ergot

severity, and seed yield decreased from 45 to 56%. The seed quality was not markedly affected. Ergot disease affected seed physiological quality by decreasing viability, vigor, and germination. Ergot-contaminated seeds were more readily invaded by larger number of fungi than the healthy seed lot (Hernández-Martínez et al. 2006).

The plant age is an important factor that significantly influences the level of susceptibility/resistance of plants to fungal pathogens. *Alternaria sesame* causes the Alternaria leaf spot disease in sesame (gingelly) in all countries. The effects of plant age on disease severity and seed infection by *A. sesami* were determined using the sesame accession SPSSIK110 at five different stages of plant growth. Rates of increase in blighted leaf area and defoliation, and areas under the disease progress curves (AUDPCs), varied among the different plant ages. Plants inoculated at 8 and 12 weeks of age were the most susceptible to the disease. The susceptibility level of 4 week old plants was the least. In order to assess seed infection, the pathogen was isolated from seeds of plants inoculated at different ages on oatmeal agar. Seed infection by *A. sesami* was the highest in plants infected at 8 and 10 weeks of age, whereas plants inoculated at 4, 6, and 12 weeks of age had the least infection levels. The results indicated that the susceptibility of plants to infection by *A. sesami* differed but without a significant relationship with plant age (Ojiambo et al. 1999).

Cladosporium variable and *Stemphylium botryosum*, causing leaf spot diseases of spinach, were responsible for reducing the quality of fresh market crops and seed crops. Spinach “seed,” as it is commonly called, is actually a fruit with a seed enclosed by a thick, corky pericarp. The true seed consists of a seed coat, embryo, and nonliving perisperm (maternal storage tissue). The entire spinach fruit consisting of the pericarp, testa, embryo, and perisperm was used in this investigation. Both pathogens were seed-transmitted and seed assays demonstrated that *S. botryosum* and *C. variable* were internal and external in spinach seed. The presence of *S. botryosum* was detected in 5–76% of the embryos of five seed lots tested, whereas *Cladosporium variable* or *C. macrosporum* was present in only up to 1% of embryos of these seed lots. The survival of the seedborne fungal pathogens is affected by storage conditions. Pathogenic isolates of *S. botryosum* were detected by freeze-blotter seed health assay in each of the 12 seed lots stored for up to 11 years at 4.4°C and 60% relative humidity. On the other hand, *C. variable* or *C. macrocarpum* could be detected in only 2 of 11 seed lots which had been stored for 3 and 8 years. Conditions favorable for long-term storage of seed also favored the survival of seedborne pathogens (Hernández-Pérez and du Toit 2006).

9.1.1.3 Environmental Factors

9.1.1.3.1 Agricultural Practices

The influence of agricultural practices on the incidence and intensity of wheat Fusarium head blight (FHB) disease on susceptible wheat cultivar “Ritmo” and moderately susceptible cultivar “Buteo” and “Dekan” was investigated. Four *Fusarium* species – *F. graminearum* (teleomorph *Gibberella zeae*), *F. culmorum*, *F. poae*, and *F. avenaceum* – were associated with FHB disease in North and Baltic areas. Genus- and species-specific primers were employed in the PCR assays for the identification of these pathogens. Higher frequencies of *G. zeae* and *F. avenaceum* were observed in Ritmo, compared with Buteo. Average leaf wetness between May and the middle of July and at the time of anthesis was positively correlated to relative frequencies of *G. zeae*, but negatively correlated to relative detection frequencies of *F. culmorum*. Temperature, precipitation (rainfall), and relative humidity during the same periods

of time did not influence the detection frequencies observed for any of the pathogen species. Relative frequencies of PCR amplicons specific for *Fusarium* spp. were positively correlated to the deoxynivalenol (DON) content of grain ($R = 0.685$, $P < 0.0001$). Tillage and previous crop might affect the severity of FHB epidemics, but they did not change the species composition significantly. In contrast, the resistance level of wheat genotype and leaf wetness affected the spectrum of *Fusarium* spp. on wheat heads in a selective manner (Klix et al. 2008).

Bread-making wheat in Japan requires a higher protein content which could be obtained by top-dressing with nitrogen near anthesis of wheat. Such N applications might increase the incidence of Fusarium head blight (FHB) disease however, and consequently increase accumulation of deoxynivalenol (DON) and nivalenol (NIV) in the grains. The effect of N application (0, 4, and 8 g m^{-2}) at anthesis on FHB infection and mycotoxin accumulation in wheat cultivars Nishinokaori and Minaminokaori was therefore assessed under greenhouse conditions and in two fields in 2006. Plants were spray-inoculated at 3, 10, and 20 days after N application. Colonized maize kernels which produced ascospores of the pathogen served as inoculum for the field experiments. The results demonstrated that grain protein level increased due to N treatment, but no significant effect on the FHB incidence and levels of DON and NIV accumulation could be seen. It was suggested that N application at anthesis might not increase the risk of FHB and mycotoxin contamination of wheat grains (Yoshida et al. 2008).

Tillage is one of the agricultural practices that may have a decisive influence on the incidence of fungal diseases. The effects of tillage (ploughing vs minimum tillage) and application of chopped maize stalks on infection by *Fusarium graminearum* and *F. culmorum* on winter wheat cultivar Hereward were investigated. Isolation on medium and quantitative PCR assays were employed to detect the pathogens in shoot bases, stem bases, and harvested wheat grains. *F. culmorum* was more frequently detected (up to 35%) than *F. graminearum* from stem bases. A lack of rainfall probably limited the dispersal of conidia from the ground surface to the ears. Ascospores of *F. graminearum* were less dependent on rainfall for dispersal, compared to *F. culmorum*. The incidence of *F. graminearum* was increased in stem bases where maize stalks were incorporated and in grain by both the presence of maize stalks and minimum tillage. The results indicated that noninversion (minimum) tillage might increase the risk of ear infection, especially by *F. graminearum*, and the consequent mycotoxin contamination of grains. The risk imposed by a susceptible preceding crop (wheat or maize) and minimum tillage was found to be significantly high for the wheat ear infection by *F. culmorum* and *F. graminearum* (Bateman et al. 2007).

Phomopsis longicolla, a major seedborne pathogen of soybean seeds, is favored by hot, humid environments. Infection and development of *P. longicolla* in vegetative and reproductive tissues of six soybean cultivars was monitored for 3 years (2002–2004). Soybean cultivars were raised without irrigation or with irrigation applied at pre- plus post-flowering or at flowering growth stage. *P. longicolla* was recovered more frequently on seed from irrigated treatments than from the nonirrigated treatments in all three years. The percentages of *P. longicolla* isolated in 2003 were significantly greater within each irrigation treatment than in the other two years (Fig. 9.1). There was a low but significant positive correlation between recovery of *P. longicolla* from pods and recovery of *P. longicolla* from seeds in nonirrigated treatments. No correlations of *P. longicolla* from leaves and stems with that from seeds could be observed (Fig. 9.2). Likewise, no consistent correlation was noted between yield and infection of plant parts.

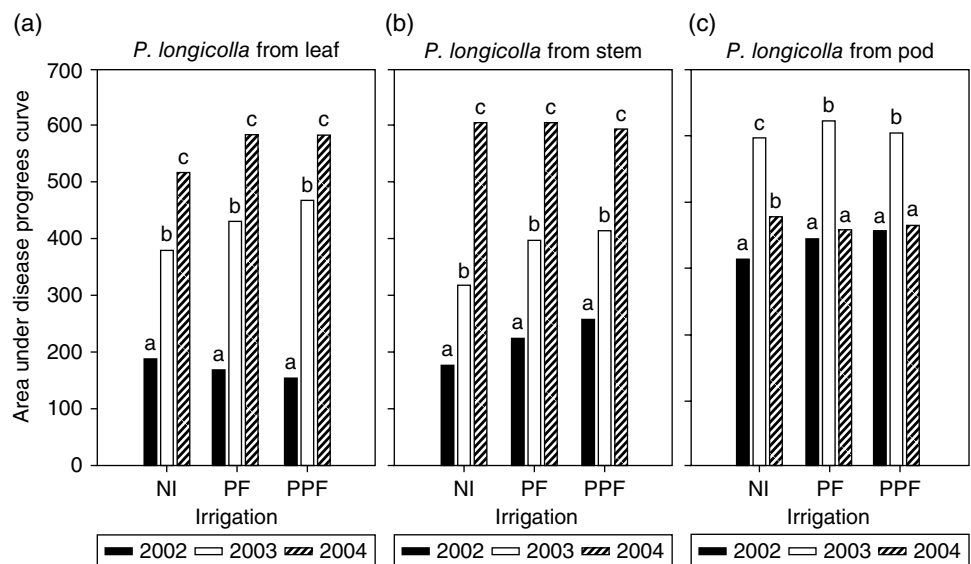


Figure 9.1 Influence of irrigation on development of *Phomopsis longicolla* (a) leaf, (b) stem, and (c) pod, represented by area under disease progress curve under nonirrigated (NI), post-flower irrigation (PF), and pre- plus post-flower irrigation (PPF) environments during 2003–2004. Means followed by the same letter within each irrigation environment are not significantly different ($P \leq 0.05$). Source: Mengistu et al. (2009). Reproduced with permission of the American Phytopathological Society, MN, USA.

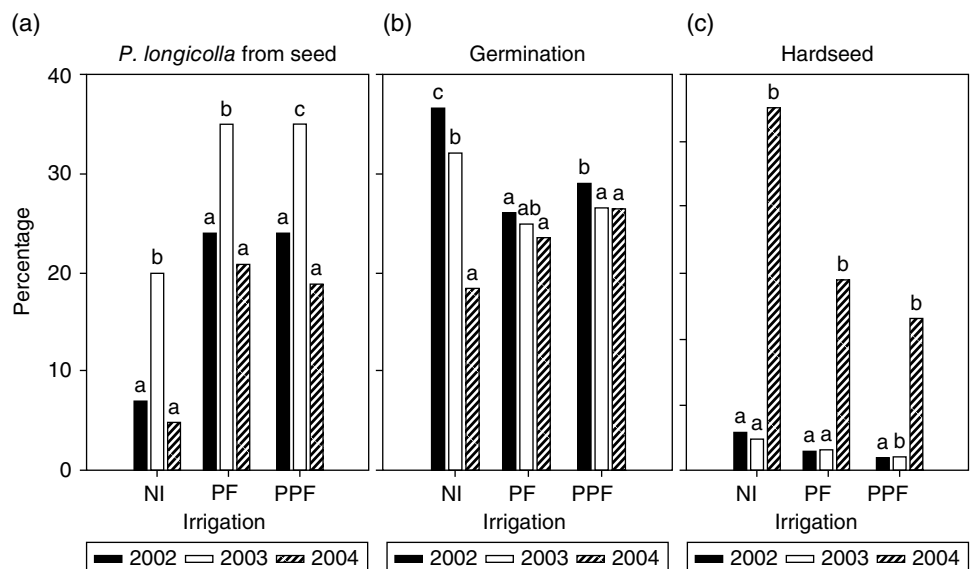


Figure 9.2 Effects of irrigation on percentage of recovery of *Phomopsis longicolla* from (a) infected seed, (b) seed germination percentage, and (c) seed hardness in soybean crop grown in nonirrigated (NI), post-flower irrigation (PF), and pre- plus post-flower (PPF) irrigation environments. Means followed by the same letter within each irrigation environment are not significantly different ($P \leq 0.05$). Source: Mengistu et al. (2009). Reproduced with permission of the American Phytopathological Society, MN, USA.

The isolation of *P. longicolla* was negatively correlated with percent seed germination in irrigated environment. The results indicated that seed infection by *P. longicolla* was moisture- and cultivar-dependent. The levels of infection of seed of some cultivars increased with an increase in moisture availability during the growing season (Mengistu et al. 2009).

9.1.1.3.2 Weather Variables

Fusarium head blight (FHB) disease infecting wheat, barley, and other small grain crops is caused by different species of *Fusarium*, the importance of which may vary depending on the geographical location. *Fusarium graminearum* is the predominant pathogen in many countries. These pathogens attract the attention of the growers, consumers, and administrators because of the grain yield loss and the health hazards of the mycotoxins produced by these pathogens contaminating the food grains and feed stuff. Concentrations of deoxynivalenol (DON), the main mycotoxin produced by *F. graminearum*, at $<1.0\mu\text{g g}^{-1}$ of grain are permitted in wheat meant for human consumption and up to $5.0\mu\text{g g}^{-1}$ for livestock feed (Miller 1994). DON can be produced in the seeds that may not exhibit any visual symptoms of infection by *F. graminearum*. Accurate measurement of DON is therefore preferred to determine the suitability of the harvested produce for consumption, rather than the loss in grain weight. Epidemiological factors that favor incidence of FHB and accumulation of mycotoxins have to be determined to understand the effects of the disease on the economy of the crop cultivation. The epidemics of Fusarium head blight (FHB) disease is generally associated with availability of inoculum abundantly, susceptible wheat or barley cultivars, and favorable weather conditions at the time of heading. Regardless of the levels of inoculum, favorable weather conditions are critical for infection to occur in wheat heads. A small quantity of inoculum may be adequate to cause concern for high concentrations of deoxynivalenol (DON) in grains, if weather conditions are favorable for infection (Miller et al. 1998).

Important weather variables and their timing were determined for predicting concentrations of DON in mature grain at wheat heading. Weather variables such as daily rainfall, daily minimum and maximum air temperatures, and hourly relative humidity were recorded for each field from nearby weather stations and normalized to the date of 50% head emergence. DON was correlatable to weather in three critical periods around heading: 4–7 days before heading with a general increase in DON with number of days with 75 mm of rain and a decrease with the number of days of $<10^{\circ}\text{C}$; 3–6 days after heading, with an increase in DON with number of days of $>3\text{ mm}$ rain and a decrease with days exceeding 32°C ; and 7–10 days after heading, with a DON increase with number of days with $>3\text{ mm}$ rain. Relative humidity did not show any relationship with DON content in grains. Based on the data collected from 399 farm fields, concentrations of DON of $<1.0\mu\text{g g}^{-1}$ could be predicted correctly on over 89% of fields (Hooker et al. 2002).

Qualitative and quantitative effects of environmental factors on initial infection and subsequent development of Fusarium head blight (FHB) disease have to be determined for assessing the disease risks. In Europe, the FHB disease may be caused by several fungal pathogens: *Fusarium graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, *Microdochium nivale*, and *M. majus*. These pathogens may differ in their requirements for infection and subsequent development (Rossi et al. 2001). Models developed, based on field data entirely, may lead to significant errors. Further, the models derived from

single-pathogen inoculations may also result in erroneous conclusions, because of interactions between different FHB pathogens. Experiments were therefore performed under controlled environmental conditions to investigate the relationship between environmental conditions, development of FHB, and mycotoxin production. A single isolate from each of *F. avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae* were inoculated onto wheat ears separately. In addition, combinations of two or three isolates were also inoculated onto wheat ears simultaneously. Inoculated ears were subjected to various combinations of duration of wetness (6–48 h) and temperature (10–30°C). Both incidence of FHB and mycotoxin concentration increased generally in all treatments, with increasing length of wetness period and temperature. There were significant positive correlations among disease incidence, fungal biomass (quantified as total amount of fungal DNA), and mycotoxins. Mycotoxin production was significantly increased by high temperatures ($\geq 20^\circ\text{C}$) during infection periods. No synergistic interaction seemed to occur between fungal isolates in causing visual symptoms. However, the results suggested that, in most cases, the pathogen isolates competed with each other as a large reduction in fungal biomass was observed compared to single-isolate inoculations, often >90% reduction for the weaker isolates. In contrast, the production of mycotoxins dramatically increased in coinoculations by as much as a factor of 1000, suggesting that competition among FHB pathogens resulted in greater production of trichothecene mycotoxins. *F. graminearum* isolate was the most competitive and isolates of the other three species had similar levels of competitiveness. The results revealed that FHB pathogens could compete for nutrients or infection sites on wheat ears (Xu et al. 2007).

Germination of ascospores is the initial step for successful infection of wheat spikes by *Fusarium graminearum*. The effects of age of spores (0–18 days after discharge), temperature (4–30°C), relative humidity (0–100), and pH (2.5–6.5) on the germination of ascospores of the mycotoxin-producing *Gibberella zeae* strain were investigated. Freshly discharged ascospores germinated within 4 h at 20°C and 100% RH and the rate of germination and the percentage of viable ascospores decreased over time after discharge from perithecia. The period of time needed to maintain 50% ascospore germination at 100% RH was 26.9 h at 4°C, 10.4 h at 14°C, 3.44 h at 20°C, and 3.31 h at 30°C, indicating the adverse effects of higher temperatures on the ascospore germinability. The time course of ascospore germination was affected by pH, the maximum being at pH 3.76. Dry atmosphere (0% RH) abolished the ascospore germination almost instantaneously. Ascospores kept at 53% RH lost the germinability rapidly from 93% to 6% within 10 min. The results indicated that age of spores, RH, temperature, and pH were vital factors affecting the percentage of germination of ascospores of *G. zeae* (Beyer and Verreet 2005).

Fusarium verticillioides (= *F. moniliforme*) infects maize (corn) plants at different stages of growth, inducing root rot, stalk rot, ear rot, and seedling blight symptoms. Maize seeds were inoculated with four different strains of *F. verticillioides* and planted in the field. The seed-inoculated strains of the pathogen could be recovered from crowns (65%), nodes (34–54%), cobs (10%), and kernels (8%), revealing the ability of *F. verticillioides* to be transmitted to seedlings and remaining dormant till the earheads are formed. This kind of infection is referred to as systemic or endophytic infection (Kedera et al. 1992). The effect of temperature on the first step in the systemic infection process was studied. Commercial maize hybrid seeds were inoculated with a strain of *F. verticillioides* transformed with green fluorescent protein (*gfp*) gene and planted in growth chambers. Three temperature regimes – low, average, and extreme

temperatures – occurring in Iowa, USA were maintained. At V2 growth stage, >90% of the root and mesocotyl tissues were infected by the transformed strain at all three regimes. At V6 growth stage, infection of root and mesocotyl tissues persisted and was detected in 97–100% of plants at all three temperature regimes. Plants remained asymptomatic and the percentage of such plants increased with increase in temperature. Seed transmission and systemic infection detected at different temperatures did not differ significantly among treatments (Wilke et al. 2007).

Relationships between Fusarium head blight (FHB) of spring malting barley caused by *Gibberella zeae* (anamorph *Fusarium graminearum*) and accumulation of deoxynivalenol (DON) and hourly weather conditions were investigated to develop predictive models. Three popular barley cultivars were planted throughout the US Northern Great Plains for six growing seasons. FHB disease incidence and DON concentration in grains were recorded. Nine predictor variables were calculated using hourly temperature and relative humidity during the 10 days preceding full head spike emergence. Simple logistic regression models were developed using these predictor variables based on a binary threshold for DON of 0.5 mg kg^{-1} . Four of the nine models had sensitivity greater than 80% and specificity of these models ranged over 67–84% ($n = 150$). The FHB incidence was significantly associated with DON accumulation in the grain and the weather conditions prior to full head emergence, which could be used to accurately predict the risk of economically significant DON accumulation for spring malting barley (Bondalapati et al. 2012).

Stagonospora nodorum (teleomorph *Phaseosphaeria nodorum*) causes Stagonospora nodorum blotch (SNB) disease of wheat. Rainfall was found to be vital for development of SNB disease because pycnidiospores of *S. nodorum* might be dispersed in splash droplets. Rainfall during stem elongation (facilitating vertical progress of the disease in the developing canopy) as well as rainfall during seed developmental stages (for splashing onto the spikes) may be important factors affecting the incidence of wheat seed infection by *S. nodorum* (Pietravalle et al. 2001). A simple model was developed to predict the regional distribution of the incidence of wheat seed infection by *S. nodorum* shortly after harvest, or even before, without having to wait for the results of seed assays. Mean monthly rainfalls in May and in June across western and central New York were used to predict seed infection. The distribution of the incidence was well reflected by beta-binomial probability distribution (parameters p and θ). The model was validated with independent data collected from New York. The predicted distribution of seed infection incidence was not statistically different from the actual distribution of the incidence of seed infection. This investigation provided a method of estimating the probability of a given seed infection incidence level of a particular seed lot produced in western and central New York in any year (Shah and Bergstrom 2002).

The effects of infection of linseed cultivar Antares by *Botrytis cinerea* or *Alternaria linicola* on seedling emergence at varying temperatures of 5–20°C and transmission of the pathogen from infected seeds to the seedlings were investigated. The maximum percentage of seedling emergence decreased linearly as the seed infection by *B. cinerea* increased from 0 to 83% at 18°C day/12°C night temperatures. Transmission of *B. cinerea* to seedlings, as revealed by the production of symptoms on the hypocotyls and post-emergence death, increased as temperature decreased from 20 to 5°C. On the other hand, a decrease in percentage of seedling emergence due to seed infection by *A. linicola* and transmission of the infection to seedlings by *A. linicola* increased as the temperature increased from 5 to 20°C. At 10°C, infection of seeds by *A. linicola*

decreased seedling emergence to a greater extent compared with infection of seeds by *B. cinerea*, even though the percent of seed infection by *A. linicola* was considerably smaller. Regression analyses indicated that maximum percentages of seedling emergence (logit transformed) against temperature were well correlated (Harold et al. 1997).

The mating types A1 and A2 of *Phytophthora infestans* were found to be in a 1:1 ratio in the highlands of Mexico, favoring the sexually derived genetic diversity of the potato late blight pathogen. The widespread occurrence of A1 and A2 mating types guaranteed successful infection of even resistant potato cultivars/wild species by *P. infestans*. Wild *Solanum* spp. present in this region could serve as alternative hosts to the pathogen. Knowledge of the external factors that influence the dynamics of potato late blight disease may facilitate the assessment and selection for genetically resistant potato cultivars, in addition to facilitating the prediction of late blight epidemics. Using 8 year weather datasets, six ideal situations were predicted with four to eight infection cycles of 6–12 h each. The observed effect of the area under disease progress curve (AUDPC) and its components was highly significant, with a low coefficient of variation among potato cultivars used in this investigation. LateBlight-LB2004 model was found to be useful for simulating and predicting late blight epidemics based on weather factors existing in Chapingo, Mexico, except for the magnitude of relative humidity threshold variable (RH-threshold) which required calibration for each potato cultivar (Diaz de la Cruz et al. 2014).

9.1.1.3.3 Soil Environment

The soil environment is always in a state of constant and dynamic variations and changes in physical, chemical, and biological constituents of the soil occur constantly, resulting in favorable or adverse conditions for the development of the pathogens as well as of the antagonistic microorganism and other microorganisms involved in the decomposition of soil organic matter. *Gibberella zeae* (anamorph *Fusarium graminearum*), the pathogen causing wheat Fusarium head blight (FHB) disease, produces macroconidia which are splash-dispersed by rain and are involved in the secondary infection of wheat earheads. Sexual fruiting bodies, perithecia, are formed in the spring on infected crop residue with rainfall greater than 5 mm. Ascospores formed in the perithecia are forcibly discharged and dispersed by wind, leading to initiation of primary infections. With increase in no-till or conservation tillage practices, large amounts of crop residues accumulate on the soil surface and form sources of inoculum of FHB pathogen. Macroconidia and perithecia could be produced from the residues on or above the soil surface up to 3 years (Konga and Sutton 1988). Survival of *G. zeae* in *Fusarium* damaged kernel (FDK) was investigated under field conditions. FDKs were either left on the soil surface or buried 5 or 10 cm deep and monitored for 24 months. Perithecia developed on FDKs from all locations and treatments, but ascospores developed only in perithecia on kernels located on the soil surface. The survival of the pathogen in kernels left on the soil surface or buried at 5 cm in heat-treated and nontreated soil at –10, 2, and 20°C was investigated. The fungus survived in 76–100% of kernels. When the kernels were incubated at 20°C, *G. zeae* was recovered from 83 and 76% of kernels in heat-treated and nontreated soils, respectively. Perithecia developed on kernels incubated at 2 and 20°C, but ascospores were formed only in perithecia on FDKs at 20°C on soil surface. The pathogen could survive for 24 months (Inch and Gilbert 2003).

Potato tubers infected by *Phytophthora infestans*, incitant of late blight disease, may remain in the soil after harvest. Growth, sporulation, and survival of *P. infestans* on the

volunteer tubers were investigated under temperatures representative of winter (4, 7, and 10°C) and spring (13, 16, and 19°C) soil conditions in western Washington. Inoculated tubers stored at 10°C for 8 days had a significantly higher percentage of disease symptoms ($P < 0.05$) on tuber surfaces and a higher number of lenticels and eyes showed infection with *P. infestans* and sporulation, compared with those stored at 4 or 7°C. Sporulation of the pathogen on cut tuber surfaces was observed following 3 week storage at three soil temperatures. At 12 week storage, tubers inoculated with a US-8 isolate had a significantly higher percentage (70%) of late blight symptoms on cut potato tuber surfaces than those inoculated with a US-11 isolates (50%). At temperatures simulating spring, tubers inoculated with US-8 isolate and held at 19°C had a significantly higher number of lenticels/tuber with *P. infestans* sporulation than the tubers held at 10°C or 16°C. Sporulation of *P. infestans* on tuber surfaces was detected on infected tubers buried at 5 cm depth in potting medium at all tested temperatures (winter and spring) for 3–6 day periods. Tubers with late blight infection that survive the winter in western Washington and support sporulation of *P. infestans* via lenticels and eyes may enable the transmission of *P. infestans* from infected tissues to sprouts of volunteer plants. However, as the mild winter conditions favored tuber breakdown in soil, the likelihood of infected tubers producing inoculum appeared to be limited (Gigot et al. 2009).

Verticillium dahliae, a soilborne fungal pathogen, infects spinach crops and is transmitted via seeds of spinach in the USA and Europe. The planting of infected seeds increased the soil inoculum density and might introduce exotic strains that contribute to epidemics of Verticillium wilt disease of lettuce and other crops grown in rotation with spinach. Further, other crop residues that are returned to the soil harbor pathogen inoculum for infection of lettuce or other crops. Detection and quantification of *V. dahliae* inoculum by employing sensitive techniques such as real-time quantitative polymerase chain reaction (qPCR) assay was therefore found to be an effective procedure for reducing seedborne inoculum of *V. dahliae* (Duressa et al. 2012).

Verticillium dahliae is the primary causal agent of Verticillium wilt of potato transmitted through tubers and soil. This disease is a monocyclic and polyetic disease and the primary inoculum consists of microsclerotia which are produced during plant senescence, and they may persist in soil for long periods of time. The disease thresholds in potato were estimated to range between 5 and 30 CFU cm⁻³ of soil for *V. dahliae* (Powelson and Rowe 1993). Microsclerotia production in potato was most abundant in aerial stems and colonized host plant debris. *V. dahliae* was isolated from the vascular system of 94% of plants grown in infested soils and 8% of plants grown from infected tubers in noninfested soil. Further, plants grown in infested soil contained microsclerotia on 46% of stems, while plants grown from infected tubers in noninfested soils exhibited microsclerotia formation only on 1% of stems, indicating that soilborne inoculum was more important for disease incidence (Dung and Johnson 2012).

Contribution of seed infection by *Pyricularia grisea* to the development of rice blast epidemic was assessed under field conditions. A marked strain of *P. grisea* (sulfate nonutilizing (*sul*) mutant) was used to determine if inoculum from infested rice grain could cause primary infections and sustain rice blast epidemic during the growing season (1996–1997) by inducing leaf, collar, and neck blast symptoms in susceptible cultivar M-201. Leaf blast symptoms were first detected in plots containing infested grain at 35 days after seedling emergence in both years. Leaf blast increased more rapidly in plots with 25 and 50 infested grains/0.1 m² than in plots with less inoculum pressure (0.5 and 2.5 infested grains/0.1 m²). At the end of both seasons, the mutant

strain could be recovered from over 90% of the leaf, collar, and neck blast lesions. Rice blast was not detected in the control plots until 65 days after planting. The results indicated that seed infection could play an important role in the development of rice blast epidemics (Long et al. 2001).

Tilletia indica, the incitant of Karnal bunt or partial bunt disease, infects wheat crops in several countries. The teliospores of *T. indica* are introduced into the soil during harvest time and persist in the soil for long time. Teliospores present on the soil surface germinate producing primary and secondary sporidia (basidiospores) when favorable conditions exist, and infect plants at flowering stage. *T. indica* teliospores survived for 3 years on the soil surface, but spores buried at 20 cm depth had reduced viability (2 years) (Krishna and Singh 1983). The teliospores survived for a longer period in dry soil than in wet soil. The viability of teliospores decreased with increase in the depth of soil from 5 to 20 cm (Ratan and Auja 1992; Sidharta et al. 1995).

In a later investigation, survival of teliospores of *Tilletia (Neovossia) indica* in soils differing in texture and other characteristics was studied. The teliospores were placed in 20 µm polyester bags which were sealed and placed at 2, 10, and 25 cm depths in polyvinyl chloride tubes containing the representative soils. The rates of teliospore recovery from soil samples were 90.2, 18.7, 16.1, and 13.1% at 1 day and 8, 20, and 30 months after incorporation of teliospores into the soils, respectively, and the recovery was significantly ($P < 0.01$) affected by soil source. The percentage of germination of teliospores was significantly affected by soil source and depth of burial over the 32 month period. The results showed that teliospores of *T. indica* could survive for more than 32 months and remain viable in conditions existing in Montana, USA (Babadoost et al. 2004).

9.1.2 Molecular Ecology and Epidemiology of Fungal Diseases

Models of disease foci and the patterns in which these foci expand in time and space have been developed in epidemiological investigations. In agricultural systems, the spatial patterns of host genotypic resistance manipulated through use of intercrops or mixed genotypes (multilines) within a crop species has been successfully adopted as a disease management strategy (Zhu et al. 2000; Mundt 2002). The genomics approach in epidemiological investigations may be useful to obtain a better understanding of multiple spatial and temporal scales as well as levels of details of genomic status of microbial plant pathogens. It is essential to determine the type of scale of variation required to include in the prediction of epidemics for the desired pathosystem. Microarray analyses have the potential to provide required information in optimal scales of variation in expression data that may be included in prediction models. Within a host plant, the local phenotype might be at the scale of a leaf or cell. Local gene expression may be at the point of infection as exemplified by the presence of a green island of host tissue formed around the initial infection site where necrotic or chlorotic lesions may appear, as in the case of bean anthracnose or rice blast diseases. Regional gene expression within an individual plant might be the expression in tissues adjacent to infection. In addition, competition between two pathogens may influence differentially depending on the time of infection and type of plant tissues infected (Al-Naimi et al. 2005).

Reverse-transcription (RT-) PCR assay is preferable for the assessment of cell viability by detecting mRNA to the DNA-based techniques, which do not differentiate dead and living cells. An RT-PCR method was developed to determine viable populations of *Mycosphaerella graminicola* in wheat presymptomatically and monitor disease

development during epidemic period. By employing a specific primer set E1/STSP2R, single fragment was only amplified from total RNA of *M. graminicola* and infected wheat leaves but not from those of healthy leaves. Further, no amplification was detected with total RNA of five other common fungal pathogens infecting wheat, indicating the specificity of the assay. This diagnostic test was applied successfully to monitor the disease development in inoculated and naturally infected plants, using the same primer set (E1/STSP2R) developed from a sequence of β -tubulin genes. The specificity and sensitivity (5 ng of total RNA) of this test was high enough to detect *M. graminicola* presymptomatically in inoculated plants as well as in field samples at 4 days after inoculation (dai), when no visible symptoms could be observed. After the production of symptoms, the increase in band intensity of the amplified RT-PCR products in the gel was in general agreement with the results of visual disease assessment (Guo et al. 2005).

Factors governing long-term disease dynamics could not be clearly understood because of a lack of long-term data. Generally surveys assess short-term responses to disease problems of immediate concern. PCR assay was applied to investigate long-term dynamics of *Phaeosphaeria nodorum* and *Mycosphaerella graminicola*, causing Septoria blotch diseases of wheat crops. An archive of data from a long-term Broadbalk winter experiment, run continuously at Rothamsted since autumn 1843, was utilized. Quantitative real-time PCR assays were applied to quantify the fungal pathogens and wheat DNAs present in a set of samples covering a 160 year period of wheat production. The relative abundance of DNA of these two fungal pathogens in a sample reflected the relative importance of the two wheat diseases they caused as determined in disease surveys undertaken in the UK. Unexpectedly, positive correlations between the abundance of *P. nodorum* DNA and SO₂ emissions, and negative correlations between the abundance of *M. graminicola* DNA and SO₂ emission, were observed, indicating a possible link between pathogen infection and extent of atmospheric pollution by SO₂ (Bearechell et al. 2005).

The mode of dissemination of fungal pathogens, a major epidemiological factor, may be through air, rain, water, soil and, in some cases, through insects. Free water or high relative humidity is required by the oomycetes for the production of sporangia. Splash droplets generated by raindrop compaction on plants have shown to be the dispersal agent of *Phytophthora infestans*, causal agent of late blight disease of potato and tomato. The traditional procedure involving collection of droplets carrying spores on glass is cumbersome and time-consuming. Further, adequate skill is required for the specific and precise identification of the pathogen structures. A new approach was therefore applied for the analysis of splash-dispersed *P. infestans*, involving the use of an engineered fluorescent strain transformed with the gene (*gfp*), encoding the green fluorescent protein (GFP). The use of fluorescent sporangia facilitated dependable detection and counting of exclusively viable sporangia of epidemiological significance by image processing. This investigation demonstrated the usefulness of GFP marker in plant disease epidemiology to provide clear evidence and a safe conclusion (Saint-Jean et al. 2005).

Potato late blight epidemics due to *Phytophthora infestans* devastated potato crops because of cultivation of single cultivar across large areas. Such uniformity contrasts with natural ecosystems in which host genotypes are usually both locally diverse and distributed in small areas. Natural ecosystems have been found to be less prone to the rapid development of severe epidemics. Intimate mixing of cultivars is a proven approach for introducing diversity to reduce incidence and intensity of crop diseases

(Akem et al. 2000; Zhu et al. 2000). Crop variety mixtures may reduce disease in three basic ways: (i) induction of resistance by avirulent isolate (spores), preventing or delaying infection by adjacent virulent isolate; (ii) resistant cultivars acting as barriers to pathogen spread; and (iii) dilution effect of resistant cultivar preventing continuous availability of susceptible plants. Potato varieties with contrasting levels of resistance to late blight pathogen were planted in pure or mixed stands in four experiments over 3 years. Late blight severity and progress in mixtures were compared with that in pure stands. Disease on susceptible (S) or moderately resistant (MR) varieties typical of those in commercial use was similar in mixtures and pure stand. On the other hand, mixtures

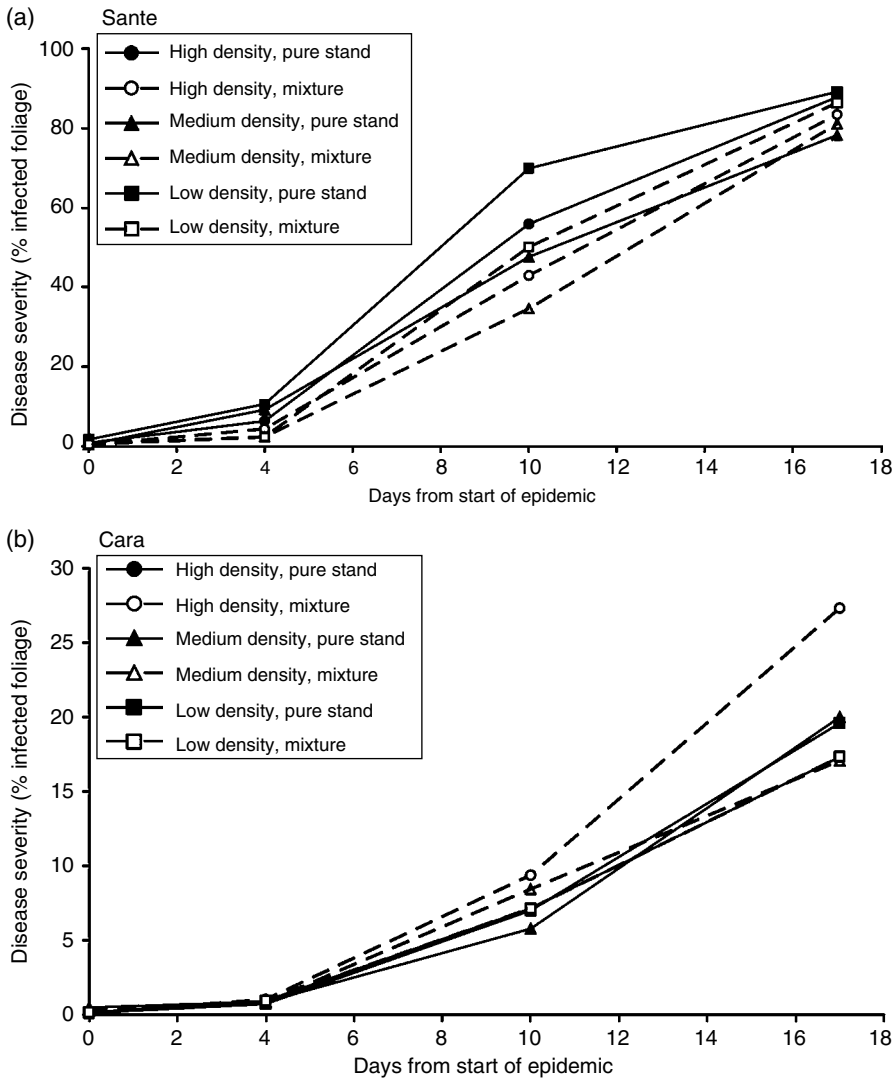


Figure 9.3 Differential disease progress curves of late blight disease in pure stands of potato (a) cultivar Sante and (b) cultivar Cara and mixtures with Sante or Cara at three planting densities. Source: Phillips et al. (2005). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

of an immune or near-immune companion with cultivars Cara (MR) or Sante (S) substantially reduced disease on the latter. The size of the plot had a distinct effect on late blight disease. Larger plots (9×7.5 m) had a greater area under the disease progress curve (AUDPC), but the average rate of disease progress was greater in smaller plots (4.5×4.5 m). This may be because most disease progress took place later, under more favorable conditions in smaller plots. Planting density did not have any effect on disease and did not interact with mixture effects. Overall, planting mixtures of potato cultivars did not offer any improvement on the average disease resistance of the components under the set of conditions of experimentation (Fig. 9.3). It was suggested that potato variety mixture should be designed to improve yield of more resistant varieties by reducing the negative effects of inter-cultivar competition and to improve the yield of more susceptible varieties by reducing disease through mixing with a resistant variety (Phillips et al. 2005).

9.2 Epidemiology of Bacterial Diseases

Phytopathogenic bacterial species may require one or more susceptible plant species and environmental conditions suitable for their proliferation to reach optimal inoculum potential in or on seeds and plant organs. Bacterial cells are surrounded by mucilage which protects them against desiccation, loss of viability, and dry weather. The bacterial cells restrict their dispersal to periods of rainfall when conditions favor splash spread and free water required for activation of bacterial cells and infection of the host. Wind-driven rain is responsible for dissemination of the majority of bacterial pathogens. Seed transmission of bacterial pathogens is a major concern for developing effective management systems to limit the introduction and spread of bacterial pathogens in different agroecosystems. Seed transmission accounts more than ever before for the movement of bacterial pathogens across long distances, natural barriers, and even political borders in the present global economy with liberal trade between countries.

9.2.1 Dynamics of Host–Bacterial–Pathogen Interactions

The number of infective propagules (pathogen population) and their pathogenic potential (virulence) constitute inoculum potential of the bacterial species pathogenic to different plant species. Disease potential depends on the level of host susceptibility which in turn is positively/negatively influenced by environment, availability of nutrition and stage of plant growth at which level of susceptibility may vary significantly. Further, host susceptibility is governed by genetic constitution. Development of epidemics is influenced by number of infective bacterial cells (propagules), virulence, host susceptibility/resistance and existence of favorable environmental conditions. Agricultural practices aimed to increase the yield of crops have been found to favor development of bacterial diseases in some crops. Incidence and spread of seed-transmitted bacterial pathogens are influenced by several factors.

9.2.1.1 Pathogen Factors

Bacterial pathogens may gain entry into the plants through different avenues, and have to reach the seed tissues and be able to tolerate the intense desiccation during seed maturation. The rate of seed infection to seed transmission or infection of seedlings

and subsequent establishment under field conditions are determined by the genetic constitution of the bacterial pathogen. Bacterial species with the ability to be transmitted through seeds at a higher rate may have greater number of foci of infection from which the disease can spread at a faster rate when favorable environmental conditions are available. In the case of some bacterial pathogens, even with a low percentage of seed infection, the disease may spread at a faster rate and cause heavy losses.

Infection of *Brassica* seeds at a rate of only 2 infected with *Xanthomonas campestris* pv. *campestris* (Xcc) per 10,000 seeds, and less than 1% of tomato seeds infected with *X. campestris* pv. *vesicatoria* (Xcv), resulted in epidemics of cabbage black rot and tomato bacterial spot diseases in the respective crops under favorable environmental conditions (Cox 1966; Schaad et al. 1978). Likewise, high levels of disease incidence occurred when bean seed lots with 0.02% infection with *Pseudomonas syringae* pv. *phaseolicola* and cowpea seeds with 1% infection by *X. campestris* pv. *vignicola* were planted (Walker and Patel 1964; Shekawat and Patel 1977). In another investigation, use of bean seed lot containing 5 infected seeds per 1000 seeds led to an epidemic of halo blight disease, while disease development was arrested when a seed lot with a single infected seed per 20,000 seeds was used, indicating the role of infected seeds in epidemics. Significant reduction in cabbage black rot caused by *X. campestris* pv. *campestris* (Xcc) was observed when the seed infection was reduced from 0.03 to 0.01% (Trigalet and Bidaud 1978; Schaad 1988).

The threshold of inoculum for seed-transmitted pathogen is influenced by several factors including inoculum concentration, inoculum location (external or internal), nature and quantum of seedborne and soilborne microflora, soil temperature, moisture, and pH, in addition to vector populations involved in the spread of some of the diseases. Information on these factors for many seed-transmitted bacterial pathogens is incomplete or these factors have not been determined. The disease transmission thresholds for *Acidovorax avenae* subsp. *citrulli* were different under field and greenhouse conditions for causing bacterial fruit blotch (BFB) disease in watermelon. The direct seeded melons in the field with a plant spacing of 1 plant/4 m² and greenhouse-grown watermelon with a spacing of 1 plant/2.54 cm² required different disease transmission thresholds. Generally, the seed infection threshold of 1/10,000 determined for cabbage black rot disease was also used for other bacterial diseases. The threshold for BFB infections in a transplant greenhouse is one plant/total populations of plants in greenhouse. If the greenhouse holds one million transplants, then the threshold is one infected plant/1,000,000 as the presence of one infected seedling might be sufficient to spread infection to all plants, resulting in rejection of all plants and a loss of 100% (Gitaitis 1993; Gitaitis and Walcott 2007).

The true inoculum density on naturally infested seed may not be correctly determined because of the nature of pathogen distribution. The efficiency of assay could be inflated if based on a naturally infested seed harboring 1×10^7 CFU of bacteria, when in reality most infested seed in the population contain only 1×10^2 CFU of bacteria. Overestimation of the sensitivity of seed health assay may result in incorrect assumptions when calculating the sample size necessary to be analyzed statistically for a predetermined infection threshold. The inoculum density on seeds is likely to affect the results of grow-out tests. For example, the mean number of *Pseudomonas syringae* pv. *pisi* (incitant of pea bacterial blight disease), CFU on the seed, and soil moisture were found to be the major factors influencing the number of diseased seedlings that emerged and the mean number of lesions/seedling (Roberts et al. 1996). The effects of

cultural conditions, pathogen strain aggressiveness, and varietal susceptibility for each disease have to be assessed to make proper assumptions for determining infection threshold on seeds for bacterial pathogens (Gitaitis and Walcott 2007).

Acidovorax citrulli, causative agent of cucurbit bacterial fruit blotch (BFB) disease, is seedborne and the infested seeds form the primary sources of inoculum for disease spread. An inoculum threshold of 1 infested seed per 10,000 seeds has been widely used in seed health testing for BFB pathogen. A later investigation showed that disease transmission to seedling occurred even with 1 infested seed containing 10^4 *A. citrulli* CFU; a zero-tolerance approach for BFB was therefore suggested for adoption for effective management of BFB disease. Seedling transmission experiments indicated that temporal spread of BFB in 128-cell seedling trays increased linearly with *A. citrulli* inoculum load ($r^2 = 0.976$; $P = 0.0037$). In addition, the frequency of spatial spread of BFB from an inoculated seedling in the center of a planting tray to adjacent healthy seedlings over 1, 2, or 3-cell distances was greater for lots with one seed infested with at least 1×10^5 CFU than for lots with one seed infested at lower inoculum loads (1×10^4 and 1×10^3 CFU/seed) (Dutta et al. 2012).

Pseudomonas syringae pv. *coriandricola* (*Psc*) causes bacterial blight disease of coriander, and epidemics of the disease resulted in significant yield reduction and consequent reduction in planting of coriander in Australia (Dennis and Wilson 1997). Coriander seeds could be inoculated by mixing them with a suspension of *Psc* wild-type strain containing methyl cellulose. Seed inoculation with *Psc* resulted in disease symptoms and the plant height was reduced significantly. The inoculum potential for seed infection and induction of disease symptoms on seedlings was $>10^5$ CFU mL⁻¹. At a concentration of 10^7 and 10^8 CFU mL⁻¹, disease developed earlier and the mortality of some plants was also observed. Disease progress was more rapid at higher concentrations, but final disease incidence was not increased at concentrations of pathogen above 10^3 CFU mL⁻¹ (Refshaug et al. 2010).

Three *Erwinia* spp., *E. carotovora* subsp. *carotovora* (*Ecc*), *E. c. atroseptica* (*Eca*), and *E. chrysanthemi*, are associated with potato tuber soft rot and black leg (stem rot). Late infection of potato tubers was found to be widespread. These bacterial species are opportunistic pathogens capable of infecting tubers when resistance was impaired. They are Gram-negative, nonsporing facultative anaerobes, characterized by the production of large quantities of extracellular pectic enzymes. They rely mainly on the production of these enzymes along with a wide range of other plant cell-wall-degrading enzymes (CWDEs) to induce disease (Collmer and Keen 1986). Soft rot *erwinias* tend to out-compete other bacteria in tuber rots, because of their ability to produce larger quantities of a wide range of cell-wall-degrading enzymes. Some pathogenicity characteristics, including motility, adhesion, siderophores, detoxifying systems, and the *hrp* gene complex, common to most bacteria including symbionts and saprophytes, have been shown to be important for infection and disease development (Pérombelon 2002).

Potato tuber-borne pathogens have the advantage of being introduced into the field at the earliest possible time, resulting in early incidence of the disease and its further spread. The effect of tuber-borne *Streptomyces* on potato common scab disease incidence in daughter tubers was investigated under microplot conditions. Visually healthy tubers, surface-sterilized healthy tubers, and tubers with 25% scab coverage were planted in pasteurized soil. Samples of soil and below-ground plant parts were obtained at 30, 57, and 93 days after planting (dap) from various zones around the mother tuber.

The total population of actinomycetes was determined by plating on a semiselective medium and colonies of pathogenic *Streptomyces* were identified using PCR assay targeting the *txtA* gene. The populations of the pathogen were below detectable levels at 30 dap. After proliferation of the pathogen, the population reached measurable levels at 93 dap in below-ground plant parts and in soil adjacent to scabby mother tubers at 10^4 – 10^5 CFU g⁻¹ of soil. Incidence of scab in progeny tubers was 89% at harvest. Progeny tubers produced by visually healthy seed tubers had an incidence of 60% scab and high populations of pathogenic *Streptomyces* were detected in the zone near the mother tuber (10^4 CFU g⁻¹ soil). On the other hand, plots planted with surface-disinfested seed tubers had a very low pathogen population in the tuber zone and yielded 100% marketable tubers. Significant correlation ($r=1.00$) between the population densities of the pathogen in the root zone and daughter tuber disease incidence was observed. After harvest, high populations of *Streptomyces* (10^6 – 10^7 CFU g⁻¹ lesion tissue) on mother tubers were observed. The results clearly showed that measurement of pathogen populations in the field could be a reliable predictor of scab disease incidence in potato (Wang and Lazarovits 2005).

Ralstonia solanacearum (*Rs*), causal agent of bacterial wilt and brown rot of potato plants and tubers, has a worldwide distribution. Based on the host range, strains of *Rs* have been classified into five races (Hayward 1991). *R. solanacearum* is considered as a heterogenous species or a species complex (Fegan and Prior 2005). Apart from races, *R. solanacearum* strains have also been grouped into biovars based on their ability to utilize and/or oxidize several hexose alcohols and disaccharides. The relationship between races and biovars could be seen only with race strains which generally correlated with biovar 2 phenotype (Hayward 1991). Diverse genes of *R. solanacearum*, such as those coding for lytic enzymes and extracellular polysaccharides (EPS), hypersensitive reaction and pathogenicity (*hrp*) genes, structural genes encoding effector proteins injected by type III secretion system (T3SS) from the pathogen into host plant cell, and genes coding for factors implicated in cell adherence are involved in colonization and symptom production (Genin and Boucher 2004). The pathogen attaches to the plant roots, infects the cortex, colonizes the xylem, and spreads to other plant organs and tubers. After the death of the plant, the pathogen returns to the soil, water, or other host plants for survival as a saprophyte or parasite. *R. solanacearum* could survive in the soil for variable periods. It survived up to 1 year in agricultural soils, even after treatment with herbicide to eliminate alternative plants. The pathogen has remarkable ability for survival in the environment in different forms such as viable, but nonculturable (VBNC) forms, starved cells, PC (physiological characteristics)-type, and biofilms (Álvarez et al. 2010).

Pantoea ananatis, incitant of onion central rot disease, was found to be seedborne in addition to its transmission via bulbs. The infected leaves of onion become bleached, followed by colonization of bulb tissues. Although *P. ananatis* cannot produce pectinolytic enzymes, secondary microbes frequently colonized infected bulbs produce these enzymes, resulting in tissue liquefaction and a foul odor. The presence of *P. ananatis* in lakes was indicated by using specific bacteriophages (Eayre et al. 1995). Further, the pathogen is capable of surviving as an epiphyte on several weed species (22) and also on Bermuda grass (*Cynodon dactylon*), cowpea, and soybean (Gitaitis et al. 2002a). *P. ananatis* was isolated from the gut of thrips (Gitaitis et al. 2002b). Sanitation by elimination of sources of inoculum of the pathogen may delay the onset of an epidemic, but may not reduce the rate. The situation becomes more difficult and challenging

when three sources of inoculums, namely seed, thrips vector, and weeds, exist as in the case of onion center rot caused by *P. ananatis* (Gitaitis et al. 2004).

Asiatic citrus canker (Acc) disease caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) (= *Xanthomonas campestris* pv. *citri* pathotype A) is widely distributed and endemic in many tropical and subtropical citrus-growing nations. International phytosanitary activities did not effectively prevent the reintroduction of infected plants or plant materials (Stall and Civerolo 1991). The inoculum density required for infection may differ depending on the mode of infection by citrus canker pathogen. For effective entry through stomata and infection, a concentration of 10^4 to 10^5 CFU mL⁻¹ was required, whereas a lower concentration of *Xac* (10^2 CFU mL⁻¹) might be sufficient for infection through wounds (Goodman 1982; Zubrzycki and de Zubrzyck 1987).

Strains of *X. axonopodis* pv. *citri* *Xac*-A, *Xac*-A^W, and *Xac*-A*, varying in pathogenic potential (virulence), were recognized (Sun et al. 2004). PCR assay was employed to detect *Xanthomonas axonopodis* pv. *citri* (*Xac*) in aqueous sonicates from 3491 mature asymptomatic fruits harvested from severely diseased Satsuma mandarin trees growing in Saga City, Japan. None of the strains of *Xac* could be recovered from the Satsuma mandarin fruits during 2005–2006, indicating that asymptomatic fruits were unlikely to harbor detectable populations of *Xac*. The survival of *Xac* on Satsuma mandarin fruits discarded in an orchard was determined in two consecutive years. Bacterial populations on the contaminated fruits declined significantly in the first 3 days after the fruits were discarded. No detectable spread of *Xac* from contaminated fruit into rainwater occurred, as shown by the results of PCR assays. The results showed that the bacterial pathogen was not transferred from the contaminated fruit to susceptible host plants, even when the fruits were placed in close proximity to plants at a receptive stage. The risk of transmission of *X. axonopodis* pv. *citri* from Satsuma mandarin fruits to plants appeared to be low (Shiotani et al. 2009).

Potato zebra chip (ZC) disease was shown to be caused by “*Candidatus Liberibacter solanacearum*” which is a putative fastidious, unculturable bacterium (Abad et al. 2009). As the pathogen is yet to be cultured in a cell-free medium, information on biology, physiology, and pathogenicity is lacking. Phylogenetic analyses of the 16S rRNA gene indicated that “*Ca. Liberibacter*” spp. belong to the α -Proteobacteria group. A new strain of “*Ca. L. solanacearum*” infected a nonsolanaceous crop carrot (Munyanza et al. 2010). Genetic diversity assessments showed that haplotypes, based on single nucleotide polymorphism (SNP) typing in the sequences of 16S rRNA, 16S/23S ISR, and 50S rplJ-rplL ribosome gene loci, were differentiated in ZC pathogen. Two haplotypes were identified in North and Central America and in New Zealand (Nelson et al. 2011). “*Ca. L. solanacearum*” has a circular chromosome which encodes a total of 1192 putative proteins coding sequence (Lin and Gudmestad 2013). The ZC pathogen is transmitted by potato psyllid *Bactericera cockerelli* (Munyanza et al. 2007).

Potato psyllid populations differed in their vector efficiency in transmitting “*Ca. L. solanacearum*” associated with ZC disease. The bacteria–vector relationship is one of the important factors influencing incidence and spread of the disease. Adult psyllids were more efficient than nymphs in transmitting “*Ca. L. solanacearum*.” Acquisition success was influenced by feeding site and was highest when psyllids had access to the whole plant. The rates of successful inoculation increased with a higher number of vectors, and plants inoculated with many psyllids had higher pathogen concentrations. The disease progress and tuber yield was independent of psyllid density. A single adult psyllid successfully transmitted the ZC pathogen (Liu and Trumble 2007; Buchman et al. 2011).

9.2.1.2 Host Factors

The ability of the bacterial pathogen to infect a few or several host plant species in addition to the primary host plant (crop species) is an important factor influencing the incidence and spread of the disease(s) caused by the pathogen under investigation. *Ralstonia solanacearum*, causing bacterial wilt and brown rot disease of potato plants and tubers, has a wide host range which appears to expand continuously. Among the host plants, crops such as banana, potato, tomato, tobacco, bean, and groundnut (peanut) and many ornamental plants are infected. Five races based on the host plant species infected have been recognized (Hayward 1991). *R. solanacearum* race 1 infects the largest number of plant species and is the most widespread. Major economic losses caused by bacterial wilt epidemics have been recorded in tomato, potato, banana, groundnut, and ginger crops (Elphinstone 2005).

The presence of alternative host plants functioning as a reservoir of inoculum and infected plant debris/residue on the soil supported survival of *R. solanacearum* in soil or water, favoring overwintering in temperate regions. Alternative host plants may be tolerant and the pathogens may invade xylem in the roots and weakly colonize at stem level (Álvarez et al. 2008). Tolerant plants did not allow further movement of *R. solanacearum* onto the stem as seen in susceptible hosts, and this might be due to the operation of defense mechanisms such as presence of inhibitory compounds, tyloses, and gums that occlude vessels whose success will depend on the rapidity of the plant response (Prior et al. 1994). Among the reservoir plants, several weed species harbored *R. solanacearum* without showing symptoms of infection. Some of them with latent infection, such as *Solanum dulcamara* (bitter sweet nightshade), *S. nigrum* (black nightshade), and *Urtica dioica* (stinging nettle), had the potential to be sources of inoculum for *R. solanacearum* (Hayward 1991; Pradhanang et al. 2000). The host range of potato zebra chip (ZC) disease pathogen "*Candidatus* Liberibacter solanacearum" included *Solanum betaceum* (tamarillo) and *Physalis peruviana* (cape gooseberry) in addition to carrot, a nonsolanaceous plant species (Liefting et al. 2008, 2009). Silver nightshade (*Solanum elaeagnifolium*), wolfberry (*Lycium barbarum*), black nightshade (*S. ptychanthum*), and jalapeno pepper (*Capsicum annuum*) were found to be hosts for ZC pathogen. The presence of "*Ca. L. solanacearum*" in these solanaceous hosts in Texas was confirmed by comparing the partial 16S rRNA gene sequences isolated from these hosts. They showed 99.8% similarity with the bacterium isolated from potato plants infected by ZC disease (Wen et al. 2009). The epidemiological significance of the perennial solanaceous plant species (wolfberry and silver leaf nightshade) has to be studied to establish their contribution to disease incidence and spread.

The incidence and intensity of Asiatic citrus canker (Acc) disease caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) were favored by wounding or mechanical damage. Feeding injuries by the larvae of the Asian citrus leaf miner (*Phyllocnistis citrella*) increased infection during the flush periods (Gottwald et al. 1997). Susceptibility of *Citrus* spp. to leaf miner is a factor influencing canker disease incidence and development. The susceptibility level may vary with the type of citrus tissues. The susceptibility of leaves and fully developed greenwood was at a similar level following wounding inoculations. Concentrations of inoculum required to cause infection were lower for younger tissues compared with mature tissues (Gottwald and Graham 1992). Citrus tissue age rating at the time of infection was found to be a good predictor of disease rating, resulting from spray inoculation on fruits and leaves and also on fruits following wound

inoculation. Mature green stems were highly susceptible after wounding, similar to leaves, but buds and leaf scars showed the lowest level of susceptibility. These variations in disease development in different tissues of citrus might have different impact on citrus canker disease incidence and severity (Vernière et al. 2003).

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) has the potential to spread in commercial greenhouses, causing epidemics of tomato bacterial canker disease. When contaminated tools were used, the disease spread only to a limited distance (<4 plants) from infected plants, but touching symptomless tomato plants bearing guttation droplets prior to touching nearby plants spread the pathogen over longer distances within rows (>22 plants). The pathogen was exuded in large numbers in the guttation fluid of infected plants. The presence of *Cmm* in the guttation fluid was not influenced by inoculation procedures, leaf age, or the volume of guttation (fluid) droplets. Population size of *Cmm* and the incidence of epiphytic bacteria on leaflets were significantly higher in plants placed in the guttation-induction chamber than those kept in a growth chamber with high humidity, suggesting that exudation through guttation contributed to the formation of epiphytic populations on leaflets. The results indicated that this simple and environmentally friendly method of avoiding contact with plants during periods when they form guttation droplets could be adopted to significantly decrease the spread of bacterial canker disease in the greenhouse-grown tomato crops (Sharabani et al. 2013).

The ability of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), causal agent of potato ring rot disease, to infect crops grown in rotation with potato in Europe was assessed. The crop plant species were inoculated by stem end inoculation with suspensions of *Cms*, and the presence of the pathogen in the inoculated plants was detected using immunofluorescence cell-staining (IF) method. *Cms* could be detected in maize, wheat, barley, oat, bush bean, broad bean, rape, and pea, but not in onion or five cultivars of sugar beet tested. Larger populations of *Cms* were detected in stems (10^5 – 10^6 cells g^{-1} of tissue) than in roots ($\leq 10^3$ cells g^{-1} of tissues). *Cms* was isolated only from IF-positive stem samples of maize, bush bean, broad bean, rape, and pea, but not from roots. Of the 12 solanaceous weed plant species tested, only *Solanum rostratum* was found to be susceptible to *Cms* which persisted in large numbers (10^8 cells g^{-1} of tissues) in stems and leaves which showed visible symptoms of infection. Different weed species (14) present in potato fields were tested for the presence of *Cms*, using immunofluorescence colony-staining (IFC) and AmpliDet RNA techniques. *Cms* was detected in the roots but not in stems of *Elymus repens* growing through rotten potato tubers, and in some *Viola arvensis* and *Stellaria media* plants in which the pathogen was detected both in stems and roots. AmpliDet RNA procedure was more sensitive than IFC in detecting *C. michiganensis* subsp. *sepedonicus* (van der Wolf et al. 2005).

Pierce's disease (PD) caused by *Xylella fastidiosa* (*Xf*) occurred in severe forms on grapevines in the Texas Gulf Coast region in the USA. As the disease pressure was intense in this region, it was expected that there might be alternative host plant species functioning as potential sources of inoculum of *Xf* for grapevine orchards. Native and ornamental plant species (101) representing 40 families were indexed for the presence of Pierce's disease pathogen by employing ELISA, standard PCR, and quantitative real-time PCR (RT-qPCR) procedures. The pathogen *X. fastidiosa* was detected in 17 plant species by two or more diagnostic methods used in this investigation (McGaha et al. 2007).

9.2.1.3 Environmental Factors

Disease development depends on several different variables involving host, pathogen, and environment in addition to a set of predictors and a set of potential responses, and they are often correlated with each other. The number of variables has to be limited in order to simplify data analysis and interpretation of results. Principal component analysis (PCA) is a statistical procedure that allows the exploration of data structure and can address variable reduction and dimensionality. PCR allows the transformation of a set of original variables into a new set of uncorrelated variables known as principal components, which may provide highly concise information. Canonical correlation analysis (CCA) is a multivariate approach which may identify environmental variables that highly influence disease expression or disease variables that are highly influenced by the environment (Vernière et al. 2003). The influence of agricultural practices and weather variables has to be investigated to estimate the extent of disease incidence and rate of disease spread.

9.2.1.3.1 Effect of Agricultural Practices

Various agricultural practices are followed primarily to enhance the quality and quantity of the produce. However, some of them provide favorable conditions for development of crop diseases. The effects of two cultural practices on the incidence and spread of onion center rot disease caused by *Pantoea ananatis* were assessed. Use of mulch and irrigation has been reported to influence disease incidence by repelling insect vectors and water-splashing by overhead irrigation. Overhead sprinkler irrigation or drip irrigation as main plots and black plastic film, wheat straw mulch, or bare ground as subplots were tested in split-plot design. The type of irrigation did not have any significant influence on the incidence of onion center rot disease. Final disease incidence was greater in plants grown on black plastic and less for those raised on straw mulch (Fig. 9.2). Further, the standard commercial practice of using black plastic hastened the onset of center rot by c. 5 or 16 days in 2000 and 2001, respectively. In contrast, straw mulch delayed the onset of center rot disease by 15 days when compared with black plastic. In both years, the least amount of disease levels was observed in straw mulch. Final disease levels among mulch treatments could be primarily attributed to a shift in the onset of the epidemic and not changes in infection rates. Plants developing infections by *P. ananatis* immediately before harvest showed significantly higher levels of rot during storage despite passing grade at harvest. This latent infection was found to be a major constraint for quality control in onion industry, since it has been not possible to grade onions on external bulb appearance alone. The results indicated the need to train workers to rogue out infected plants before harvest or during harvest to reduce losses due to center rot disease in storage (Gitaitis et al. 2004).

9.2.1.3.2 Weather Variables

The interactions between a set of environmental and epidemic variables associated with expression of Asiatic citrus canker (Acc) disease caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) were studied. Moisture is not a limiting factor in the tropics and is present in different forms daily. The quantity of rain and relative humidity (RH) therefore showed little effect on disease development and symptom expression. The moisture provided a very conducive environment and fulfilled the most immediate requirement of *Xac* for proliferation. In the treatment where wounding preceded inoculation, pathogen cells were in direct contact with free water due to the prevalence of high RH.

Disease incidence was adequately represented by a single integrated variable – area under disease progress curve (AUDPC) – which took into account many of the characteristics associated with the other epidemic variables that were eliminated. AUDPC as the strongest dependent variable and combinations of the environmental parameters as independent variables were used in multiple regression analyses. The AUDPC offered a quantitative measure of symptom development. The disease as represented by AUDPC could be predicted by a minimum set of environmental variables. The age of tissues at the time of infection was a good predictor for disease resulting from spray inoculation on fruits and leaves, representing natural rain-splash deposition of inoculum. Temperature, denoted $T_{\min}$ or $T_{\max}$, was also a significant factor influencing disease development reflected by AUDPC. *Xanthomonas axonopodis* pv. *citri* (*Xac*) could grow at the temperature range of 6–36°C. At the minimum temperature of 6°C the pathogen growth was minimal, just being able to establish in plant tissues. The growth of *Xac* in planta resulted in lesion formation between 15 and 36°C. The optimum temperature for lesion development was approximately 30°C. The range of $T_{\min}$ and $T_{\max}$ was 15.5–23.5°C and 19.7–31.8°C in Tahiti lime and 12.2–22°C and 19.7–30.7°C in pineapple sweet orange plots, respectively. Relative humidity did not have a significant effect on *Xac* survival on leaves and leaf infection. Estimates of the quantity and availability of inoculum in the orchard and the ratio of young and mature tissues will be useful for determining an effective strategy for Asian citrus canker disease (Vernière et al. 2003).

9.2.2 Molecular Ecology and Epidemiology of Bacterial Diseases

The structure, host specificity, and phylogenetics of microbial populations associated with plant diseases may be understood better by comparing the DNA sequences of the target pathogen(s). The identity of the pathogen up to species level may be efficiently established by determining the sequences of specific genes. The number and types of resistance genes in natural populations of plant hosts may be determined by identifying the sequences associated with resistance (R) genes. Similarly, estimates of the number and types of effector proteins in a single bacterial pathogen, such as the number of gene products transferred into plant cells by the type III secretion system (TTSS) of *Pseudomonas syringae* pv. *tomato* DC3000, could be obtained (Collmer et al. 2002; Alfano and Collmer 2004; Chang et al. 2005).

The effector proteins may function as virulence proteins and also targets of plant disease resistance proteins. Genetic and pathogenic variation of *Xylella fastidiosa* (*Xf*), the xylem-limited bacterial pathogen of almond, grapevine, and several other crops, is an important factor affecting the epidemiology of diseases caused by this pathogen. The almond leaf scorch disease (ALSD) induced by *Xf* has become a serious threat to almond production in the San Joaquin Valley, California. Pure culture-dependent DNA polymorphism-based methods such as random amplified polymorphism DNA (RAPD) and random fragment length polymorphism (RFLP) technique were employed to differentiate the strains of *X. fastidiosa*. A conserved genomic region may be used for recognition of bacterial species. On the other hand, a single nucleotide polymorphism (SNP) within the conserved region had to be used for differentiation of genotypes below the species level. Based on SNPs in the 16S rRNA gene (16S rDNA), *Xf* strains (G- and A- types) were found to coexist simultaneously in the same infected almond orchard. The assumption that one genotype of *X. fastidiosa* caused ALS in one location might be erroneous. The epidemiological investigation has to consider the concept of mixed

genotype infections and modify disease management strategy to limit the incidence and spread of multiple genotypes with differing pathogenic potential, survival ability, and other biological characteristics (Chen et al. 2005).

Xanthomonas axonopodis pv. *allii* (*Xaa*), incitant of bacterial blight of onion (BBO) disease, also infects garlic, leek, and Welsh onion. Seedborne inoculum was considered to contribute to the development of BBO epidemic. The extent of seed contamination with *Xaa* in experimental plots was assessed and the relationship between disease status of leaves and inflorescences and seed infestation was investigated by employing molecular techniques. Seedborne inoculum associated with naturally contaminated seeds was likely to be the primary source of BBO outbreaks in the tropical environment, as indicated by this investigation. The onion plants in experimental plots, established in an area where no other commercial crops were present, were inoculated with a rare bacterial haplotype that has unique AFLP fingerprint. The haplotype had not been observed earlier at the experimental site or in its vicinity, so the occurrence of exogenous inoculum in the experimental plots could be quantified by AFLP analysis. Most of the strains of *Xaa* isolated from onion leaf or floral stem lesions had fingerprints identical to that of the inoculated LMG16528 strain, as indicated by the AFLP analysis. However, a single haplotype different from the LMG16528 strain was also found during the experimental duration of 2 years. This variant CFBP6369-like haplotype was detected in two to five foci per plot, making a total of 12 foci of infection. The application of nested PCR assay revealed the seed contamination with *Xaa* which could be ascertained by using the semiselective medium NCTM1. Both tests showed similar levels of seed contamination with *Xaa*. This indicated that seedborne *Xaa* population were composed, at least in part, of live cells. Seed contamination by *Xaa* was observed at rates of 0.01 and 0.05% only from plots with high disease incidence in 2004. The systematic AFLP analysis showed the presence of a haplotype different from the inoculated haplotype in all experimental plots. The proportion of strains recovered from diseased plants that were genotyped by AFLP represented 18.4% of the total number of diseased plants in 2003 and 3.5% in 2004, due to the high levels of disease incidence in that year. This investigation demonstrated that AFLP analysis was very effective in identifying exogenous inoculum in epidemiological studies at a microgeographic scale, and stressed the need for an accurate assessment of the biological significance of the putative inoculum sources for a sustainable disease management system. The usefulness of a genotyping procedure involving application of AFLP and rep-PCR assays was demonstrated for assessing the epidemiological significance of multiple inoculum sources on a pathosystem involving plant bacterial pathogen for the first time (Humeau et al. 2006).

Isolates (42) of *Xanthomonas axonopodis* subsp. *citri* (*Xac*), causative agent of Asiatic citrus bacterial canker (CBC) disease, were collected from diverse *Citrus* spp. across 17 different citrus orchards in Argentina. Molecular, biochemical, and pathogenicity tests were performed to assess the extent of variations among the isolates. Based on the analysis of genome-specific *X. citri* markers and DNA polymorphisms repetitive elements-based PCR assay, all the 42 isolates were identified as *X. citri*. However, one isolate that shared >90% genetic similarity to the reference strain *X. citri*^{A^T} exhibited host range specificity. This strain, designated *X. citri*^{A^T} and deficient in xanthan production, induced an atypical, noncancerous chlorotic phenotype in *Citrus limon* and *C. paradisi* and weak cankerous lesions in *C. aurantifolia* and *C. clementine* leaves. Suppression of canker development in *C. limon* was concomitant with an oxidative burst.

It was suggested that bacterial factors other than xanthan might be involved in triggering the defense responses (Chiesa et al. 2013).

“*Candidatus Liberibacter asiaticus*” (CLA), the causal agent of citrus huanglongbing (HLB) disease, is a phloem-limited bacterial pathogen and its principal vector is the Asian citrus psyllid *Diaphorina citri*. In order to have a better understanding of virulence mechanisms of CLA, a procedure was developed to detect live bacterial populations of CLA in the citrus and vector tissues using a novel DNA binding dye, propidium monoazide, in conjunction with real-time PCR (PMA-qPCR). Chinese box orange (*Severinia buxifolia*), a common ornamental plant in Florida, could be a host for both CLA and *D. citri*. The changes in live CLA populations in graft- and psyllid-transmitted Valencia orange (*Citrus sinensis*, Valencia) and *S. buxifolia* plants were monitored by PMA-qPCR procedure. The results indicated that the live CLA population was significantly lower in the months of December, January, and February than in the rest of the year in both host plant and psyllid vector. Different patterns in total bacterial population growth of “*Ca. L. asiaticus*” depending on the growth of the host plant were observed (Hu et al. 2014).

9.3 Epidemiology of Virus Diseases

9.3.1 Dynamics of Host–Virus Interactions

Epidemiology is an aspect of plant diseases dealing with different dynamic factors influencing disease incidence, severity, and spread. An epidemic is the culmination of the major interacting components: the pathogen, host plant, and environment. In the case of most virus diseases, the additional component of vector(s) is also involved, making the interactions more complex. Except for a few, most viruses have one or more species of vectors which show varying vector efficiency and specificity with the virus(es) transmitted by them. The type of relationship of the virus with its vector, namely nonpersistent (styletborne), semipersistent (circulative), or persistent (propagative), is determined by the virus. Structures of virus disease epidemics show different and complex patterns which may vary with the virus characteristics, relationship with vector, geographical location, and weather.

The complex interactions between viruses, vectors, host plants, and the environment may be highlighted by appropriate epidemiological models. However, the value of the model's predictions depends on the quality of the data from which the model is constructed and the validity of the assumptions made. Models may be classified according to their main objectives as conceptual (also known as explanatory or analytical), predictive, or descriptive (Kranz and Royle 1978). Predictive models describe and forecast occurrence and severity of epidemics, identify effective control measures, and provide risk assessments (van Maanen and Xu 2003). Statistical modeling techniques for predicting epidemics involve calculations such as simple, linear regression, and multivariate analysis time series. Statistical models are empirical and describe relationships between factors by equations without theoretical foundation, and are correlative if the descriptions are based on theoretical relationships. Predictive simulation models are usually dynamic and may predict changes in an epidemic over time. Simulation models can also be termed deterministic if they make definite predictions for quantities such as virus incidence without any associated probability distribution (Miyai 1993; Bertchinger 1997).

Extensive knowledge of the factors (virus, host, vector, and environment) and the dynamics of these factors have to be incorporated into the predictive model. The development of an epidemic commences with the introduction of the virus into the field or a geographical location via various modes. In addition, the nature and disposition of the primary infection source(s), mode of spread of the virus from infected plant to healthy plant within a crop, survival of the virus in the absence of primary (crop) host plant, type of vector and its relationship with the virus, ability to survive in the soil in a free state or in plant debris/volunteer plants, and other relevant information have to be gathered. Furthermore, some of the viruses are seed- and/or pollen-borne and the propagules may carry the viruses asymptomatically. Viruses infect annuals and perennials, wild relatives and weeds, and crops grown in rotation in the same farm. Virus epidemics have been observed under varying crop-growing conditions ranging from monocultures to mixed cultivation, rainfed to irrigated, intensive high input to extensive low input, under controlled greenhouse conditions, and hydroponics. Sources of virus infection may be internal or external (brought from outside the field concerned) (Jones et al. 2010).

9.3.2 Quantitative Epidemiology

Factors influencing the spread of virus diseases from infected plants are investigated. The host plant may contract virus inoculum from germinating infected seeds which introduce the disease into the field. The host plant is static, whereas viruses carried by infected seeds and/or viruliferous vectors are dynamic. The spread of the virus may depend on the characteristics of the virus itself. Virus strains that become systemic rapidly are more easily spread by their vectors compared with the strains that spread slowly in the infected plant or that cause necrotic lesions. The spread of viruses has two dimensions – spatial and temporal – considered in quantitative epidemiological investigations.

The spatial distribution of infected plants may indicate whether the disease spread from internal sources or external sources. The appearance of infected plants in groups may indicate spread of the disease from internal sources such as plants growing from infected seeds or infected volunteer plants left in the field after harvest. In contrast, the random distribution of infected plants may suggest the possibility of disease spread from external sources by the vectors carrying the inoculum from nearby plants or plants that are far away. The wind direction and speed also influence spatial distribution of virus diseases, especially when vector movement is significantly altered by wind. The spatial distribution patterns of *Tomato mosaic virus* and its vector *Myzus persicae* were investigated. The pattern of appearance of infected plants varied with time. In the initial stages, the diseased plants were randomly distributed; with increasing time, a clustering of infected plants followed by an even distribution towards the end of crop growth was observed. On the other hand, the presence of aphids on adjacent plants was noticed, indicating that alate aphids were mostly involved in the spread of tomato mosaic disease (Zhang and Wang 2002).

The disease progress with time indicates temporal distribution of the disease(s) under investigation. When the cumulative incidence of the disease is plotted against time, the curves generally tend to be sigmoid. However, within the same overall pattern, differences due to locations, seasons, and disease onset, rate, duration, and total amount of spread may be observed. A rapid increase in diseased plants after the initial appearance

of disease may be observed followed by a decline in new infections, possibly because of unfavorable weather conditions or a reduction in the vector population or development of adult plant resistance to the viruses. Young plants are generally more susceptible to viruses and the level of susceptibility decreases as the plants become older. A differential increase in the populations of vectors, when two or more species of vectors are involved in virus spread, becomes an important factor affecting temporal distribution of diseases. Virus transport by different aphid vector species occurs to different extents, as in the case of *Soybean mosaic virus*-soybean pathosystem. Different aphid species with different flight activities transmit the viruses, increasing the complexity of the epidemiological conditions (Irwin 1999). The activity of aphid species associated with lettuce and transmission of *Lettuce mosaic virus* (LMV) was studied in Spain. Aphid flight during spring and autumn growing seasons was monitored. Populations of *Aphis spiraeicola* and *Myzus persicae* varied over different seasons and also in different areas in the country. Differences in the populations of aphid species may affect the rate of virus disease spread (Kuchne 2004).

The pattern of current season spread of *Potato virus Y* (PVY) was studied in 11 potato fields planted with six different cultivars in New Burnswick, Canada during the 2009–2010 growing season. In all, 100 plants/field were selected and the current season spread of PVY infection was monitored using ELISA and real-time RT-PCR assay. Average PVY incidence increased from 0.6% in 2009 and 2% in 2010 in leaves to 20.3% in 2009 and 21.9% in tubers at the time of harvest. In individual fields, PVY incidence in tubers reached as high as 37% in 2009 and 39% in 2010 at the time of harvest. Real-time RT-PCR assay detected PVY in greater number of leaf samples compared with ELISA test. Real-time RT-PCR also detected PVY in a larger number of samples from growing tubers than in leaf samples collected from the same plant at the same time. PVY incidence determined from the growing tubers showed a significant positive correlation with PVY incidence of tubers after harvest. The results suggested that when the PVY incidence was low the crop might be top-killed immediately to secure seed market; when the incidence of PVY was high, the tubers might be allowed to develop further for table or processing purposes (Fageria et al. 2013).

9.3.3 Molecular Ecology and Epidemiology of Virus Diseases

Over the past decades, molecular ecology including epidemiology has emerged as a fast expanding branch of biological sciences. The need for an interdisciplinary approach to unravel and obtain solutions to intricate problems in biology, ecology, epidemiology, molecular evolution, and genetics was realized. Molecular techniques have been applied to study complex patterns and the processes of biological diversity at different levels of organization from genes to organisms, populations, species, and ecosystems, with a focus on genomes of organisms. Molecular ecological principles have the potential to shed light on unresolved questions raised by earlier studies. Molecular epidemiology examines the processes that may result in a successful state of disease in host plant, facilitating identification of a weak link in the biotic and molecular interactions or process that may be useful to develop effective systems for plant disease management. Various crops are affected by different viruses and other microbial pathogens, each one requiring a specific set of conditions for population buildup to reach levels required for infection and spread in different geographical locations. A basic requirement for epidemiological investigations is the availability of efficient, sensitive, and reliable diagnostic

technique(s) that can provide results rapidly. When a virus or its variants appear at a location, it should be possible to establish its identity and also quantify the population at different stages of disease development as early as possible. Information about the viral genomics may provide a range of tools for choosing the appropriate technique. Nucleic acid-based techniques have been demonstrated to be preferable to gather precise information about the viral genomes required for identification and differentiation of target virus and its strains (Narayanasamy 2008).

9.3.3.1 Molecular Biology of Virus Infection

The DNA microarray technique allows the resolution of specific hybridization events between nucleic acid in a sample and known nucleic acid probes bound to a solid phase. Nucleic acid from the sample is extracted and labeled. The labeled nucleic acid (target) is then hybridized to the array which is scanned to resolve the presence of target nucleic acid. Potato viruses and cucurbit-infecting tobamoviruses were detected either individually or in mixed infections by array technique. The sensitivity levels of array and ELISA techniques were similar (Boonham et al. 2003; Lee et al. 2003). Further, microarrays could detect a range of virus isolates and strains and discriminate them at species level by employing synthetic oligonucleotide-based arrays. The serotypes and subgroups of *Cucumber mosaic virus* (CMV) that differed by only 8% in an amplified PCR product larger than 700bp were discriminated by employing an oligonucleotide-based assay (Deyong et al. 2005).

Investigations of the association of satellite viruses/RNAs laid the foundation for the development of molecular epidemiology. Experiments on epidemiology may commence with models within individuals, predicting infection levels based on the particular gene(s) and then expand on these to predict infection rates in plant populations based on gene expression rates in individuals. The genetic structure of populations of *Cucumber mosaic virus* (CMV) satellite RNA (sat-RNA) and its evolution were analyzed during the course of a CMV epidemic in tomatoes in eastern Spain. RNase protection assay (RPA) was employed to characterize 62 variants of CMV-sat-RNA from three epidemic episodes during 1987–1991. Sixty haplotypes were differentiated by RPA patterns. High genetic variability of CMV-sat-RNA resulted in very heterogeneous populations. The high diversity of the population was maintained through time by the continuous generation of variants by mutation, counterbalanced by negative selection. Because of this, the replacement of certain haplotypes might occur from year to year. The sequential accumulation of mutations in CMV-sat-RNA may result in fast genetic divergence, possibly reaching the upper permitted threshold level (Aranda et al. 1993). In a further study, molecular epidemiology of CMV and its sat-RNA in locations where CMV was endemic in vegetable crops was investigated. The genetic structure of CMV populations (about 300 isolates) representing 17 outbreaks (subpopulations) in different crops, regions, and years was compared. The RPA for genetic analyses of CMV isolates was performed using cRNA probes representing RNA-1, RNA-2, and two ORFs in RNA-3. The results indicated that heterologous genetic combinations were not favored. About 30% of CMV isolates were supporting sat-RNA. Molecular analyses of CMV-sat-RNA isolates revealed high genetic diversity due to the accumulation of point mutations and to recombination. Further, CMV-sat-RNA might have spread epidemiologically on the existing virus population from an original reservoir in eastern Spain (Garcia-Arenal et al. 2000).

The diversity of genetic characteristics may be due to recombination and mutation in viral nucleotide sequences. Natural variations in the strains of *Citrus tristeza virus* (CTV) have been studied. A sudden outbreak of CTV in Israel was attributed to a new form of the virus. Uneven distribution of genomic variants of CTV was observed. Individuals of *Aphis gossypii* could transmit the new variant of CTV occurring in Israel. As citrus plants were continuously exposed to aphids which might carry different variants at different periods of time, changes in CTV population might be possible. It was presumed that continuous genetic recombination might take place, resulting in divergence of nucleotide sequence of CTV with varying time and space (de Orso et al. 2000). The passage of variants through host plants may lead to the selection of variants that find suitable environment in the infected host plant species. Evolutionary analysis of genetic variation in CTV isolates after host passage revealed that genetic variability occurred in two genes (*p18* and *p20*) in two groups of CTV isolates. One group of isolates (T385, T318, T317, and T305) was derived from a Spanish source by successive host passage. On the other hand, the group of isolates (T388 and T390) was obtained using aphid vectors that transmitted these isolates from a Japanese source. Sequences derived from the severe isolates T318, T305, and T388 clustered together and differed from those of mild or moderate CTV isolates, as indicated by phylogenetic analysis, regardless of the geographic origin. Molecular variance based on hierarchical analyses indicated that up to 53% of the total genetic variability in *p18* and up to 87% variability in *p20* might be due to differences in the pathogenic potential (virulence) of the isolates and different selection forces might act differentially on isolates and their genes involved in pathogenicity and other characteristics (Sentandreu et al. 2006).

In another investigation, the structure and genetic diversity of a California CMV population were studied by using single-strand conformation polymorphism (SSCP) and nucleotide sequence analyses of genomic regions of 2b, CP, MP, and 3' nontranslated region of RNA-3. Low genetic diversity and the presence of one to three predominant haplotypes for specific genomic regions were revealed in California CMV population. Phylogenetic analysis suggested that genetic exchange by reassortment contributed to the evolution of the CMV population genetic parameters, whereas the distribution of synonymous and nonsynonymous mutations indicated that different coding regions and even different parts of coding regions were under different evolutionary constraints, including a short region of the *2b* gene (Lin et al. 2004).

9.3.3.2 Molecular Biology of Virus Transmission

The development of virus disease epidemics depends on several factors, of which transmission of the virus from infected plant to healthy plants is a vital factor. The frequency of transmission and population and efficiency of vectors involved in the transmission may influence the development of epidemics. As viruses cannot infect plants independently by themselves, they depend on plant-feeding organisms such as insects, mites, nematodes, or fungi which function as agents of transportation from plant to plant. Three steps in transmission of viruses by vectors have been recognized: acquisition, retention, and inoculation of the virus by the vector. The mechanisms of virus–vector interaction (relationship) may vary widely depending on the nature of the virus. Most of the viruses transmitted through seeds are noncirculative (mechanical or nonpersistent) viruses. In these viruses, two distinct molecular strategies regulate the

virus–vector interaction: (i) the capsid strategy in which the virus coat protein (CP) interacts directly with the vector; and (ii) the helper strategy in which at least one nonstructural viral gene product, the helper component (HC), is required for effective transmission of the virus (Foissart et al. 2002).

The characteristics of viral coat protein (CP) determine the vector transmissibility of viruses belonging to genus *Cucumovirus*, whereas the vector transmissibility of the viruses belonging to genera *Potyvirus* and *Caulimovirus* depends on the characteristics of both CP and the virus-encoded nonstructural accessory factors such as helper component (HC). The CP of *Cucumber mosaic virus* (CMV) has multiple functions such as the encapsidation of viral genomes involved in the systemic movement, host range, and determinant of vector transmission. Two domains in the CP amino acids are involved in aphid transmission of CMV (Palukaitis et al. 1992). Variations in the amino acid sequences of CP may influence the vector specificity, while vector efficiencies for CMV by one aphid species may not have any effect on transmission rates with another aphid species (Perry et al. 1998).

The ease of binding and/or release of virions in the mouthparts or foregut of the vector or instability of virus particles during the transmission of noncirculative viruses was assessed by Lopez-Moya (2002). A bridge hypothesis was proposed to explain the helper strategy adopted by the genera *Potyvirus* and *Caulimovirus*. The nonstructural HC protein molecules may be capable of engaging two types of interactions. The HC protein molecules interact with the virus CP on one side and with a putative receptor present in the mouthparts of the vector on the other side, creating a reversible “molecular bridge” allowing virus retention in the vector. The loss of vector transmissibility may possibly be due to either the mutations in the HC rendering it nonfunctional or defects in the CP abolishing of HC–CP interaction. This phenomenon was designated HC-transcomplementation involving sequential acquisition of HC and virus particles. The HC acquired by the aphid assisted in the transmission of virus particles present in the same cell, in other cells, or even other host plants probed by the vector (Foissart et al. 2002).

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10

Crop Disease Management

Exclusion of Pathogens

Crop disease management is primarily based on three principles, namely: exclusion of pathogens, eradication of sources of inoculum of pathogens, and immunization of host plant for enhancement of resistance to pathogens. Information on pathogen biology, infection processes, and epidemiological factors may provide the basis for development of strategies for effective management of diseases transmitted through seeds and propagules. As soon as the incidence of a disease is observed, the basic step for development of disease management system is the detection and identification of the pathogen(s) involved in the disease by employing rapid, reliable, sensitive, and specific diagnostic techniques. The presence of microbial pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses, and viroids – in/on seeds and propagules has to be detected and identified precisely. Use of disease-free seed/propagules is the basic requirement for successful crop cultivation to achieve potential yield of cultivars both quantitatively and qualitatively.

10.1 Health Status of Seeds and Propagules

It is well known that seeds infected by microbial pathogens are potential sources of primary inoculum, introducing and spreading the diseases into near and distant locations efficiently. Pathogens can multiply rapidly in the plants growing from infected seeds and secondary spread may lead to the development of epidemics when optimal environmental conditions or natural biological vectors of the pathogens are available. In addition to the reduction in yield from infected seed lots, the quality of produce may be substantially affected. As a result of removal of restrictions in the import of seeds, propagules, and plants following international agreements set by the World Trade Organization, the frequency of pathogen introduction to various geographical locations has dramatically increased. The need to ensure a supply of disease-free seeds and propagules has been emphasized to prevent the introduction of new or more virulent strains of the pathogen through them. Certified seeds pass through tests at different stages prior to sowing, during crop growth, at harvest, and in storage of seeds and propagules.

The management of plant diseases through exclusion consists of three phases: sanitation, quarantine, and certification. Sanitation programs aim at recovering healthy plants from selected local cultivar(s). The recommended procedure for sanitary

programs involves the selection of mother plants/trees of local cultivars, indexing mother plants, recovery of pathogen-free plants by shoot-tip grafting (STG) *in vitro*, indexing micrografted plants, evaluation of genetic characteristics of healthy plants generated, and maintenance of healthy source plants. Quarantine programs are entrusted with the responsibility of preventing/avoiding the introduction of new pathogens through seeds and propagules. The imported plants, seeds, and propagules may be kept under observations or indexed using appropriate diagnostic methods under controlled greenhouse conditions. The certification programs are responsible for producing seeds and propagules or oversee the agencies given this responsibility. The sanitary status and trueness-to-type on the initial material is maintained during the process of commercial propagation at the nurseries. They are given the authority to control the quality of nursery plants. These programs have to enforce legal regulations for different steps of nursery operations and periodic indexing and inspection. A certification program should be established in all countries for the production of high-quality and healthy nursery plants (Navarro 1993).

10.1.1 Certification of Seeds and Propagules

The freedom of seeds and propagules from diseases, insects, and weeds has to be ensured to obtain high-quality grains and other food materials. Certain quality standards such as genetic purity, percentage of seed germination, and seedling vigor are also applied. The International Seed Testing Association (ISTA) and Official Seed Certifying Agencies (AOSCA) have suggested minimum seed certification standards. Certification procedures aim to maintain genetic purity and quality of seed production in the field and during harvest, processing, storage, and inspection of the commercial seed lots in the market. Microbial pathogens significantly reduce the quantity and quality of the seeds and propagules. Standards (tolerance limits) have therefore been fixed for adoption by seed producing agencies. The standards are based on aggressiveness and potential of the pathogen to cause epidemics, resulting in the adverse impact of the pathogen(s) on the quality and quantity of the produce.

10.1.1.1 Application of Diagnostic Techniques

Various diagnostic techniques have been applied for the detection and identification of oomycetes, fungi, bacteria, phytoplasmas, viruses, and viroids in seeds and propagules. Traditional methods, namely direct seed examination, plating, or grow-out assays, have been found to be time-consuming, require a large space, and often the results are inconsistent. Immunoassays have been shown to be more efficient and sensitive than conventional methods. However, they have some limitations such as nonavailability of antisera, and lesser sensitivity for detection of many viruses which are present in low concentrations and unevenly distributed in infected plants (which in some cases may remain asymptomatic). Nucleic acid-based techniques have higher levels of sensitivity, specificity, and reliability and can provide the results rapidly and reproducibly. However, the major drawback of the immunoassays and nucleic acid-based techniques is their inability to differentiate live and dead propagules of fungal, bacterial, and phytoplasmal pathogens. The need to obtain results rapidly and cost-effectively may determine the selection of tests(s) for detection of pathogens and subsequent selection of healthy seed and propagules (Chapters 2, 5 and 7).

Plants are exposed to numerous plant pathogens, but they are susceptible to only a few pathogens. Resistance of plants to pathogens is more common than susceptibility. The frequency of transmission of microbial pathogens from mother plants to seeds and from seeds to seedlings may vary widely depending on the cultivar–pathogen combinations. Cultivars differ in their resistance to seed transmission and the infected seeds may or may not show symptoms of infection. Diagnostic techniques should be sensitive enough to detect the pathogen in seed lots, especially in the case of viruses and viroids. Soybean plants/seeds may be infected by different pathogens causing anthracnose (*Colletotrichum lindemuthianum*), soybean bacterial blight (*Pseudomonas syringae* pv. *glycinea*), Phomopsis seed decay (*Phomopsis longicolla*), and soybean mosaic disease (*Soybean mosaic virus*). These pathogens did not induce any visible symptoms on seeds and in the plants until they became older. Field inspections may be useful in eliminating the infected plants and rejecting the seed lots from fields with high levels of seed infection (Sinclair and Backman 1989). The importance of many diseases that were previously responsible for heavy losses has been addressed by adopting efficient certification programs which restricted the incidence and spread of those diseases effectively. Certification standards have been prescribed based on several factors such as the relative role of seedborne infection in disease development, perpetuation of seedborne inoculum by other means, factors affecting seed transmission, economic loss due to the target pathogen(s), planting area of the crop, influence of seed treatment on seedborne infection, and treatments of seed during processing procedures (Agarwal and Sinclair 1996). Certification standards are essential to determine seed and plant health status for different diseases infecting different crops. Different certification standards are enforced by different countries to restrict/prevent the introduction of pathogens through seeds, plants, and planting materials, based on the perceived importance/potential risks of allowing the plant materials. The standards may be altered as and when necessary to make the certification programs more efficient and comprehensive. The certification standards for the pathogens carried in or on the seeds have been suggested for adoption in the UK (Rennie 1993).

Certification programs are usually applied to maintain genetic purity primarily for breeders' seeds through successive multiplication. Detection methods have been employed to assess the extent of seed infection by plant pathogens and to reject the seed lots with levels of infection above the prescribed standards. Seed testing for seedborne pathogens in seed certification programs was emphasized because of the substantial quantitative and qualitative losses caused by the plant pathogen concerned when uncertified seeds with greater levels of infection were used. Effective control of wheat and barley loose smut disease could be achieved through seed certification programs based on the results of diagnostic assays in India, UK, and Sweden (Agarwal 1983; Rennie 1993). Different levels of disease incidence under field conditions are fixed for certification of seeds. Other criteria included are seed production isolation, field inspection at required intervals, laboratory evaluation by the embryo count method, and seed treatment with recommended fungicide. For loose smut, the standard for field approval for wheat and barley was 1 smutted ear per 10,000 ears. In the case of susceptible cultivars of wheat and barley, the permissible level was not more than 1 smutted ear in 2000 in commercial crops and 1 in 5000 in crops raised for multiplication. Field assessment of loose smut disease incidence is unreliable, unless the crop is inspected at the appropriate time (heading stage) and adequate ears are taken for counting diseased and healthy ears. Later, the embryo count test permitting 0.2% infection

was preferred to field assessment of loose smut infection (Doling 1966; Neergaard 1977). In India, seed lots with seed infection up to 0.5% (assessed by seed embryo test) were certified without fungicide treatment; whole seed lot with infection levels of 0.51–2.0% had to be treated with fungicide for certification. Adoption of this standard resulted in significant reduction in loose smut incidence in wheat (Agarwal 1983). In Australia, seed lots of *Lupinus angustifolius* with >0.5% infection by *Cucumber mosaic virus* were not permitted for sowing (Jones and Proudlove 1991).

Benefits of strict enforcement of certification standards have also been evident in the reduced disease incidence in other crops. In bean (*Phaseolus vulgaris*), bacterial blight disease caused by *Pseudomonas syringae* pv. *phaseolicola* infection up to 0.005% was permitted under field conditions. Under laboratory conditions however, zero tolerance in seeds using the seedling infection test with leachates from 2.27 kg of seeds was applied for this bacterial disease (Copeland and Adams 1975). Dramatic reduction in losses in yield could be achieved by using *Lettuce mosaic virus*- (LMV-) free seeds in the Imperial Valley, CA, USA. Seed lots without a single LMV-infected seed were grown in insect-free greenhouses, followed by careful inspection to rogue out all infected seedlings. Seeds from the healthy plants were certified after grow-out infectivity tests. The certified seed lots were distributed to growers only when they did not have a single infected seed in 30,000 seeds. In Europe, the tolerance limit was fixed at 0.1% seed infection (Marrou et al. 1967; Grogan 1980). Seed certification was instrumental in reducing the incidence and severity of *Barley stripe mosaic virus* (BSMV) disease. Immunoassays were employed to detect the presence of BSMV in barley embryos. Foundation, registered, and certified seeds were tested rigorously to produce BSMV-free seeds by employing sodium dodecylsulfate-disk test/latex flocculation test. The certification program saved the crops from losses worth millions of dollars (Carroll 1983).

Phaseosphaeria nodorum (anamorph *Stagonospora nodorum*), causing glume blotch disease in wheat, barley, and oats, is transmitted through seeds. The extent of seed transmission of *P. nodorum* was 70% or more on the coleoptiles from germinated winter wheat seeds which might be exposed above the soil. Transmission of the pathogen from infected seed was also noted on the first leaves at frequencies of 2–37%. The infected wheat seeds were likely to be the primary sources of inoculum of *P. nodorum* (Shah and Bergstrom 2000). Barley net blotch disease caused by *Drechslera teres* was shown to be seed-transmitted. Transmission of the pathogen from infected barley seeds to plumule was up to 18.8% with mean values of 14.5% under glasshouse conditions. In the absence of other sources of inoculum of *D. teres*, plumule infection after seedling emergence indicated that the infected seeds formed the primary sources of inoculums, resulting in higher levels of plumule infection. The alarm threshold for net blotch disease control was determined to be 60% for incidence of disease on leaves (Carmona et al. 2008).

Seedborne obligate pathogen *Plasmopara halstedii*, causative agent of sunflower downy mildew disease, is difficult to isolate and to study its biological characteristics. The quantitative polymerase chain reaction (PCR) assay was found to be specific and sensitive in detecting the presence of 1 infected seed in a sample of 1000 seeds. The assay could provide consistent results and the procedure was adaptable to routine analysis of seed samples (Ioos et al. 2012). Use of disease-free seeds is the most effective strategy for the management of rice grain rot disease caused by *Burkholderia glumae*. The reliable and sensitive SYBR Green real-time quantitative diagnostic assay was therefore applied for the detection and identification of *B. glumae* in rice seeds.

This test not only detected *B. glumae* efficiently, but also differentiated *B. glumae* from 6 other species of *Burkholderia* and 18 other reference pathogenic bacterial species. The SYBR Green real-time PCR was highly sensitive and it was possible to detect the pathogen even at a concentration of 1 CFU/reaction. The diagnostic technique has the potential for detecting the pathogen present in very low concentrations, facilitating the elimination of infected seed lots to achieve disease-free seed stocks (Kim et al. 2012).

Several diagnostic tests based on biological, biochemical, immunological, and nucleic acid characteristics of the pathogen(s) have been developed for the detection of seed- and propagule-borne pathogens and have different levels of sensitivity and specificity. Further, they are only able to detect a single pathogen in seed or propagules. This may lead to longer periods to obtain results when the plant materials are infected by two or more pathogens. To save time, labor, and cost of testing, it is preferable to choose technique(s) that have the potential to detect multiple pathogens present in the seeds or propagules. Barley crops are infected by *Pyrenophora graminea* and *P. teres* which are seedborne. DNA primer sequences were designed based on the unique sequences of these pathogens and were used to detect the target pathogens. PCR was performed in a Lightcycler with real-time measurement of the product using a fluorescent dye. *P. teres* infection was quantitatively assessed and the assay provided the results within 48 h. This rapid seed health testing technique provided the results rapidly, facilitating application of disease management strategies to restrict disease incidence by seed treatment with fungicides (Thomas et al. 2000). The presence of *Pyrenophora teres* and *Rhynchosporium secalis* in barley seeds was detected by employing a PCR-based assay using species-specific primers. The PCR primers RS1-RS2 and RS8-RS9 were able to detect *R. secalis* in barley seeds and leaves, whereas the primers PTT-F/PTT-R and PTM-F/PTM-R could detect *P. teres* in barley seeds effectively (Gubis et al. 2004). *Fusarium circinatum*, incitant of pitch canker disease affecting many *Pinus* spp., was detected using a real-time PCR assay. The real-time PCR assay was found to be highly specific and more sensitive than conventional PCR format. The real-time PCR assay could detect *F. circinatum* in samples containing 1 infected seed among 1000 seeds, preventing long-distance movement of the pathogen (Ioos et al. 2009).

A multiplex PCR assay was developed using probe color in real-time PCR analysis to detect *Tilletia indica*, the quarantinable Karnal bunt pathogen which has significant impact on the international wheat trade. A single-tube five-plex fluorescent assay was employed to detect and identify five different *Tilletia* spp. This fluorescent assay could detect even one spore of *T. indica* and therefore bypass the germination step which is mandatory for testing seeds/grains for the presence of seedborne fungal pathogens. This protocol was found to be effective and economically sustainable because of the high specificity and sensitivity of the assay, requiring less labor and reagents (Tan et al. 2009). Magnetic capture hybridization (MCH) in combination with multiplex real-time PCR technique was developed for simultaneous detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*), causative agent of bacterial fruit blotch (BFB) disease, and *Didymella bryoniae*, incitant of gummy blight disease in cucurbit seeds. The comparative frequencies of detection of *Aac* for direct real-time PCR assay and MCH real-time PCR formats were 25% (1 of 4 samples) and 100% (4 of 4 samples) at the same pathogen DNA concentration, respectively. However, a combination of MCH and real-time PCR procedures did not improve the sensitivity of detection of *D. bryoniae* relative to the direct real-time PCR format using seed washes from infested cucurbit seed. The MCH real-time PCR procedure could be effectively applied for the detection of pathogens present

in watermelon and melon seed samples ($n = 5000$ seeds/sample) in which 0.02% of the seeds were infested with *Aac* and also at the same level (0.02%) by *D. bryoniae* (Hay et al. 2009). Likewise, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) and *Pepino mosaic virus* (PepMV) could be detected in tomato seeds. The combination of MCH and multiplex real-time PCR had a detection threshold of 10^5 cells for *Cmm* and 10^4 fold dilution of total RNA extracted from infected tomato tissues. The results showed that MCH facilitated the simultaneous detection of *Cmm* and PepMV by multiplex real-time PCR format (Johnson and Walcott 2012).

The extent of seed transmission and endophytic activity of the seedborne bacterial pathogen *Pseudomonas fuscovaginae*, causal agent of rice seed rot disease, were investigated using a rifampicin-marked strain of *P. fuscovaginae* (*Pf^R*). Rice seeds with five different levels of contamination, namely 0, ≤ 25 , ≤ 50 , ≤ 75 , and ≤ 100 , with rifampicin marked strain were tested. The relative seedborne bacterial concentration increased significantly with increasing levels of seed lot infection/discoloration. The *Pf^R* strain was recovered from all growth stages of rice plants, the maximum recovery being at seedling stage. Drastic reduction in bacterial cell recovery was observed at 75 days after sowing (das) and at milk stage (100 das). Crushed discolored seeds contained a greater bacterial population than the noncrushed discolored seeds. The use of clean or healthy-looking seed is not an assurance of disease-free seed, since low levels of bacterial pathogen cells could be detected in visibly clean seeds. Application of sodium hypochlorite (5%) for seed disinfection reduced the bacterial population present externally. However, endophytic bacterial contamination persisted since the pathogen *Pf^R* strain could be recovered from rice plants up to milk stage. The disease-free nature of the rice seeds therefore has to be ensured by treatment with effective chemicals (Adorada et al. 2015).

The effectiveness of seed health testing depends on a test that can provide timely sensitive and broad-spectrum detection of all genetic variants of a pathogen or, in some cases, of several different pathogens infecting the same crop. Soybean seeds are predisposed to infection by fungal pathogen *Phomopsis* spp. because of infection of soybean plants by *Soybean mosaic virus* (SbMV). Both SbMV and *Phomopsis* spp. were found to be associated with soybean seed coat mottling. Seed infection with *Phomopsis* spp. was determined by isolation and SbMV accumulation was assessed by enzyme-linked immunosorbent assay (ELISA) and nucleic acid hybridization techniques. *Phomopsis* spp. could be isolated from four categories of seed mottling, with infection percentage increasing with increasing levels of mottling. On the other hand, SbMV was detected in both mottled and nonmottled seed coats, with generally higher levels of SbMV in mottled seed coats. However, the relationship of SbMV concentrations with seed coat mottling was inconsistent across cultivars and years (Koning and Tekrony 2003). A sensitive, reverse-transcription (RT-) PCR-based qRT-PCR was developed for broad-spectrum detection of both serotypes of *Squash mosaic virus* (SqMV) that could be incorporated into the simultaneous detection of three pathogen types in a single PCR reaction. SqMV RNA was converted into cDNA using reverse transcriptase prior to multiplexing to stabilize the viral template, which was then mixed with two other DNA templates of *Acidovorax avenae* subsp. *citrulli* (*Aac*), causative agent of bacterial fruit blotch (BFB), and *Didymella bryoniae* (DB), incitant of gummy stem blight disease. A generic plant nucleic acid extraction procedure was also developed to facilitate seed health testing for cucurbit seeds. Nucleic acids extracted from seed samples gave strong signals for each of the three target pathogens (a fungus, a bacterium, and a virus)

infecting cucurbits in a multiplex qPCR format. The ability to use a common general nucleic acid extraction procedure with subsequent PCR to detect three kinds of plant pathogens formed the foundation for a universal system for cucurbit seed health testing (Ling et al. 2011).

Stone fruits are infected by several economically important viruses belonging to different genera: *Apple mosaic virus* (ApMV), *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), and *American plum line pattern virus* (APLPV) belonging to *Ilarvirus*, *Plum pox virus* (PPV) belonging to *Potyvirus*, and *Apple chlorotic leaf spot virus* (ACLSV) belonging to *Trichovirus*. Two viruses *Apricot latent virus* (ApLV) belonging to *Feavirus* and *Plum bark necrosis stem pitting-associated virus* (PBNPaV), belonging to *Ampelovirus*, have also been reported to infect stone fruits. A sensitive and reliable one-step RT-PCR assay with an internal control was employed to detect and differentiate the abovementioned viruses affecting stone fruit trees. The primer cocktail was used to analyze samples from 38 stone fruits originating from nine countries and six fruit tree species. A large number of virus combinations and mixed infections by up to three different viruses in five samples were detected by the multiplex RT-PCR assay. The use of five different primer pairs in a cocktail resulted in a decrease in sensitivity of detection. However, comparative analyses showed that the multiplex RT-PCR containing eight virus primer pairs was even more sensitive than ELISA or molecular hybridization assays. The multiplex RT-PCR procedure could allow routine and reliable detection of eight different viruses infecting stone fruit trees (Sánchez-Navarro et al. 2005). *Apple stem pitting virus* (ASPV), *Apple chlorotic leaf spot virus* (ACLSV), and *Apple stem grooving virus* (ASGV) infect apple and pear, but these viruses remain asymptomatic in commercial pear and apple cultivars. Two multiplex RT-PCR techniques with coamplification of an internal control from a plant mRNA sequence (mitochondrial *nad5* gene) were employed to detect these viruses simultaneously. The internal control was effectively coamplified with multiple I and II RT-PCR assays to detect ACLSV, ASGV, and ASPV in Korla pear in Xinjiang, China. Detection assays, using bark chips of 1- or 2 year old pear branches, could be performed both in the growing season and in dormant trees. Purified RNA of viruses as amplification template was not required. Three virus-specific primer pairs were designed to allow two multiplex RT-PCR amplifications accompanied by one pair of primers of the mitochondrial *nad5* gene. Four amplification products specific to three viruses and the internal RNA control were employed for specific detection of the corresponding viruses. Use of internal control reduced the risk of false negatives attributable to inhibition of conventional RT-PCR due to presence of inhibitors coextracted with target viral nucleic acids (Ma et al. 2008).

Prune dwarf virus (PDV) and *Prunus necrotic ringspot virus* (PNRSV) naturally infect the majority of *Prunus* spp. and have a worldwide distribution. They have multiple modes of transmission through mechanical inoculation, grafting, pollen, and seeds. Use of healthy plant material for propagation is the primary requirement to prevent virus spread in fruit crops. Certification programs have been established in many countries with the aim of identifying healthy sources for propagation. Several molecular techniques have been developed to detect the viruses precisely even at low virus titers in plant tissues and also in asymptomatic plants. Certification programs specify standards of fewer than 5% of virus-infected plants. ELISA tests were applied for the detection of PNRSV and PDV in *Prunus* seed lots planted in Montana and Washington (Mink and Aichele 1984). The operational capacity of the duplex RT-PCR

procedure for the detection of PDV and PNRS simultaneously was established by nine European laboratories. Samples (576) from *Prunus* trees with known sanitary status (corresponding to 32 samples in two repetitions for each laboratory) were analyzed. The assay had a level of sensitivity of 98.3% for PDV and 90.4% for PNRSV, whereas the specificity levels were 87.4% for PDV and 94.3% for PNRSV. This protocol showed high potential for large-scale application to determine health status of mother plants (Massart et al. 2008).

The sanitary status of the clonal Genebank collection of stone fruit was assessed by employing ELISA, tissue-printing (TP) hybridization, and GF305 woody indexing for detecting the presence of *Plum pox virus* (PPV), *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Peach latent mosaic viroid* (PLMVd), and *Hop stunt viroid* (HSVd). A total of 645 trees (197 peach and nectarine, 183 sweet and sour cherries, 106 plum, 106 apricot, and 53 other cherries) were indexed. PNRSV and PDV were frequently detected in single and mixed infections by ELISA tests and PPV was not detected in any of the fruit trees tested. Tissue-printing (TP) assay detected PLMVd and HSVd after testing a total of 336 trees. The presence of PLMVd in peaches (28/116 peach and nectarine) and HSVd in apricots (2/44) was detected by TP assay. Infection of 114 (38.4%) of 297 accessions tested by at least one virus and/or viroid could be detected by the assays (Michelutti et al. 2005).

Assessment of the health status of plum planting materials was carried out to establish plum mother plantations according to the recommendations of European Plant Protection Organization (EPPO) and Certification Standards of the Republic of Serbia. Propagules from pomologically selected trees in commercial and experimental orchards were grafted onto virus-free Myrobalan rootstock. Candidate clones were maintained in a screenhouse to protect the plants from external inoculum. Fifteen plum cultivars and a prospective hybrid (14/21) were included in this investigation. The selected clones were tested on woody indicators *Prunus tomentosa*, *P. persica*, and *P. serrulata* cultivar Shirofugen. The ELISA test was applied for the detection of possible presence of *Plum pox virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Apple chlorotic leaf spot virus*, *Apple mosaic virus*, and *Myrobalan latent ringspot virus*. Immunocapture (IC-) RT-PCR procedure was also applied for greater detection sensitivity. Nested PCR format was used for the detection of the phytoplasma "*Candidatus* Phytoplasma prunorum." The ELISA tests indicated the presence of *Apple chlorotic leaf spot virus* in four plants of cultivar Jelica. Latent infection with *Plum pox virus* was detected by IC-RT-PCR assay in four candidate clones. All other selected clones were free of all other viruses and the phytoplasma infection was not found in any planting materials tested. The investigation showed that ELISA test was suitable for routine diagnosis of viruses for which antiserum was available. Detection of ACLSV in cultivar Jelica by ELISA reduced the time required by biological testing. IC-RT-PCR format effectively detected PPV in plants with low virus titer and in young plants with uneven distribution of PPV. Based on the results, four plants were eliminated to maintain a higher level of health status in the collection (Jevremović and Paunović 2010).

Grapevine is the host for several viruses and viroids. A multiplex RT-PCR assay was developed for the simultaneous detection of *Grapevine leaf roll-associated virus 1, 2, and 4* (GLRaV-1, -2 and -4), *Grapevine viruses A and B* (GVA, GVB), *Grapevine fleck virus* (GFV) and *Grapevine fan leaf virus* (GFLV). These viruses were efficiently detected up to a 10^{-3} fold or higher dilution of the original plant extract. Two viroids infecting grapevines, *Hop stunt viroid* (HSVd) and *Grapevine yellow speckle viroid* (GYSVd), were also amplified to

the same level as the viruses by employing viroid-specific primers. This protocol showed potential for providing reliable diagnosis of grapevine virus and viroid diseases (Nakaume and Nakano 2006). In another investigation, a multiplex RT-PCR (mRT-PCR) procedure involving the use of primers specific to target viruses and a plant RNA as internal control was employed. The viruses *Arabid mosaic virus* (ArMV), *Grapevine leaf roll-associated virus 1, 2, 3* (GLRaV-1, 2 and 3), *Grapevine fleck virus* (GFV), and *Grapevine fan leaf virus* (GFLV) were detected (Gambino and Gribaudo 2006).

Degenerate and species-specific primers were designed for the simultaneous detection of GFLV, ArMV, *Tobacco ringspot virus* (TRSV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine deformation virus* (GDefV), *Grapevine Anatolian ringspot virus* (GARSV), *Artichoke Italian latent virus* (AILV), *Grapevine Bulgarian latent virus* (GBLV), and *Grapevine Tunisian ringspot virus* (GTRSV). Four different virus species could be detected in single grapevine samples containing mixtures of viruses of the same subgroup, using sense and antisense primers. PCR products amplified for TRSV, GFLV, ArMV, and GDefV (subgroup A) were 190, 259, 301, and 371 bp, respectively, whereas GCMV, AILV, GARSV, and GTRSV produced amplicons of 190, 278, 425, and 485 bp. This one-step RT-PCR procedure could rapidly resolve the components of mixed infections in grapevine (Digiario et al. 2007). In another investigation, simultaneous detection of GLRaV-4, -5, and -6 and isolates of GLRaV-De and -Pr was reported by Maliogka et al. (2008). These investigations indicated the need to apply multiplex assays to assess the health status of mother plants rapidly in the case of crops which are frequently infected by two or more pathogens simultaneously.

The occurrence of many viruses on strawberry crops leads to complex symptoms and heavy losses in many countries. Multiplex RT-PCR assays were therefore employed for simultaneous detection of isolates (18) of *Strawberry crinkle virus* (SCV), *Strawberry mild yellow edge virus* (SMYEV), *Strawberry mottle virus* (SMoV), and *Strawberry vein banding virus* (SVBV), which infected strawberry plants in different combinations. The upper limit of detection of the target viruses in plant tissue extracts was 1/200 (Thompson et al. 2003). In a later investigation, a multiple RT-PCR format was applied for the detection of *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV), and *Tomato bushy stunt virus* (ToBSV), employing virus-specific primers along with a plant internal amplification control (PIAC). Interactions between primers and coamplification of inhibitors of PCR amplification were avoided. This protocol had similar levels of specificity and sensitivity as conventional PCR applied for detection of individual viruses (Wei et al. 2008).

Total nucleic acid extracted from strawberry leaves was compared with total RNA as the template for detection of two RNA and one DNA viruses by both single and multiplex RT-PCR assays. Primer pairs D1/D3, 12/SM2, and YT1 and Y2 were employed for the amplification of expected fragments from *Strawberry mottle virus* (SMoV), *Strawberry vein banding virus* (SVBV), and *Strawberry mild yellow edge virus* (SMYEV), by multiplex RT-PCR using total nucleic acid as the template. When total RNA and total nucleic acid extracts were used as templates, detection efficiency for SMYEV and SMoV was in equivalence and the intensity of band amplified by single or duplex RT-PCR format was also similar. On the other hand, with total nucleic acid as template, detection results for SMYEV and SMoV by multiplex RT-PCR showed good correlation with those of single and duplex RT-PCR assays. The protocol developed in this investigation could be effectively used for detecting both RNA and DNA viruses simultaneously (Chang et al. 2007).

Multiple infections by viruses have been frequently observed in *Citrus* spp. In order to detect the presence of two or more viruses simultaneously, a multiplex RT-PCR (mRT-PCR) assay was employed for the detection of seven viruses, namely *Citrus leaf rugose virus* (CLRV), *Citrus psorosis virus* (CPsV), *Citrus tatter leaf virus* (CTLV), *Citrus tristeza virus* (CTV), *Citrus yellow mosaic virus* (CYMV), and *Indian citrus ringspot virus* (ICRSV). Based on the sequences of the respective viruses, degenerate primers were designed. PCR products with different sizes varying from 245 bp to 943 bp specific to each virus were amplified, facilitating the identification of individual virus. The protocol offered the advantages of reduced contamination risk and cost of testing, reduction in time and labor, and rapidity in providing results (Roy et al. 2005). The simultaneous infection of viruses is commonly seen in *Citrus* spp., leading to rapid decline and ultimate death of affected trees in India. *Citrus tristeza virus* (CTV) with an RNA genome and *Citrus mosaic badna virus* (CMBV) with a DNA genome infected sweet orange simultaneously. Primers targeting ORF 8 of CTV genome and ORF 3 of CMBV genome amplified PCR products of expected sizes, 510 and 245 bp respectively, enabling the detection and identification of CTV and CMBV in a single duplex PCR format (Ghosh et al. 2008).

Potato plants are infected by several viruses belonging to the genera *Alfavirus*, *Carlavirus*, *Luteovirus*, *Potexvirus*, *Potyvirus*, *Tobravirus*, *Tospovirus*, and *Tymovirus*. Infected progeny tubers are likely to become sources of virus inoculums, contributing to the spread of diseases induced by these viruses in new geographical locations where they are nonexistent or less important. Potato plants, generated through tissue culture technique, have to be indexed for the presence of viruses before their vegetative propagation. Multiplex assays are preferable to methods that can detect only a single virus in plant tissues, saving time, labor, and cost of testing. A multiplex nonradioactive nucleic acid spot hybridization (NASH) assay was applied for simultaneous detection of *Potato virus S* (PVS, *Carlavirus*), *Potato virus Y* (PVY, *Potyvirus*), and *Potato virus X* (PVX, *Potexvirus*) in potato tissue-cultured plantlets. The sensitivity of NASH procedure was determined by hybridization of PVS-, PVX-, and PVY-specific probes labeled with nonradioactive digoxigenin (DIG) on membranes spotted with a dilution series of extracts of tissue-cultured plantlets. The viruses could also be detected individually with specific probes. The probe which was amplified from plantlets infected with PVY^O could also detect other strains of PVY in plantlets infected with PVY^N, PVY^{NTN}, and PVY^{N:O}. In addition, the multiplex NASH assay was effective in detecting PVS, PVX, and PVY in a single hybridization assay by employing a cocktail of different probes specific to the respective viruses to be detected (Janczur et al. 2006).

The effectiveness of the 18S ribosomal RNA (rRNA) was compared with the commonly employed *nad2* mRNA as internal control for enhancing the sensitivity of detection of five potato viruses using a multiplex RT-PCR assay. The protocol simultaneously amplified cDNAs from *Potato virus A* (PVA), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV), *Potato virus S* (PVS), and 18S rRNA as well. This multiplex RT-PCR assay could detect all five viruses in different combinations. The multiplex RT-PCR format had a 100-fold greater sensitivity than DAS-ELISA for detection of PVX, in addition to its ability to detect the viruses in ELISA-negative samples. The internal control 18S rRNA was found to be better than *nad2* mRNA as internal control in terms of detection sensitivity. Multiplex RT-PCR was more convenient and less expensive compared with several individual RT-PCR assays required for detecting individual viruses (Du et al. 2006). A multiplex RT-PCR assay

was developed for the detection of three viruses, namely *Potato yellow vein virus* (PYVV), *Tomato infectious chlorosis virus* (TICV), and *Tobacco rattle virus* (TRV), including the plant internal amplification control (PIAC). The multiplex RT-PCR assay had a detection sensitivity of the same order as the conventional RT-PCR format, revealing that the multiplex RT-PCR protocol was a simple cost-effective, sensitive, and convenient tool for the detection of multiple infection of potato by two or three viruses (Wei et al. 2009).

Garlic crops are infected by viruses belonging to different taxa, collectively designated the virus complex including *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV) (potyviruses), *Shallot latent virus* (SLV), and *Garlic common latent virus* (Gar CLV) (carlaviruses). Recognizing the need for a sensitive and rapid diagnostic technique, a duplex RT-PCR assay was standardized for the simultaneous detection of OYDV and SLV (synonym *Garlic latent virus*) in ten selections of garlic. The protocol employed was sensitive enough for the detection of OYDV and SLV in cloves and leaves of garlic. The titer of OYDV was greater both in leaves and bulbs compared with SLV. This protocol was cost-effective, sensitive, and showed potential for use in certification and quarantine programs (Majumder et al. 2008).

Ornamental plants are infected by different viruses, resulting in substantial reduction of their market values. *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* (ORSV) are economically important viruses adversely affecting orchid industry. They induce less conspicuous foliar symptoms and cannot be detected by visual inspection. A multiplex RT-PCR assay was employed to detect CyMV and ORSV in *Phalaenopsis* orchid plants, using specific primers targeting the sequences of the coat protein (CP) of the respective viruses and one primer pair derived from the plant mitochondrial NADH dehydrogenase gene (*nad5*) as the internal control. The presence of CyMV and ORSV in *Phalaenopsis* orchids from the flower market in Taiwan was detected by multiplex RT-PCR protocol. The expected fragments of 666, 474, and 185 bp specific for CyMV, ORSV, and plant *nad5* mRNA, respectively, were amplified only in the orchid plants infected by CyMV or ORSV or by both viruses. The multiplex RT-PCR assay was shown to reduce the time, labor, and cost of testing compared with single assays, in addition to minimizing the risk of false-negative results required for routine large-scale orchid virus disease diagnosis in certification programs (Lee and Chang 2006).

Leaf yellowing of *Phalaenopsis* orchid was investigated to determine the identity of the virus(es) involved. A multiplex RT-PCR was employed for simultaneous detection of the commonly prevalent viruses, *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CyMV), present in the total RNA extracts. Both commercial kits and the simple-direct tube (SDT) technique were tested, using the primers designed in this investigation. ORSV was detected in a greater number of samples (55%) compared with CyMV (34%). Severe symptoms were observed in plants coinfecting by both viruses. The SDT procedure was efficient in extracting RNA suitable for the RT-PCR assay, which was cost-effective in undertaking screening of *Phalaenopsis* plants on a commercial scale in orchid nurseries. Another virus *Orchid fleck virus* (OFV) could also be detected in Australian orchid using virus-specific primers (Yamane et al. 2008). The usefulness of a multiplex RT-PCR format for the simultaneous detection of *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TuMV), and *Konjac mosaic virus* (KoMV) in *Calla lily* (*Zantedeschia* spp.) was demonstrated. This multiplex RT-PCR assay detected virus infection in 72% (36/50) of *Calla lily*, and about two-thirds (23/36) of infected samples showed mixed infections (Hu et al. 2010).

10.2 Plant Quarantines for Preventing Entry of Microbial Pathogens

Regulatory methods aim to prevent the import and spread of microbial pathogens into a country, state, or province. Legislative measures are formulated to regulate the cultivation of crops and distribution of planting materials between countries or states within the country. Regulatory control is enforced by establishing quarantines, inspections of crops in field/greenhouses/warehouses and, in some cases, by voluntary or compulsory eradication of host plants of certain pathogens. The primary aim of establishing plant quarantines is to prevent/restrict the introduction and spread of diseases and pests into new areas, where they have little or no chance of spreading naturally. Quarantines therefore enable importing countries to protect agriculture and the environment from avoidable damage that may be induced by diseases introduced by humans inadvertently. The appearance of epidemics caused by introduced pathogens indicates the important role of plant quarantines in international trade and the need for international standards. After the adoption of the General Agreement on Tariffs and Trade (GATT) there has been a dramatic increase in the movement of plant products, necessitating the enforcement of sanitary and phytosanitary measures at the global level. Legislative measures have become necessary to prevent or delay the establishment of plant pathogens in locations where they are absent or unimportant. The International Plant Protection Convention (IPPC) came into existence on 4 April 1991, following the acceptance of GATT by the majority of nations. An Expert Consultation on the Harmonization of Plant Quarantine Principles was convened by the Food and Agriculture Organization (FAO) to identify the basic principles necessary for formulating standards for plant quarantine procedures in relation to international trade (FAO, 1991).

10.2.1 Principles of Plant Quarantines

The principles of establishing plant quarantines recognize the sovereignty of the importing country which has the right to implement the phytosanitary measures deemed fit by that country. Restrictive measures for a minimum impact on the international movement of plant materials are ensured by different member countries. There is freedom to effect necessary modifications as and when required, and plant quarantine regulations are transparent so that there is harmonization of phytosanitary measures with no discrimination between countries. Plant quarantine activities vary based on the hazard and risk associated in allowing the import of plant materials likely to carry pathogens. Hazard is the danger that a specified pathogen may present to the agriculture of the importing country and the possibility of becoming established at the new location. Risk is the chance that a hazardous organism will enter and become established. The levels of risk of the pathogens may be low, medium, or high depending on their aggressiveness and survival capacity in a wide range of environmental conditions. Safeguards include rules and regulations, phytosanitary certificates, inspection treatments, isolation, passage through a quarantine greenhouse (post-entry), seed health evaluation, and other measures formulated by the importing country. The importing country has the option of either permitting or denying the entry of high-risk material for commercial purposes or to issue a permit for plant material imported for scientific purposes (Kahn 1988, 1991). Plant quarantines have been established by most countries, with regulations ensuring that exporting countries adhere to the measures

prescribed by the importing country to protect their country against possible invasion by more aggressive pathogens or their strains.

10.2.2 Functions of Regional Plant Quarantine Organizations

In compliance with the International Plant Protection Convention (IPPC) in 1951, the following eight regional plant protection organizations were set up to advise and assist the member governments on the technical, administrative, and legislative measures concerned with plant protection activities: (i) Comité Interamericano de Protection Agricola (CIPA); (ii) Caribbean Plant Protection Commission (CPPC); (iii) European and Mediterranean Plant Protection Organization; (iv) Interafrican Phytosanitary Council (IAPSC); (v) Near East Plant Protection Organization (APPO); (vi) North American Plant Protection Organization (NAPPO); (vii) Organismo Internacional Regional de Sanidad Agropecuaria (ORISA); and (viii) Plant Protection Committee for the South East Asia and Pacific Region (SEAPPC). The member countries governed by the respective regional plant protection organizations disseminate information on changes in the quarantine regulations and requirements and examine the possibilities of simplification and unification of phytosanitary regulations and issue of certificates.

A pathogen or insect pest is considered to be of quarantine significance (QS) if its exclusion is perceived as important enough to the agriculture and natural vegetation of the importing country for its government to provide necessary infrastructure and personnel to prevent or delay its entry along manmade pathways (Kahn 1991). Plant pathogens/pests coming under plant quarantine regulations are selected based on the following parameters: (i) the pathogen is capable of causing serious losses in economically important crops grown in the country/region; (ii) the pathogen(s), if introduced, will not be able to spread rapidly by natural means; (iii) the pathogen(s) may survive under conditions existing in the field/glasshouses; and (iv) current trade practices may introduce the pathogen(s). The pathogens selected are grouped into three categories as follows.

- List A: dangerous quarantine organisms. This is further subdivided into two categories:
 - List A1: pathogens absent from all member countries (exotic pathogens); and
 - List A2: pathogens present only in some countries in the region.
- List B: pathogens included by individual countries under regulations; comparatively less stringent measures are enforced by the country concerned.

The necessity for inclusion of additional pathogens or deletion of pathogens from the current list is constantly reviewed and modified, if required. Various countries have enacted Acts that authorize the Plant Protection Organization of the respective country to take necessary steps for the establishment of plant quarantines in seaports and airports, and for intercepting organisms of quarantine importance. A list of quarantine pathogens are prepared and the countries from which the plants and plant products are imported are informed of the regulations and requirements to be satisfied. Each country may have certain specific acceptable entry procedures. For example, in the USA, a procedure known as “preclearance” is insisted upon. This procedure entails removal of the pathogen/pest risk at the point of origin of plants/plant products by technical personnel at the port of entry. Inspection of plants/plant products by technical personnel of the USA at the country of origin is necessary, and the cost of

such inspections/examinations have to be met by the exporting country. Deciduous fruits from South Africa, apples from New Zealand, Unshu (Satsuma) oranges from Japan, fern plants and bulbs from the Netherlands, grapes from Chile, mangoes from Haiti, and bulbs from Germany, France, Israel, Italy, and South Africa are allowed entry into the USA after adoption of preclearance procedure pertaining to the crops concerned (Rohwer 1979). Domestic quarantines operate at the boundaries between states/provinces to restrict the movement of plants and planting materials as per the specifications.

10.2.3 Impact of Introduced Pathogens

The number of plant pathogens brought along with new crops/varieties increased steadily from the nineteenth century, and many crops in different countries were ruined as the pathogens found suitable environmental conditions and available susceptible plants in large areas. The need to regulate the entry of plants/plant materials into the country was recognized. Some of the destructive diseases of economically important crops that were primarily responsible for the establishment of plant quarantines in various countries are discussed in the following sections.

10.2.3.1 Potato Late Blight Disease

Potato late blight disease caused by *Phytophthora infestans* destroys all infected plant parts and also infects the tubers which spread the inoculum to various locations. The center of origin of potato (*Solanum tuberosum*) and its wild relatives is considered to be the mountains of Mexico and Guatemala, and the pathogen *P. infestans* might also have coevolved with its host plants (Leppik 1970). The potato late blight disease is one of the earliest recorded plant diseases for which the association of a fungus *Phytophthora infestans* was established as the causative agent, and was primarily responsible for the infamous Irish famine in 1845. Potato crops were entirely devastated, leading to one of the greatest migrations of impoverished humans to other European countries and North America. The pathogen was introduced with seed potatoes from Peru to Belgium or France by about 1842 to 1844 (Agarwal and Sinclair 1996). Potato was introduced along with the pathogen into Europe and other countries. As the potato cultivars were selected for higher yield and attributes other than resistance to pathogen(s), conditions became increasingly favorable for the pathogen as the area under potato cultivation enlarged year after year. The population diversity of *P. infestans*, differing in their virulence and sensitivity to fungicides, had to be investigated since genotypes with a greater level of virulence and resistance to fungicides may be introduced through infected seed tubers. Clonal propagation and migration through seed tubers contributed to long-distance transport of *P. infestans*. Both A1 and A2 mating types of *P. infestans* were found in the same potato seed tuber production areas in Canada. This situation might increase the potential for sexual recombination within the pathogen populations, leading to the production of oospores (sexual spores) that could survive in the absence of host tissues and create new strains of *P. infestans* (Peters et al. 2014).

10.2.3.2 Citrus Canker Disease

Xanthomonas axonopodis pv. *citri* (Xac) causes citrus canker disease in all parts of the plants, including fruits, reducing the market value of the fruits of *Citrus* spp. The pathogen was considered to have been introduced into the USA from Asian countries

through infected budwood materials. The application of quarantine regulations effectively prevented the establishment of the disease. Federal quarantine laws were enforced in 1912 as soon as the first incidence of the disease was observed. State and federal regulatory personnel initiated programs to eradicate 20 million citrus trees at a cost exceeding US\$ 20 million. The infected trees and all plant materials within 125 feet (41 m) were required to be burnt as per quarantine regulations. Clothes of workers, tools, machinery, and vehicles were thoroughly cleansed (Schumann 1991). The eradication of infected plants, combined with quarantine regulation to prevent further entry of the pathogen, has made the disease management system very effective (Fry 1982). The preclearance program operated by the personnel of the USA and Japan has created a formidable barrier preventing the pathogen spreading from other countries (Rohwer 1979). However, reintroduction of the pathogen through infected fruits and plants carried by travelers remains a distinct possibility.

10.2.3.3 Panama (*Fusarium*) Wilt Disease of Banana

Panama (*Fusarium*) wilt disease of banana caused by *Fusarium oxysporum* f.sp. *cubense* is one of the most destructive diseases of banana, named after the country where the first epidemic of the disease was recorded. The disease incidence progressively increases in subsequent generations causing heavy losses if proper care is not taken to select healthy suckers (corms). In Costa Rica, banana production was drastically reduced from 11 million bunches in 1923 to 1.4 million bunches in 1941 (Schumann 1991). The pathogen primarily spreads through infected suckers and survives in the soil in new areas after introduction through infected suckers. This fungal pathogen may also be introduced into new areas by contaminated soil, footwear (or feet), and agricultural tools. Once the pathogen reaches the soil, it is very difficult to eliminate the pathogen entirely from the field. When the crop production becomes unprofitable because of the death of infected plants, infested areas have to be abandoned since the pathogen can survive in the soil for several years. Fallowing for long periods may be required. As the quarantine regulations were ineffective, the disease incidence is seen in all banana-growing countries in the world. This situation underscores the need for strict enforcement of regulatory measures by both domestic and international quarantines on the movement of planting materials from one state to another and from one country to another, to restrict the further spread of the disease.

10.2.3.4 Banana Bunchy Top Disease

The causative agent of banana bunchy top disease (BBTD), *Banana bunchy top virus* (BBTV), has been responsible for ruining banana production in Australia, Fiji, India, and several other countries in the Asia-Pacific basin and Africa. The virus spreads primarily through infected suckers which may or may not exhibit any visible symptoms of virus infection, and secondary spread occurs through the banana aphid *Pentalonia nigronervosa* in a persistent manner. However, the new infections due to aphid transmission in a growing season are far less compared to introduction of the disease by infected suckers obtained without indexing for the presence of BBTV. Successful management of BBTD was achieved in Australia (Queensland) by inspection of suckers for health status, eradication of infected plants completely in old plantations, and planting virus-free suckers in new plantations. It is an excellent model for emulation by other banana-growing countries. Use of suckers from BBTV-infected plantations was prevented by legislation and the infected plants were destroyed with the cooperation of growers.

Virus-free suckers were planted in new plantations (Magee 1936; Eastwood 1946; Colborne 1953). Because of strict enforcement of these regulations for over 50 years, bunchy top disease incidence was reduced from a mean of 6.6 plants ha⁻¹ in 1937 to 0.1 ha⁻¹ in 1967. Following the application of the set of operations required for the management of BBTD, a rather unique situation developed: it became difficult to find even a few infected banana plants required to train inspection personnel (Allen 1983). This example of banana bunchy top disease reveals the importance and effectiveness of plant quarantines in preventing the introduction and subsequent spread of destructive diseases of economically important crops.

10.2.3.5 Strawberry Anthracnose Disease

Phytosanitary legislation was considered to be the most effective means of restricting the entry of alien pathogens into the country without impacting trade. However, the limitations and weaknesses of the phytosanitary systems worldwide were revealed by the entry and establishment of *Colletotrichum acutatum* in the UK in the 1980s. Datasets of disease incidence gathered by personnel involved in plant health programs between 1984 and 2008 were analyzed to determine how and why the pathogen entered the UK in 1982 in plants originating from the USA, 1 year earlier than the first UK record of the disease was reported. At least 54 further cases of infested plant materials breached the phytosanitary procedures and entered the UK, 75% of which originated from EU countries. Further, internal trade transmission was found to be highly influential in the successful spread of the disease. The nurseries that had a track record of selling infested plant material were blacklisted, even if they were accompanied by a plant passport (certificate). The results revealed the huge responsibility of personnel concerned in the nurseries throughout the EU in limiting the spread of quarantine diseases (Calleja et al. 2013).

10.2.3.6 Potato Virus Diseases

Seed potato certification programs were first established in Europe and the concept was extended to North America in 1913. At that time, the purity of potato varieties and the use of fewer well-characterized cultivars were considered as the primary goals. State-based potato grower associations were formed prior to 1913 in many regions and these associations were responsible for setting up seed certification schemes. Each state in the USA has different regulations and certification agencies are managed by the state department of agriculture, universities, or grower groups. Later, control of necrotic strains of *Potato virus Y* (PVY) was one of the main reasons for the establishment of federal seed potato certification standards (Trank 1991). Seed potato certification in North America is based mainly on visual inspection of growing plants, since it was considered effective and the small benefit of large-scale laboratory testing for the pathogen was not worth the added cost. Varieties that did not exhibit early recognizable symptoms of important pathogens were therefore discouraged (de Souza-Dias and Betti 2003). However, the evolution of new PVY strains that elicited few symptoms, regardless of the potato genotype, made identification of infected plants difficult. This situation changed the views of potato seed certification in favor of using sensitive, specific, and reliable techniques based on immunological and nucleic acid properties of viruses.

Potato virus X and *Potato leafroll virus* (PLRV) have limited host ranges and spread by mechanical transmission and aphids. They can be effectively dealt with by eliminating seed potato lots carrying these viruses. The recent emergence of soilborne viruses

Tobacco rattle virus (TRV) and *Potato mop-top virus* (PMTV) has become a challenge for seed potato certification programs (Johnson 2008). PVY incidence in seed lots was one of the main characteristics used to determine which class a seed potato lot would fall into. The virus incidence threshold for each seed class varies by seed potato certification agency. There is a zero tolerance for every virus in tissue-culture-derived plantlets and in greenhouses and this could be ensured by visual inspection and mandatory laboratory testing for growers of certified seed potatoes. The cost of extensive laboratory testing, while providing more accurate data, is prohibitively expensive (Halterman et al. 2012).

10.2.4 Assessment of Efficiency of Quarantines

Plant quarantines should be rational and based on biological and sociological data on pathogens/pests. Restrictions have to be imposed on the entry of exotic pathogens likely to cause substantial losses if introduced into the country concerned. The efficiency of plant quarantines can be increased by a proper choice of effective strategies. The expected economic impact should be estimated so that the data constitute a basis for prioritizing quarantine activity. The plant protection organization of the country should be alert and responsive to initiation of appropriate investigations on reports of possible new introduction of pathogens. The rapid action taken by the USA may possibly serve as a model in enforcing quarantine regulations. Wheat Karnal bunt caused by *Tilletia indica* infects seeds and the pathogen presence was detected by the personnel of the US Department of Agriculture in Arizona on 8 March 1996. The presence of *T. indica* was confirmed by PCR assay and quarantine activities immediately accelerated. Within a period of less than 2 weeks, the Secretary of Agriculture announced a “Declaration of Emergency” to contain the spread of the disease. Growers and handlers were adequately compensated for losses due to quarantine action. Information on the disease was disseminated through media and the internet (Bonde et al. 1997). Such urgent and coordinated actions may be necessary to prevent the introduction and subsequent spread of the disease in other countries. Cooperation between countries and importers of plant materials would enhance the efficacy of plant quarantines. Adequate care and attention should be exercised by the exporting countries to ensure the required level of phytosanitary status of the plant materials. Travelers form a potential source of immigrant pathogens and pests. It has been reported that about 80% of all uncertified plant materials with potential pathogens/pests were carried in traveler’s baggage in Canada. It is probably worthwhile to thoroughly examine baggage in view of the potential risks involved. According to Schumann (1991), a dog was employed to sniff out citrus fruits to prevent the reintroduction of citrus canker pathogen into the USA.

The use of efficient, sensitive, and reliable diagnostic techniques for the detection of pathogens in plant materials becomes indispensable, especially when asymptomatic infection has to be detected to enhance the effectiveness of plant quarantine measures in preventing the introduction of exotic pathogens. Constant vigilance and coordinated efforts of all personnel are required because of ever-increasing travel and liberalization of trade among countries. Conventional methods involving the isolation of pathogens and their identification based on classical taxonomic characteristics require a long time to obtain results that are sometimes inconclusive. Molecular methods have been shown to be more sensitive, specific, rapid, and reliable. These methods are more useful and effective in detecting phytoplasma, virus, and viroid pathogens which have not been

isolated in cell-free media so far. The choice of technique for diagnosis of crop pathogen(s) carried in seed and propagules depends on the sensitivity, specificity, and reliability of the results obtained, in addition to the cost-effectiveness of the technique. Multiplex assays that can detect two or more pathogens simultaneously have been developed. These techniques offer the advantages of saving time, labor, and cost of testing significantly.

The arrival and subsequent establishment and spread of nonindigenous pathogens and other organisms may result in substantial economic loss of crops, either directly or through trade barriers. To be a successful invader, a pathogen must negotiate a sequence of events that include arriving, surviving, and thriving in a new environment. Limiting the survival of a pathogen after successful entry is the principal goal of regulatory activities, such as eradication and quarantine. In the USA, the exclusion, eradication, and quarantine of plant pathogens is the responsibility of the USDA Animal and Plant Health Inspection Service (APHIS). The increase in the volume of international trade and travel has increased the likelihood of immigration of nonindigenous, invasive pathogens into several countries. Identification of the pathways of introduction of nonindigenous pathogens may significantly assist efforts to curtail their entry into the country concerned.

Karnal bunt of wheat caused by *Tilletia indica* was first detected in the USA in Arizona in 1996. The seed lots of infected, spring-habit, durum wheat associated with the initial detection were traced to planted fields in California, Arizona, New Mexico, and Texas. In 1997, the bunt disease incidence was observed at several locations about 700 km away from the potentially contaminated wheat which was destroyed prior to reinfection. The pathways of introduction of *T. indica* were sought by analyzing the USDA/APHIS Port Information Network (PIN) database from 1984 to 2000. All interceptions were made on wheat transported from Mexico, with the majority (98.8%) being intercepted at land border crossings. Karnal bunt was not intercepted from any other country over the 17 year period analyzed. Most interceptions were on wheat found in automobiles, trucks, and railway cars. The interceptions peaked in 1986 and 1987, although the pathogen was intercepted during the entire 17 year period. Averaged over the years, a significantly greater number of interceptions were made during May (19.2% of interceptions) and the fewest interceptions were in July (4.0%). The relatively unaggressive nature of the disease and its reliance on rather exacting weather conditions for infection may be the possible reasons for the long period between the arrival of the pathogen and establishment at the invaded agroecosystem (Marshall et al. 2003).

A dot-blot hybridization procedure using nonradioactive digoxigenin (DIG-) labeled riboprobes and chemiluminescent detection could be employed for the diagnosis of infections by *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), *Tomato yellow leafcurl virus* (TYLCV), *Alfalfa mosaic virus* (AMV), and *Tomato mosaic virus* (TMoV) in tomato seedlings. This procedure was successfully applied for phytosanitary purposes in Italy (Salderelli et al. 1996). Multiplex tissue-print immunoassay (TPIA) and dot-blot immunoassay (DBIA) were evaluated for their efficacy for the detection of *Potato leafroll virus* (PLRV), *Potato virus S* (PVS), *Potato virus X* (PVX), and *Potato virus Y* (PVY) in the extracts of green and/or tuber tissues. In TPIA, the specific precipitation patterns in infected tissues of leaf petioles or stem cross-sections observed with each virus allowed the specific virus or mixed infections to be identified in a single multiplex assay. DBIA was more sensitive (50 times) than ELISA for the detection of PVY in green tissues. TPIA and ELISA tests were suitable for rapid detection of PVY and PVS in the

extracts from tuber stem end or eyes without prior germination of tubers. PVS was evenly distributed in potato tuber tissue, whereas PVY was localized in the vascular tissue beneath the epidermis, with irregular distribution along the periphery of the potato tuber. TPIA technique was recommended for monitoring the incidence of PVS and PVY as a suitable alternative to ELISA tests for laboratories lacking the time and facilities for tests based on tuber germination (Samsatly et al. 2014).

The procedure for the detection of viral infection based on the presence of double-stranded (ds-) RNA in plants was developed. Plants infected by *Cucumber mosaic virus* (CMV) and *Potato virus X* (PVX) could be identified by detecting the ds-RNAs which show characteristic patterns. This procedure has the potential for the first screening of infection by those widely distributed viruses for plant quarantine regulations (Yamashita et al. 1996). Although molecular assays have been shown to be preferable for the rapid detection and identification of microbial pathogens, conventional (biological) assays should remain as the fundamental assay for the continued success of clean stock/certification programs because numerous uncharacterized graft-transmissible agents (GTAs) infect *Vitis* spp. and *Prunus* spp. For example, the Red Globe virus, described as *Grapevine leafroll-associated virus 2RG* (GLRaV-2RG), did not induce leafroll symptoms in the standard indicators (Cabernet France or Cabernet Sauvignon) used for viruses infecting grapevine. On the other hand, GLRaV-2 caused an acute reaction on certain grape rootstock (Uyemoto et al. 2001). GLRaV-2RG was serologically related to GLRaV-2 and both shared 74% nucleotide sequence homology. Red Globe strain passed the required assays of the California Department of Food and Agriculture (CDFA) and California Registration and Certification (CRC) program. However, when Red Globe scion materials were bench-grafted onto different rootstocks and planted in commercial orchards, all plants of acutely sensitive rootstocks died within two growing seasons. The molecular technique enabled the identification of GLRaV-2RG strain and its pathology in sensitive rootstocks was useful to study the variations in host–virus interaction (Rowhani et al. 2005).

The extent of the presence of viruses was assessed in the samples of imported propagation materials with phytosanitary certificate pertaining to the Turkish Regulation on Agriculture Quarantine. A total of 114 fruit propagation materials (rootstock and seedling varieties) were tested by DAS-ELISA and compound direct ELISA formats. Apple rootstock samples (26) and apple seedling samples (17) were tested for the presence of *Apple mosaic virus* (ApMV) and *Apple chlorotic leaf spot virus* (ACLSV), while pear rootstock samples (5) and pear seedling samples (6) were tested for the presence of ACLSV. Apple and pear rootstocks and seedling samples were found to be free of the viruses tested. *Prunus necrotic ringspot virus* (PNRSV) was detected in one plum rootstock sample. *Prune dwarf virus* (PDV) infection was detected in two cherry seedling samples and one sample each from peach rootstock and cherry rootstock and seedlings were positive for *Plum pox virus* (PPV). One cherry seedling sample showed mixed infection by PDV and PPV. Overall, 6.13% of fruit propagation materials intended to be imported were found to be infected by one or both viruses (Özdemir and Kaya 2008).

Apple chlorotic leaf spot virus (ACLSV), the type member of the genus *Trichovirus*, *Flexiviridae*, has a worldwide distribution infecting a wide range of rosaceous hosts, and is an important pathogen targeted in certification and quarantine programs. Detection of ACLSV was accomplished based on biological indexing, ELISA, and RT-PCR assays. The RT-PCR assay was evaluated for its suitability as a reliable molecular indexing tool, using a broad spectrum of virus isolates from various hosts. Oligonucleotide

primers targeting the viral CP and replicase regions of ACLSV were tested for the detection of virus isolates from natural hosts originating from domestic and imported sources of plant germplasm. The primers CLS6860 and CLS7536 amplifying a 676 bp fragment in the CP coding region were the most reliable, detecting all isolates in all tested hosts. Comparison of the RT-PCR data with ELISA and biological indexing results revealed the advantages of using a reliable molecular tool for problematic hosts (Spiegel et al. 2006). The phytosanitary status of the sources should be precisely assessed before nursery propagation or for germplasm exchange.

The phytosanitary status of *Prunus* spp. in the National Clonal Germplasm Repository (NCGR) in California was evaluated. A total of 221 trees representing 185 different cultivars of cherry, almond, peach, apricot, and plum were sampled. Conventional RT-PCR assay was employed for the detection of *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Plum bark necrosis stem pitting-associated virus* (PBNSPaV), *American plum line pattern virus* (APLPV), *Cherry virus A* (CVA), *Cherry leafroll virus* (CLRV), *Cherry rasp leaf virus* (CRLV), *Cherry green ring mottle virus* (CGRMV), *Cherry necrotic rusty mottle virus* (CNRMV), *Apple chlorotic spot virus* (ACLSV), *Tomato ringspot virus* (ToRSV), *Little cherry virus 1* (LChV-1), *Little cherry virus 2* (LChV-2), *Peach latent mosaic viroid* (PLMVd), and *Hop stunt viroid* (HSVd). Although the majority of the trees were asymptomatic, all tested viruses (11 of 13; ToRSV and CRLV excluded) and two viroids were detected. Two of the viruses ACLSV and LChV-2 had never been found in California previously. The incidence of PNRSV was the highest (55 trees), followed by the two viroids (PLMVd and HSVd) with 33 and 32 infected trees, respectively. The incidence of other viruses ranged over 0–19 trees. The infection rate of all tested samples ranged over 0.5–24.9%. The results revealed the need to evaluate the mother plants for their phytosanitary status periodically to restrict the spread of the virus(es) and viroid(s) through propagules (Osman et al. 2012).

Citrus spp. are infected by many viroids, in addition to different viruses. Detection of viroids through biological indexing on sensitive indicator plants followed by sequential polyacrylamide gel electrophoresis (sPAGE) was widely adopted previously, but this procedure was found to be time-consuming and the test plants had to be maintained at optimum conditions. A conditional use of RT-PCR assay was shown to be an efficient and alternative detection method for testing plant materials in the Texas virus-free citrus budwood certification program. Prior to final biological indexing, RT-PCR assay was used to detect, clone, and sequence full-length viroids of *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) (both cachexia and non-cachexia variants), *Citrus viroid-III* (*Citrus dwarfing viroid*), and *Citrus viroid-IV* (*Citrus bark cracking viroid*) from a collection of viroid-inoculated grapefruit plants. The presence of viroids in source plants was checked earlier by biological indexing in Etrog citron plants. The results indicated that RT-PCR could be a conditional substitute for biological indexing of mother trees in the virus-free budwood program. Trees that are positive for RT-PCR may be discarded from the pool before extensive biological indexing (Kunta et al. 2007).

All known grapevine viruses are graft-transmissible and are spread over long distances through infected propagative materials (buds/cuttings) or by using rooted cuttings from infected canes. An important strategy for the management of grapevine viruses is therefore through clean stock programs. In accordance with the European Legislation and national system for production of certified vine-growing materials, 1295 samples from grapevine cultivars and rootstocks were tested for their health status during 2004–2006. Application of ELISA tests showed that 30.5% of tested grapevine cultivars

and rootstocks were infected by one (86.1%) or more viruses (13.9%). *Grapevine fleck virus* (GFKV) was the most widespread (23.3%) among grapevine viruses, followed by *Grapevine fan leaf virus* (GFLV) (5.05%) and *Grapevine leafroll-associated virus-1* and *-3* (GLRaV-1 and -3) (3.34% and 2.78%, respectively). *Grapevine Bulgarian latent virus* (GBLV) (0.37%) was detected occasionally. *Arabid mosaic virus* (ArMV), *Grapevine leafroll-associated virus-2* and *-7* (GLRaV-2 and -7), *Grapevine virus A* (GVA), or *Grapevine virus B* (GVB) were entirely absent from the locations where the diagnostic tests were carried out. Mixed infections by eight virus combinations were observed. The most widespread virus combinations were GFKV + GLRaV-1 (8.3%) and GFLV + GLRaV-3 (8.3%). The wild relatives of *Vitis vinifera* ssp. *sylvestris* were free of ArMV, GFLV, and GBLV. The results showed that ELISA tests were effective in detecting grapevine viruses in source plants (Kamenova et al. 2007).

10.3 Production of Disease-Free Seeds and Propagules

As seeds and propagules carry the microbial pathogens, they form the primary sources of inoculum for the pathogens; natural biological vectors or environmental conditions such as wind or rain may be responsible for the secondary spread of the disease(s). Use of pathogen-free seeds and propagules is therefore the basic requirement for successful and profitable crop cultivation. The role of certification and plant quarantine programs in providing pathogen-free seeds and propagules was discussed in Sections 10.1 and 10.2. These approaches aim to select the mother plants or seed lots which satisfy the limits of pathogen presence, and those that are either free of pathogens or carry the pathogens not exceeding tolerance limits are distributed to the growers. On the other hand, concerted efforts have been taken to eliminate the pathogen(s) present in and/or on the seeds and propagules. Plant viruses are known to be transmitted through true seeds of some crops such as lettuce, barley, peanut, and soybean at higher frequencies compared to other crops. In contrast, transmission of viruses occurs through propagules of all vegetatively propagated crops. Different approaches have been made to contain the virus diseases depending on the virus distribution in infected plants, susceptibility to heat, and antiviral chemicals.

10.3.1 Production of Virus-Free Plants

10.3.1.1 Meristem-Tip Culture

Microshoot-tip tissue culture is a preferred method to eliminate virus(es) and other plant pathogens from many plant species. This method provides the advantage of regenerating a single plant from a single minuscule (approximately 0.5 mm) shoot. This technique avoids the production of plants from callus which may lead to regeneration of an off-type plant. The combination of low hormone levels combined with a minimum time in culture reduces the chance of mutation and regeneration of an off-type plant. At the same time, many pathogens including viruses are eliminated by this technique. As the meristem is able to grow faster than it can be infected by viruses that may be present in the mother plants, this technique can be useful to build up virus-free stocks. The apical meristems of systemically infected plants are generally free of the virus infecting the plant species. Meristem-tip culture may be effective in obtaining virus-free plants, even when all the plants of a valuable cultivar or germplasm are

infected by the virus. The meristem piece (0.5–1.0 mm long) is planted in an appropriate medium such as Murashige-Skoog (MS) medium under aseptic conditions, and incubated at the required temperature–light conditions. The plantlets growing from the meristem are indexed for the presence of virus by various assay methods. Viruses infecting members of Araceae and strawberry could be eliminated by such procedures (Hartman 1974; Tong et al. 1994).

In China, sweet potato viruses including *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato latent virus* (SPLV), and *Sweet potato caulimo-like virus* (SPCLV) were observed in all sweet potato production regions in China, which is the largest cultivator of sweet potato in the world. Shoot-tip cultures of sweet potato have been effective in eliminating sweet potato viruses. An effective propagation and delivery system of virus-free sweet potato plants was established and carried out successfully in Shandong and Jiangsu provinces. The use of virus-free sweet potato plants could restore high yield and quality, in addition to improving the resistance against other pathogens such as the fungal pathogens *Monilochaetes infusans* and *Ceratocystis fimbriata* and the nematode *Pratylenchus coffeae*. Further, the area of virus-free sweet potato production reached 70,000 ha in 1994, 90,000 ha in 1995, and 466,000 ha in 1998. The effectiveness of meristem-tip culture for the elimination of sweet potato viruses has therefore been amply demonstrated in China (Feng et al. 2000).

Cucumber mosaic virus (CMV) infection in chrysanthemum significantly reduced production. By using meristem-tip culture method, CMV was eliminated and virus-free plants were produced by planting meristem tips (0.3 mm) planted in MS medium. When indexed by RT-PCR assay, 72% of the plants generated were found to be free of CMV (Verma et al. 2004). *Onion yellow dwarf virus* (OYDV) infects onion and garlic crops severely, causing serious losses. The basic requirement to restrict the incidence and spread of OYDV is to use virus-free planting materials; propagation materials were therefore generated by meristem-tip culture to build virus-free nuclear stocks. The absence of OYDV in the tissue-culture-generated plantlets was ensured by applying RT-PCR assay. Specific primers from conserved region of RNA-dependent RNA polymerase (RdRp) gene and 3' UTR region of RNA of OYDV isolates were designed and employed in the RT-PCR assay. The primers successfully amplified virus-specific fragments (c. 1.1 kb) in naturally infected garlic bulbs. The RT-PCR protocol developed in this investigation provided rapid and reliable detection of OYDV in propagation materials generated through meristem-tip culture, facilitating production of virus-free stocks (Arya et al. 2006).

The introduction of micro- and minitubers revolutionized potato tuber production, resulting in a shortening of the field production cycle to obtain an adequate number of seed potatoes, thus guaranteeing a high level of healthiness of base materials. Minitubers are small tubers produced on in vitro plantlets or microtubers under covers. The possibility of multiplying the materials produced in vitro directly under field conditions was examined. The extent of infection by *Potato virus Y* (PVY), *Potato virus M* (PVM), and *Potato leafroll virus* (PLRV) of potato tubers derived from plants grown directly from in vitro plants, microtubers, minitubers, and traditional seed potatoes planted in the field at different times was assessed. The levels of susceptibility to the viruses using aphid vectors were determined. Infection of tubers derived from in vitro plants and microtubers was greater than that of seed potatoes and minitubers. However, the greater susceptibility was largely considered to be due to unknown causes. Multiplication of microtubers and in vitro plantlets under field conditions could be very economical if

cultivars with relatively higher level of resistance are used. Adopting a later-than-usual planting period (end of June) and applying an additional protective cover, such as non-woven agricultural fabric, in the first phase of plant growth promoted multiplication of minitubers and in vitro plantlets in the field conditions for cultivars with low level of resistance (Wróbel 2014).

Cucumber mosaic virus (CMV) and *Cymbidium mosaic virus* (CyMV) were successfully eliminated from vanilla (*Vanilla planifolia*) through meristem culture. Apical meristems (0.1–0.25 mm) were planted in Murashige-Skoog medium supplemented with nutrients and hormones. The regenerated plantlets were hardened in an insect-free glasshouse and checked for the presence of viruses by RT-PCR assay using virus-specific primers. The frequency of CMV elimination was 79.4%, whereas CyMV was eliminated in 82.4% of the regenerated plants. Simultaneous elimination of both CMV and CyMV was up to 75% (Retheesh and Bhat 2010).

Phalaenopsis, a very popular orchid plant species in the global market, is frequently coinfectd by *Odonotoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CyMV) in Taiwan. A relatively simple procedure for eliminating ORSV and CyMV using shoot-tip culture was developed. Early isolation and selection of virus-free protocorm-like body (PLB) through subcultures was accomplished. The indexing of viruses in the field-grown plants, PLBs, and tissue-cultured plants was carried out by indirect ELISA and one-step multiplex RT-PCR assay. Induction of PLBs in shoot-tips was achieved on $1/2 \times$ Murashige-Skoog (MS) basal medium supplemented with 1% sucrose, 6% potato pulp, 0.5 g L^{-1} tryptone, 0.25% activated charcoal, and 0.6% Bactoagar. The regenerated plants from virus-free PLBs acclimatized easily in the greenhouse and showed 100% survival rate. All the *Phalaenopsis* plants raised through shoot-tip culture tested negative for both viruses. At least three subcultures were necessary to authenticate that the shoot-tip culture-derived plants were virus-free. The protocol developed in this investigation showed potential for reliable elimination of viruses and mass propagation of genetically uniform and virus-free *Phalaenopsis* plants (Chien et al. 2015).

Viruses infecting grapevines could be eliminated by adopting microshoot-tip culture, involving culturing of growing tips (less than 0.5 mm) excised from the grapevine shoot tip. Many viruses and the crown gall bacterium were eliminated by this procedure. *Grapevine leafroll-associated viruses*, *Grapevine fan leaf virus*, *Grapevine virus A*, *-B*, and *-D*, and *Rupestris stem pitting-associated virus* were eliminated successfully by microshoot-tip culture. In addition, *Agrobacterium vitis*, causing crown gall disease, could be effectively eliminated by adopting microshoot-tip culture technique (using growing tip of about 5–10 mm in length) (Susan et al. 2010).

A combination of thermo (heat) -therapy and meristem-tip culture has been demonstrated to be more effective than the use of meristem-tip culture alone. The effect of selective virus elimination on vigor, yield, and fruit quality of *Vitis vinifera* cultivar Chardonnay was assessed under field conditions over 6 years. The test materials were propagated from three clones infected by multiple combinations of *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine virus B* (GVB), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine fleck virus* (GFkV), GVB and GRSPaV, GLRaV-2, GLRaV-3, and GRSPaV and virus-like diseases (vein mosaic and vein necrosis). Progenies were selected after virus elimination by heat therapy and in vitro apex grafting. It was not possible to eliminate all grapevine viruses in the progeny, as revealed by graft indexing and DAS-ELISA. Elimination of GLRaV-2 was achieved to the highest level and the progenies showed a marked increase in cumulative weight growth (21%),

fresh fruit yield (22%), and sugar content of fruit juice (9%). Selective elimination of GLRaV-1, GLRaV-3, and GVB also provided a beneficial impact in terms of growth and yield, but maturity index was variably affected. The results suggested that GLRaV-2 should be included in certification programs because of detrimental effects on the grapevine growth and yield (Komar et al. 2007).

In vitro thermotherapy and tissue culture technique were combined for elimination of *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) from *Vitis vinifera* cultivar Agiorgitiko. GRSPaV was found to be quite resistant to elimination, whereas GLRaV-1 was more easily eradicated. In vitro thermotherapy was performed in a growth chamber for 6 weeks with a 16 h photoperiod. The initial temperature of 26°C (day) and 23°C (night) was gradually increased by 3°C/week; during the final week, the temperatures maintained were 40°C (day) and 37°C (night). Regeneration rates from meristem- and shoot-tip culture were 55.7% and 80.3%, respectively. The combination of thermotherapy and tissue culture effectively eliminated both GLRaV-1 and GRSPaV-1. Both viruses were more readily eliminated when thermotherapy was followed by meristem-tip rather than shoot-tip culture procedure. The respective values were 91.2% and 73.8% for GLRaV-1 and 67.6% and 50.8% for GRSPaV-1. On the other hand, 61.8% meristem-tip and 37.7% of shoot-tip culture-derived plants were free from both viruses. Elimination of GLRaV-1 was easier than that of GRSPaV-1 with both meristem-tip and shoot-tip culture methods. The ratio of virus elimination to explants survival was higher for meristem-tip than for shoot-tip culture (1.108 and 0.469, respectively). Freedom of regenerated plantlets from GLRaV-1 and GRSPaV-1 was confirmed by employing nested RT-PCR assay (Skiada et al. 2009).

The elimination of viruses and viroids has been shown to be possible by employing micrografting technique in vitro from local citrus cultivars and/or clones in Cyprus during 2000–2005 through a special program. All mother plants were tested for viruses and viroids by biological indexing and ELISA for *Citrus tristeza virus* (CTV) and by RT-PCR assay for viroids. All mother plants were found to be free of CTV and *Citrus viroid V*, but were found to be infected by *Citrus exocortis viroid* (CEVd) and/or other viroids including *Citrus cacahexia viroid* (CaVd) and *Citrus bent leaf viroid* (CBLVd). Some mother trees were infected by *Citrus psorosis virus* (CPsV). Shoot-tip grafting technique was employed for the elimination of viroids and CPsV in the selected citrus isolates. The average micrografting success was 40–60% from the 500 micrografts. Micrografted plants were regrafted on sour orange seedlings in vivo and were established successfully and transferred to the glasshouse. They were tested at 6–9 months later for the viruses and viroids present in the meristem of donor mother plants. The micrografted plants free of CPsV and viroids were used as primary source materials for the basic citrus plantation and for further propagation for distribution through government agencies, private nurseries, and private growers. The micrografting technique was successfully employed to produce healthy planting materials of valuable traditional varieties in Cyprus, and the healthy plants were made available to citriculture (Kaparia-Isaia et al. 2007).

The sanitation procedure of shoot-tip grafting (STG) has been used extensively in citrus improvement programs to restrict *Citrus tristeza virus* (CTV) incidence and spread. Shoot tips of 0.14–0.18 mm in length, aseptically isolated from diseased citrus plants, were grafted (inverted T graft) onto young etiolated seedlings grown in vitro. About 1 month after grafting, plants were transferred in vivo and these plants

set fruits 2–3 years later. To increase explants survival, bigger shoot tips were collected from thermo-treated buds at 32–35°C. This thermotherapy treatment reduced virus replication and increased meristematic cell activity, facilitating production of clean shoot-tips for use in shoot-tip grafting (STG). The combination of thermotherapy and STG was useful to eliminate *Citrus psorosis virus* (CPsV) which was found to be difficult to eliminate by STG alone. Safe introduction of citrus germplasm could be achieved by micrografting in vivo shoot tips excised from imported budsticks in vitro cultured at 32°C. The number of successful grafts depended on *Citrus* spp. varieties and type of virus strain, in addition to the operator's skill which was considered a crucial factor for survival and sanitation rate (Navarro et al. 1975, 1991; Carvalho et al. 2002; Meziane et al. 2009).

Citrus somatic embryogenesis from stigma and style in vitro culture is another technique used for total recovery of healthy plants from most of infected *Citrus* spp., except for clementines and pomelos. Styles and stigmas were dissected from freshly collected closed flowers under laminar flow. Explants were vertically placed in petridishes with the cut surface in contact with the medium supplemented with N6-benzyl-aminopurine (BAP). A creamy-white callus appeared on the cut surface of the style base at about 2 weeks after culture initiation, whereas green somatic embryos developed at 2–7 months after several subcultures. After germination, embryos developed into plantlets which were transferred in vivo. Regenerated plants produced fruits on some branches after 3 years. All embryos formed were totally free of CTV. The regenerated plants could be used in the safe exchange of citrus germplasm with very little manipulation. The crucial point in the successful regeneration of plant by embryogenesis was the requirement of collection of flower explants before opening or storing at 4°C for 5–6 days (De Pasquale 1999).

Thermotherapy followed by the microbudding technique along with indexing by ELISA test was applied to produce and multiply *Citrus tristeza virus* (CTV-) free Musambi plants. Source plants were maintained under the greenhouse conditions for thermotherapy. The budwood of 3 and 4 mm in size was budded at two different heights (15 and 23 cm) of the plants. Source plants of sweet orange, both infected and apparently healthy were collected from orchards. The microbudded Musambi sweet orange plants were indexed for CTV by DAS-ELISA test. Maximum (50%) success of microbudding was achieved when budwood of 4 mm was microbudded at a height of 23 cm. The results showed that thermotherapy combined with microbudding and indexing by ELISA test might significantly reduce the time to obtain CTV-free Musambi plants (Abbas et al. 2006).

Indian citrus ringspot virus (ICRSV), infecting Kinnor (*Citrus nobilis* x *C. deliciosa*) plants, is widespread in India. The effect of thermotherapy coupled with shoot-tip grafting (STG) in vitro on the elimination of ICRSV from infected plants was assessed. Nodal segments from infected mother plants (indexed by ELISA test and RT-PCR assay) were treated both in water bath and moist hot air at the different temperatures of 40, 45, and 50°C for 30, 60, and 120 min, and cultured on Murashige-Skoog medium (MS). Mother plants from which the explants were taken were found to be ICRSV-positive by indirect ELISA format. Application of RT-PCR assay showed the presence of ICRSV-specific 539bp fragment of partial coat protein gene in mother plants. On the other hand, no virus-specific product was detected in some of the in vitro raised plantlets through thermotherapy coupled with STG procedure. Shoot tips were excised from nodal

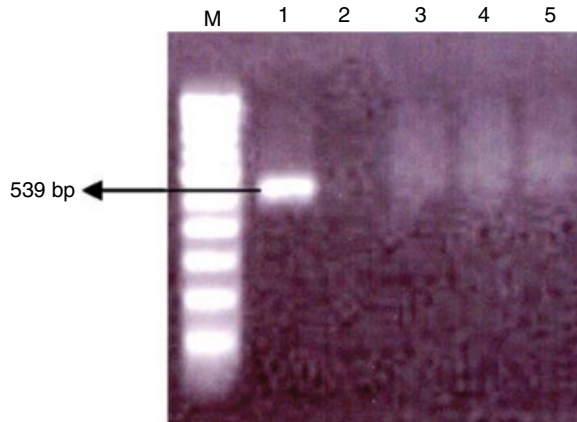


Figure 10.1 Effectiveness of in vitro thermotherapy coupled with shoot-tip grafting for elimination of *Indian citrus ringspot virus* (ICRSV) from citrus plantlets. Note the absence of ICRSV-specific amplicons in treated plantlets. Lane M: DNA size standards; lanes 1 and 2: positive and negative controls, respectively; lanes 3–6: samples from treated citrus plantlets. Source: Sharma et al. (2008). Reproduced with permission of Springer Science, Heidelberg, Germany.

sprouts and grafted onto rough lemon (*C. jambhiri*) root stock under aseptic conditions. Water bath treatment was more effective compared to moist hot air treatment, as the maximum number of ICRSV-free plants (36.84%) was obtained by grafting the tips (0.7 mm) taken from the nodal segments treated at 50°C in the water bath for 120 min. With treatment of 1 year old infected plants, a maximum of 60% ICRSV-free plants could be obtained by grafting the tips (0.7 mm) from plants treated at 40°C. Plants/plantlets negative to both ELISA and RT-PCR assays were considered as virus-free (Fig. 10.1) (Sharma et al. 2008).

Thermotherapy coupled with shoot-tip grafting technique was applied for the elimination of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), *Apple mosaic virus* (ApMV), and *Apple scar skin virus* (ApSSV) from apple plants infected by one or more viruses. The mixed infections were detected by RT-PCR assay in 95.8% of the potted apple plants of four cultivars, and these plants were subjected to thermotherapy (37 + 1°C) followed by shoot-tip grafting. The sprouts of cultivar Dahongrong (DHR) and Qiyuetianxian (QYT) were inhibited by high-temperature treatment. However, some buds of these cultivars were able to sprout and grow slowly. The survival rate of shoot tips cut after thermotherapy was 41.2% (61/148). The survival rate of DHR (63.2%) was highest among the four cultivars tested. An average virus elimination rate of 65.6% (40/61) for new leaves and 37.7% (23/61) for dormant branches was attained. The results indicated that the virus detection and elimination was more difficult in the dormant branches than in actively growing plants (Hu et al. 2015).

Sweet potato plants are infected by many viruses; infection by *Sweet potato feathery mottle virus* (SPFMV) is more frequently observed in various countries. Apical cuttings (65, each 15 cm in length) from the cultivar Abees plants were collected and tested for the presence of SPFMV using spot-ELISA test. The cuttings were found to be infected by SPFMV and kept under controlled glasshouse conditions. Infected plants were

subjected to thermotherapy (42°C by day and 39°C by night for 3 weeks). Meristem-tip culture method was followed and regenerated plants were grown in vitro. Of the 65 nodal cuttings infected by SPFMV, 62 were found to be virus-free after thermotherapy and meristem-tip culturing. RT-PCR assay was employed to ensure freedom of rooted plantlets from SPFMV virus (Fig. 10.2). The tissue-culture-generated plants were tested for two successive years using dot-ELISA format and the plants remained virus-free under in vitro conditions (El-Far and Ashoub 2009).

A system of production of virus-free lilies involving repeated shoot meristem excision, before and during in vitro culture and thermotherapy applied to bulblets in vitro, was developed. *Lily symptomless virus* (LSV) causes no distinct symptoms, but decreases plant growth, vigor, and the quality of cut flowers. Stock bulbs of pollenless Asiatic hybrid lily (*L. x elegans*) lines (499 and 599) were used as explants. Excised shoot meristems were micropropagated. In vitro grown bulblets were subjected to thermotherapy at 35°C for 42 days and a second meristem cut was made from heat-treated material. Leaf tissues from bulblets formed prior to post-heat treatments were tested by ELISA test or RT-PCR assay. Line 499 produced LSV-free plants without heat treatments. On the other hand, heat treatment was needed to eliminate LSV from line 599. The amplified banding patterns after electrophoresis in agarose gel followed by staining with ethidium bromide revealed the absence of the virus-specific band in line 499 after first meristem isolation. The virus-free bulblets grew vigorously in vitro and acclimatized well. Thermotherapy applied to in vitro growing bulbs might effectively eliminate the virus, resulting in the production of fast-growing mother plant stocks (Nesi et al. 2009).

Standard thermotherapy employed to eliminate *Potato virus X* (PVX) from potato (*Solanum tuberosum*) microplants of the Mexican National Potato Program was modified to improve the effectiveness of thermotherapy. Microplants were cultured with or without 10^{-5} M salicylic acid (SA) for 4 weeks, then subcultured without SA and exposed to 42°C for 30 days. Survival was more consistent in SA-treated microplants. Among seven genotypes, 40–100% of SA-treated plants and 0–96% of controls survived at the end of thermotherapy. In addition, SA also improved survival of post-thermotherapy subculture. Among the surviving microplants, SA increased the yield of virus-free microplants up to 100% as against 40–65% in the controls. Further, 98% of the surviving SA-treated microplants of 30 PVX-positive genotypes became free of PVX after thermotherapy treatment, compared to 75% of virus-free control plants. Assessment of physiological activities of SA-treated microplants indicated that SA treatment induced higher hydrogen peroxide levels, while catalase activity was reduced by SA treatment (López-Delgado et al. 2004).

Auxins or antiviral chemicals have been applied to virus-infected plants to accelerate stem elongation or to inactivate the virus to obtain virus-free plants. Sweet potato plants infected by leaf spotting, internal cork, and feathery mottle viruses were treated with a combination of kinetin and indole acetic acid (IAA). The meristems of treated plants elongated at a rate faster than the virus could invade newly formed tissues, providing a longer piece of meristem free of virus (Alconero et al. 1975). Among the several antiphytoviral chemicals tested, ribavirin (also known as virazole, 4(5)-amino-1H-1,2,3-triazole-5(4)-carboximide) was found to show higher inhibitory activity against many animal and plant viruses (Matthews 1991). A combination of thermotherapy and incorporation of ribavirin as a component of growth medium in which the apical meristem was grown enhanced the effectiveness of virus elimination. This approach

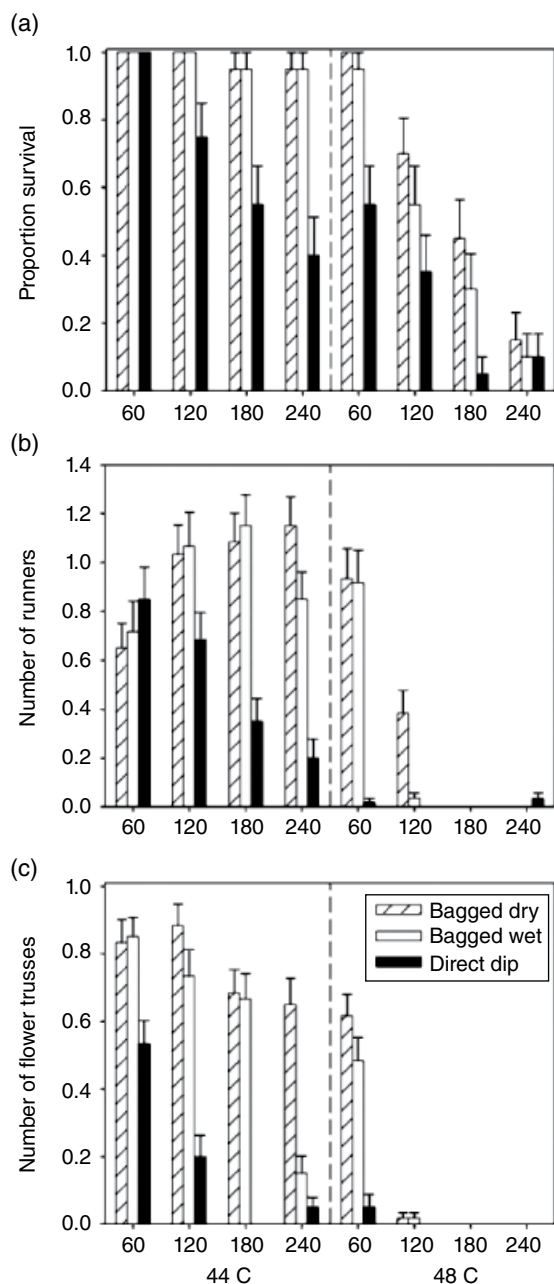


Figure 10.2 Efficiency of hot water treatment (HT) in eliminating *Xanthomonas fragariae* from strawberry plants. (a) Survival of plants (percentage) after different periods of heat treatment (min) at 44°C and 48°C; (b) average number of runners in survivors after HT; and (c) average number of flower trusses formed in plants exposed to HT. Source: Turechek and Peres (2009). Reproduced with permission of the American Phytopathological Society, MN, USA.

was adopted for the successful elimination of *Peanut stripe virus* (PStV) and *Tomato spotted wilt virus* (TSWV) from peanut plants (Dunbar et al. 1993). A similar approach was successfully applied for the elimination of *Potato virus S* (PVS) from potatoes. However, seven subcultures were required to eliminate PVS from the potato cultivar Red Pontiac. The potato plantlets were free of PVS, as revealed by ELISA tests. The combined treatment of thermotherapy and chemotherapy demonstrated a synergistic effect on virus elimination (Park et al. 1994; Kim et al. 1996).

Carnation latent virus (CLV) remains latent in several carnation cultivars, but causes considerable loss in yield and quality. CLV induced distinct chlorotic spots and/or mosaic mottling in the carnation cultivar Scania. The infected plants were placed in the heat therapy chamber at $35 \pm 2^\circ\text{C}$ and $40 \pm 2^\circ\text{C}$ for 1, 2, 3, and 4 weeks. Axillary bud explants from the heat-treated and untreated plants were cultured on MS medium. Regenerated plants were indexed by indirect ELISA and indicator plants. The survival of plants had a negative correlation with temperature treatment. Plants exposed to $35 \pm 2^\circ\text{C}$ for 4 weeks and those treated at $40 \pm 2^\circ\text{C}$ for 3 weeks were freed from CLV. ELISA tests confirmed the virus-free nature of these carnation plants following thermotherapy (Mangal et al. 2004).

Plum pox virus (PPV) causes the sharka disease, which is considered to be one of the most devastating virus diseases of plum in many countries in the Europe. One of the preventive measures suggested for the management of PPV is the use of virus-free planting materials to establish healthy orchards. Different methods such as meristem-tip culture and thermotherapy have been applied to produce virus-free planting materials. The effectiveness of chemotherapy in vitro with ribavirin to produce PPV-free plum plants (*Prunus domestica* cultivar Cacanska Lepotica) was evaluated. The infected shoots were multiplied in MS medium amended with different concentrations of ribavirin for 6 weeks. Regenerated and rooted plants were grown under greenhouse conditions for 2 years. The plants were tested by DAS-ELISA and IC-RT-PCR assay for the presence of PPV. Chemotherapy with ribavirin at 4 and 6 mg L^{-1} was effective in producing PPV-free plants to the extent of 15.38 and 16.66%, respectively. Phytotoxicity symptoms due to higher concentrations (80 and 100 mg L^{-1}) were observed. The DAS-ELISA test was less efficient in detecting PPV in ribavirin-treated plantlets, and IC-RT-PCR assay could detect PPV in several ELISA-negative plantlets (Paunovic et al. 2007).

10.3.1.2 Chemotherapy

Potato viruses *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Potato leafroll virus* (PLRV) were present in potato plantlets singly or in a mixture of *Potato virus M*, PVS, and PVY. Infected plantlets were raised in the MS medium amended with different concentrations of ribavirin (0 – 200 mg L^{-1}), an antiviral chemical. Phytotoxicity due to ribavirin increased with increasing concentration of the chemical, as reflected by plant height and fresh weight of treated plantlets. At the maximal concentration (200 mg L^{-1}), the plantlets could not survive. Ribavirin was more efficient in eliminating PVA, PVM, PVS, and PVX than PVY and PLRV, as revealed by ELISA tests. Healthy plantlets could be generated with high frequency (up to 100%) by culturing the plantlets in the MS medium containing 75 – 150 mg L^{-1} ribavirin after 2–3 subcultures. On the other hand, PVY- and PLRV-free plants could be obtained by using ribavirin at 75 – 150 mg L^{-1} in the medium for growing plants after three subcultures. Reduction in virus content following ribavirin treatment

was indicated by quantitative RT-PCR (qPCR) analysis. Healthy seedlings could be generated from infected stocks at the first or end of propagation and no virus could be detected at the post-eradication stage. No genetic variation in plantlets could be discerned in ribavirin-treated plantlets. The results indicated the possibility of eliminating viruses from potatoes for the propagation of virus-free planting materials using antiviral chemicals (Yang et al. 2014).

10.3.1.3 Electrotherapy

Electrotherapy is a simple method of virus eradication without the need to use any special or expensive equipment. In this technique, an electric current is applied to plant tissues in order to disrupt or degrade viral nucleoprotein and eliminate its pathogenic potential. This technique was first comprehensively employed for the elimination of *Potato virus X* (PVX) from potato plants. Potato stems infected with PVX were treated with electrical current at 15 milliamperes (mA) for 5 min and the axillary bud tips planted in growth medium in vitro immediately. The tissue temperatures in treated stems increased from 4 to 10°C during exposure to electric current. The therapy efficiency was determined as the product of percentage of virus-free plants after a 60 day growing period in vitro. Both organogenesis and virus elimination were stimulated by electrotherapy. The treatment resulted in 40–80% regeneration of buds and 60–100% of the regenerated plantlets were free of PVX (Lozoya-Saldaña et al. 1996).

Successful elimination of several viruses such as *Potato virus X*, *Potato virus Y*, *Potato virus A*, *Potato virus S*, and *Potato leafroll virus* (Pazhouhande and Mozafari 2001), *Onion yellow dwarf virus*, and *Leek yellow stripe virus* from garlic (Hernández et al. 1997) and *Banana streak virus* from banana (Hernández et al. 2002) using electrotherapy procedure, has been reported. *Bean common mosaic virus* (BCMV) is a major seed-transmitted virus infecting common bean (*Phaseolus vulgaris*). Production of BCMV-free seeds is the basic requirement for certification of seeds.

The efficiency of electrotherapy in eliminating BCMV and producing virus-free plants of two common bean cultivars Khomein and Capsouli was evaluated. Nodal cuttings of infected plants were exposed to electric currents of 5, 10, and 15 mA for 10 min. The cuttings were cultured in test tubes containing MS5 medium. The test tubes were maintained in a growth chamber under light conditions of 16 h light and 8 h darkness at 23–25°C. One month old regenerated plants were indexed by DAS-ELISA test for the presence of BCMV. The three intensities of electric current resulted in mean virus elimination percentages of 37.2%, 45.5%, and 71.9% and mean plant regeneration of 79.5%, 68.6%, and 58.4%, respectively. The therapy efficiency index (TEI) for three electrotherapy treatments was determined to be 28.9, 29.2, and 39.1 in cultivar Khomein and 26.9, 33.3, and 44.8 in cultivar Capsouli. The results showed that the electric current of 15 mA for 10 min most effectively eliminated BCMV from both common bean cultivars tested. Regenerated plants after exposure to electrotherapy were morphologically identical to those regenerated from nontreated control explants. Electrotherapy was found to be more effective, simpler, faster, easier, and less demanding than other techniques applied for elimination of viruses from seeds (Hormozi-Nejad et al. 2010).

10.3.1.4 Cryotherapy

Cryopreservation of shoot tips is a process in which shoot tips are exposed to ultra-low temperature, stored, and regenerated for multiplication. A critical step in this process is dehydration of cells before immersing tissues into liquid nitrogen. Crystallization of

intracellular water inside cells damages them lethally, because the crystals penetrate membrane structures. In cryotherapy, lethal injury of cells during cryotreatment is utilized to kill infected tissues/cells that are located far from the apical dome (AD), as they are more likely to be infected by viruses or phytoplasmas and are more sensitive to injury than the cells that are located in the meristematic zone of AD. Cells in the AD and in the first-leaf primordia are therefore likely to survive and regenerate into pathogen-free shoots. Consequently, a larger proportion of plants regenerated following cryotherapy may become pathogen-free than what could be obtained through traditional meristem culture. The effectiveness of cryotherapy may be due to enhancement of pathogen eradication by combining with thermotherapy of shoots before excision of shoot tips. Cryotherapy provides an alternative, efficient strategy for eradication of plant pathogens in many crops. Cryopreserved shoot tips may be considered to be safer for exchange of germplasm between countries and regions within a country, since the cryogenic methods may reduce the amount of viable infected tissues (Panis et al. 2005; Wang et al. 2008b).

Banana crops are seriously affected by *Cucumber mosaic virus* (CMV) (genus *Cucumovirus*) and *Banana streak virus* (BSV) (genus *Badnavirus*), reducing the fruit yield significantly. Cryotherapy by vitrification was applied to eradicate CMV and BSV from in vitro cultures of meristematic clumps of desert banana cultivar Williams. The regenerated plantlets were free of CMV and BSV up to 30% and 90%, respectively (Helliot et al. 2002). *Plum pox virus* (PPV), causative agent of the most destructive sharka disease, infects stone fruits of *Prunus* spp., including plum, peach, almond, nectarine, sweet cherry, and sour cherry. By using cryotherapy, virus-free plants were generated from in-vitro-grown shoot tips taken from inoculated plants. A vitrification protocol was applied for elimination of PPV. Virus-free plants were obtained with high frequencies (45–60%), regardless of the size of the shoot tips used. Larger shoot tips (0.5–2.0 mm) had better regrowth (56–70%) as against the regrowth of 11% with smallest shoot tips (0.3–0.5 mm) (Brison et al. 1997).

Notable success in eradicating viruses from infected plants has been reported in grapevine and raspberry plants. *Grapevine virus A* (GVA) could be eliminated from cultivar Bruti. About 96% of plants regenerated from cryotreated shoot tips were virus-free, while only a low percentage of plants (12%) could be freed from GVA by following meristem-tip culture method (Wang et al. 2003). It was difficult to eliminate *Raspberry bushy dwarf virus* (RBDV) from infected plants by thermotherapy. By combining thermotherapy of RBDV-infected shoot cultures with by cryotherapy of shoot tips excised from heat-treated shoots, RBDV could be eliminated from plantlets up to 33–35% (Wang et al. 2008a).

Potato virus Y (PVY) (genus *Potyvirus*) and its strains and *Potato leafroll virus* (PLRV) (genus *Polerovirus*) occur in most potato-growing countries around the world, and they are important factors limiting production. Three cryogenic procedures (encapsulation-dehydration, encapsulation-vitrification, and droplet-vitrification) were employed to eliminate PVY and PLRV. Naturally doubly infected plants were treated with the cryogenic methods. Both viruses were efficiently eradicated by all three procedures resulting in 83–86% and 91–95% plants free of PLRV and PVY respectively, with no significant differences in their efficacy (Wang et al. 2006). Sweet potato feathery mottle virus (SPFMV) belonging to the genus *Potyvirus* (transmitted by aphids) infects all types of tissues of sweet potato. *Sweet potato chlorotic stunt virus* (SPCSV) belonging to the genus *Crinivirus* (transmitted by whiteflies) is restricted to the phloem tissue of

infected sweet potato plants (Wang and Valkonen 2008b). These two viruses are found to infect sweet potato plants simultaneously causing the complex sweet potato virus disease (SPVD), the most serious limiting factor for production (up to 90% losses) (Mukasa et al. 2006). As sweet potatoes are clonally propagated, the need for using virus-free planting materials was realized. Shoot tips (0.5–1.5 mm) from sweet potato plants infected by SPFMV or SPCSV singly or in combination and treated by cryotherapy were propagated in MS medium. These two viruses could be eradicated from all (100%) shoots by cryotherapy treatment. On the other hand, meristem-tip culture produced only 7–10% SPFMV-free regenerants from the coinfecting plants (Wang and Valkonen 2008b; Section 10.4.1).

10.3.2 Production of Viroid-Free Plants

Viroids are transmitted through seeds and propagules, as for plant viruses. They may coinfect plants along with viruses, causing complex symptoms. The presence of viroids in seeds and propagules may be detected by various techniques described elsewhere (Chapter 7), and the infected seeds and planting materials can be freed of the viroids to obtain viroid-free nuclear stocks by eliminating infected mother plants. The presence of *Potato spindle tuber viroid* (PSTVd) in the propagating materials may be detected by inoculating an intermediate tomato plant to amplify PSTVd to enhance the analytical sensitivity of detection based on the PAGE technique (Morris and Smith 1975). Return gel electrophoresis (RGE) was more effective in detecting PSTVd in potato true seeds and was comparable with or superior to the nucleic acid hybridization procedure (Singh et al. 1988).

Citrus spp. infected by different viroids and mixed infections by two or more viroids have been observed. Sequential polyacrylamide gel electrophoresis (sPAGE) method was effective in detecting *Citrus exocortis viroid* (CEVd), *Citrus viroid II* (CVd-II), and *Citrus viroid III* (CVd-III) in the extracts of young bark tissues. CEVd, CVd-II and CVd-III were detected during warm season (June to November), but CEVd could also be detected in other months (Tessitori et al. 1996). The sPAGE technique was successfully applied for the detection of *Hop stunt viroid* (HSVd), CEVd, and *Citrus viroid III*. Two or more viroid-like RNAs with similar electrophoretic mobility as CEVd, *Citrus bent leaf viroid* (CBLVd), HSVd, CVd-II, and/or CVd-III were also detected. The sPAGE detection results were similar to those obtained with Northern and dot-blot analyses. The techniques had the potential to eliminate infected source plants and select healthy mother plants for propagation of planting materials (Malfitano et al. 2005). Northern blot hybridization procedure was applied for the detection of *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CEV-III), and *Citrus viroid IV* (CVd-IV) in inoculated Etrog citron used as biological indicator (Barbosa et al. 2005). A total of 113 samples from commercial citrus orchards and nurseries were tested. Washington Navel and Naveline oranges (11 of 32) were found to be infected by CTV, whereas Clementine trees (4) were infected by *Citrus infectious variegation virus* (CIVV). All of these virus-infected trees were also coinfecting by HSVd, and 50% were also infected by *Citrus exocortis viroid* (CEVd) (Barbosa et al. 2007).

Tissue-printing hybridization (TPH) technique was effective in detecting viroids infecting stone fruit trees. Of the 352 stone fruit samples tested, 88 were found to be infected by viroids. *Peach latent mosaic viroid* (PLMVd) infection was detected in

61 peaches and apricots. The remaining 27 samples showed infection by *Hop stunt viroid* (HSVd). Mixed infection by PLMVd and HSVd were detected in two samples. The TPH assays provided a relatively clear assessment of the sanitary status of stone fruit crops in Turkey (Gümüş et al. 2007). In another investigation, tissue-printing hybridization (TPH) was demonstrated to be effective for the detection of *Apple scar skin viroid* (ASSVd) and *Pear blister canker viroid* (PBCVd) in field-grown plants. Using nonradioactive probes specific to the respective viroids, all three viroids were effectively detected by TPH as well as by dot-blot hybridization techniques in experimentally inoculated apple plants. Each probe hybridized strongly and exclusively with the complementary viroid RNA. Petioles collected from apical parts of the shoots reacted by producing the best TPH signals, suggesting that petioles of young leaves are most suitable tissue for detection of viroids and differentiation of viroids by TPH procedure (Lolic et al. 2007).

Peach latent mosaic viroid (PLMVd) and *Hop stunt viroid* (HSVd), both belonging to the family Pospiviroidae, were listed as quarantine pathogens by EPPO because of the heavy losses caused by them. An optimized RT-PCR protocol was developed for detecting PLMVd and HSVd that was suitable for a certification system in operation in the Czech Republic. The primers employed for detection of the viroids were specific and sensitive, capable of detecting nucleic acid equivalent to 10 ng of leaf tissue. PLMVd was detected in several peach cultivars of different ages (5–12 years). HSVd was also detected in leaves, petioles, and bark tissues obtained from field-grown cultivar NJC103 plants (Hassan et al. 2004). RT-PCR assay was efficient in detecting PLMVd in peach and pear trees, HSVd in pear, peach, and almond trees and *Pear blister canker viroid* (PBCVd) in pear trees. Mixed infections of pear with PBCVd + HSVd and PLMVd + HSVd were detected. This protocol had the potential for use in routine indexing of plants and planting materials in certification programs (Hassani et al. 2006).

10.3.3 Production of Bacterial and Phytoplasmal Pathogen-Free Plants

Seeds and planting materials carry a large number of bacterial pathogens. They may reduce the seed quality and germination capacity and seedling vigor. Depending on the extent of seed-to-plant transfer, they are capable of causing significant losses; infected plants growing from infected seeds/propagules may become potential infection foci for a secondary spread of the disease(s). Different heat treatments have been applied for the elimination of bacterial pathogens from seed, although the seed germination is affected to varying degrees. A combination of seed dry heat treatment and thermotherapy of seedlings followed by meristem- and shoot-tip culturing was evaluated for elimination of common and halo blight pathogens *Xanthomonas campestris* pv. *phaseolicola* (*Xcp*) and *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) from bean seeds that form the primary sources of inoculum for these diseases. The bean seeds exposed to dry heat at 47–48°C for 3 days had significant reduction of germination. However, the contamination rate of explants excised from seedlings of dry-heat-treated seeds and cultured for 3 weeks in MS medium was significantly lower than that recorded in seedlings of untreated seeds. Seedlings growing from heat-treated seeds were exposed to thermotherapy for 10–14 days at 37°C. Meristem- and shoot-tips from plants that were exposed to thermotherapy treatment were cultured. The bacterial pathogens *Xcp* and *Psp* were eliminated effectively from bean seeds. Although common bean was recalcitrant in culture, the regeneration of shoots and transfer of a few plantlets for acclimatization was successfully accomplished.

Tissue cultures showed lower levels of bacterial contamination because of the dry heat treatment of seeds and thermotherapy of seedlings grown from heat-treated bean seeds, resulting in the elimination of *X. campestris* pv. *phaseoli* and its fuscous variant (Grum et al. 1998).

Citrus huanglongbing (HLB) disease occurs in more than 40 countries in Asia, Africa, and the Americas and is reported to be caused by three different bacterial species, namely "*Candidatus Liberibacter asiaticus*," "*C. L. africanus*," and "*C. L. americanus*." The HLB pathogen could be eliminated from infected in vitro plantlets by cryotherapy of shoot tips and HLB-free plants were produced at a frequency of 98%. Cryotherapy of shoot tips of sweet orange accession Luogang (*Citrus sinensis*), mandarin Ponkan (*C. reticulata*), Shatianyou Pumelo (*C. grandis*), and Beijing lemon (*C. limon*) resulted in production of almost all regenerated plants being HLB-free to the extent of 93%, 93%, 94%, and 91% respectively, indicating the effectiveness of cryotherapy in eradicating this important citrus pathogen (Ding et al. 2008).

Pecan (*Carya illionoinensis*) bacterial leaf scorch (PBLs) disease caused by *Xylella fastidiosa* (Xf) reduced the nut yield significantly when the disease became persistent for several years in the infected trees. Graft transmission through infected scion wood is one of the principal modes of Xf transmission, because pecan cultivars are clonally propagated. Submersion of pecan scion wood in 46°C for 30 min reduced transmission of Xf to a great extent following grafting. During a 2 year investigation, potted rootstock was grafted to either hot-water-treated or nontreated scion wood collected from limbs of "Cap Fear" pecan infected with Xf. The pathogen was detected in 21% of the trees that developed from nontreated scion wood and 0.7% of the trees from hot-water-treated scions. Only 1 of the 140 trees produced from hot-water-treated scions was infected with Xf. Hot water treatment (46°C for 30 min) did not affect graft success and the scion diameter did not affect graft establishment or efficiency of the hot water treatment. No increase in the number of pecan plants showing symptoms in plants grafted with hot-water-treated scion was observed during the 2 year period (2005–2006). The results indicated that hot water treatment of scion wood material was very effective in reducing transmission of PBLs disease through scion wood. Elimination of Xf infection was also successfully achieved in grapevine (Sanderlin and Melanson 2008).

Xanthomonas fragariae, incitant of strawberry angular leaf spot disease and listed as an A2 quarantine pathogen, affects nursery production seriously. This pathogen is transmitted to production fields almost exclusively through infected nursery stock. As the pathogen is absent from European countries, nurseries have to maintain specific phytosanitary standards to avoid rejection. Planting materials should be derived from mother plants certified free of *X. fragariae*. The effectiveness of heat treatment for killing or reducing the number of viable bacterial cells in the crown tissue was assessed. Plants were treated by immersing them in hot water at 44 or 48°C for 0, 60, 120, 180, and 240 min. The plants were then potted after thermotherapy treatment and rated for growth characteristics. Results showed that bacterial populations in cultures exposed to 56°C and 52°C were killed completely after 15 min and 60 min of exposure, respectively; however, both treatments killed the plants. Bacterial populations exposed to 44°C for 4 h or 48°C for 2 h were reduced by 10^5 or 10^6 CFU mL⁻¹. These treatments minimally affected vegetative growth of plants bagged dry (sealed in plastic bag which was immersed in water). Flowering in treated plants was adversely affected. These heat treatments were applied for several cultivars in field trials established in two locations in successive years. The survival rate among cultivars was similar to that observed in

Table 10.1 Effect of heat treatments on survival of strawberry plants infected by angular leaf spot disease under field conditions (Turechek and Peres 2009).

Cultivar	Temperature (°C) ^a	Duration (min) ^a	Disease rating	
			Survival ^b	LSM ^c
Ventana	UTC	UTC	20.00	0.692a
Camino Real	UTC	UTC	19.75	0.268bc
Camarosa	UTC	UTC	20.00	0.302b
Festival	UTC	UTC	20.00	0.083de
Festival (dry)	UTC	UTC	20.00	0.153cd
Ventana	44	240	10.00	0.024def
Camarosa	48	120	13.75	0.006ef
Ventana	48	120	18.00	0.013ef
Festival	44	240	14.50	0.000ef
Festival	48	120	14.75	0.000ef
Camino Real	48	120	15.75	0.000ef
Camino Real	44	240	14.75	0.000ef
Camarosa	44	240	15.00	0.000f
Festival (dry)	48	120	19.50	0.012ef
Festival (dry)	44	240	19.75	0.000f

^a For each cultivar, treatments were 44°C for 4 h bagged dry, 48°C for 2 h bagged dry, or an untreated control (UTC); ^b Mean number of plants per plot (maximum = 20) surviving heat treatment; ^c Least square means (LSM) are derived from the interaction of cultivar treatment; means followed by the same letter are not significantly different from each other.

greenhouse trials. The thermotolerant cultivars might be considered for heat treatment to reduce the pathogen population to acceptable levels and consequent reduction in disease incidence (Table 10.1). However, careful attention should be paid to monitoring the disease development in the strawberry cultivar(s) selected for raising nurseries (Turechek and Peres 2009).

Phytoplasmas cause several important diseases affecting many crops, especially horticultural and floricultural plants. The infected asexually propagated plant materials are the primary sources of inoculum and carry the infection to subsequent generations if the planting materials are not properly indexed and healthy materials are not selected for planting. Budwood materials, setts, and tubers therefore have to be thoroughly indexed for the presence of phytoplasmas by employing efficient and sensitive techniques. Among the phytoplasmas infecting potatoes, phytoplasmas causing potato purple top (PPT), potato witches' broom (PWB), and potato stolbur diseases are important and occur in North and South America and eastern Europe. Eight batches of seed potatoes collected from different locations were planted under glasshouse conditions. A total of 625 plantlets were indexed as they were asymptomatic. Nested PCR assay of both single and grouped samples with general ribosomal primers without spacer region allowed detection of phytoplasmas. Phytoplasmas belonging to diverse ribosomal

groups were identified after RFLP analysis. Ribosome subgroups 16 SrII-D (related to stolbur) were identified in different percentages. The protocol used in this investigation reflected OEPP/EPPO 2004, which was found to be reliable and sensitive for the elimination of phytoplasma-infected seed tubers when the plantlets growing from them were asymptomatic which might escape visual examination. Nested PCR could be employed to screen potato seed tuber lots for high-quality seed potatoes (Paltrinieri and Bertaccini 2007).

Phytoplasma-free plants could be obtained by the stem culture technique. Mulberry stem segments of nursery plants infected by dwarf phytoplasma were cultured on MS medium for 2–3 months. The regenerated plants were grown in the greenhouse for 3 years and then indexed by employing PCR assay, DAPI staining, and visual examination for the development of disease symptoms. A high percentage of regenerated plants (70–80%) were found to be free of mulberry dwarf phytoplasma (Dai et al. 1997). Elm yellows (EY) disease occurring in North America and Europe are caused by different strains of the phytoplasma. The effectiveness of hot water treatment (HWT) on the elimination of EY phytoplasma was assessed using an experiment device at $50 \pm 0.5^\circ\text{C}$ for 45 min. The plants were forced in the climatic chamber. None of the treated plants developed any adverse effects due to treatment, and they showed earlier bud-burst and better growth characteristics. None of the plants revealed the presence of EY phytoplasma in PCR assay. Plants grown from cuttings taken from EY-infected clones soaked in hot water (50°C for 45 min) did not develop disease symptoms and tested negative for EY phytoplasma. In contrast, untreated control plants developed disease symptoms and were EY-positive in PCR assay. The results showed that HWT was effective in eliminating EY phytoplasma (Boudon-Padieu et al. 2004).

Sugarcane grassy shoot disease (SGSD) is caused by the phytoplasma which is classified as a member of the 16Sr XI-B group. The disease may become severe in ratoon crops if the planting materials (setts) are not prepared from healthy mother plants. Sugarcane setts are traditionally treated by different thermotherapy methods involving the use of hot air, hot water, or aerated steam to inactivate/reduce the pathogen inoculum. The effectiveness of apical meristem culture technique for producing phytoplasma-free planting material was assessed. Apical meristems (2, 3, and 4 mm) were aseptically excised and placed on the MS medium amended with benzylaminopurine (BAP) and kinetin. The developing shoots were subcultured at weekly interval and transferred to multiplication medium. Freedom from GSD phytoplasma was verified using PCR assay and those free of the phytoplasma were multiplied and transferred to pots in the greenhouse. The elimination frequency of the phytoplasma was 80% and 60% for plantlets derived from 2 mm and 3 mm meristems at the first multiplication stage, respectively. The results showed that GSD phytoplasma could be effectively eliminated from planting materials by employing meristem culture techniques (Tiwari et al. 2011).

Grapevine flavescente dorée (FD) phytoplasma, causing flavescente dorée (FD) disease of grapevine, is an EU listed A2 pathogen as included in Annexe II/A2 of the EU Plant Health Directive (2000/20/EC) and as an EPPO A2 Pest recommended for regulation as a quarantine pest. Two types of hot water treatment have been applied as prophylactic and quarantine measures for grapevine propagation material for all countries in the world. A short duration of hot water treatment at 52°C for 5 min was used against the external pathogens. A long-duration hot water treatment was recommended for both external and internal pathogens such as FD pathogen. Hot water treatment may result in a loss of varying quantities of treated planting materials. The planting materials

have to be placed at room temperature (for 12–24 h) both prior to and after hot water treatment at 50°C. After completion of hot water treatment, the treated planting materials should be grafted onto healthy plants. Hot water treatment against FD phytoplasma was found to be a reliable technique and was mandatory for all basic propagation materials in France and Australia (Ministère de l'Agriculture, France 2003; ICA 2007).

Another phytoplasma disease caused by the Bois Noir (BN) phytoplasma is one of the most widespread yellows diseases in grapevines in European and Middle Eastern countries. Propagation of infected plants by nurseries widely contributed to the introduction and spread of BN disease in locations that had previously been free of the disease. The strategy of producing phytoplasma-free planting materials therefore assumed greater importance for the management of BN disease. The efficiency of procedures using tissue culture techniques associated with or without heat treatment or hot water treatment was evaluated for producing phytoplasma-free vines cultivar Chardonnay. Nested PCR assay was employed to ensure the presence or absence of BN phytoplasma in the infected grapevine plants from which young shoots were taken for hot water treatment, and also in shoots derived from tissue cultures. Stem cuttings with or without thermotherapy at $38 \pm 1^\circ\text{C}$ for 4 days were used for culturing. Young shoots (0.4–0.5 mm in diameter) were excised from single-node stem cuttings and grown in MS medium. The nested PCR assays at the end of the first subculture showed sanitation rates varying over 36–100%. All tested shoots regenerated from the stem cuttings treated in hot water bath at 50°C for 15 or 30 min were phytoplasma-free, whereas the sanitary rates dropped to 76% for the shoots regenerated from stem cuttings exposed to heat treatment. In the case of shoots regenerated from shoot tips, heat treatment increased the sanitation rate from 36% to 76%. The second nested PCR assays carried out at the end of the third subculture (after 120 h of culture) revealed the absence of BN phytoplasma in the shoots that were negative in the first test. The hot water bath treatment for 15 min only was also effective in eliminating the BN phytoplasma. Despite the lower explants survival rate at the beginning, hot water treatment was considered as the most efficient sanitation technique since it yielded 100% BN-free shoots. The protocol based on tissue culture followed by micropropagation of sanitized explants appeared to be a good candidate for producing phytoplasma-free vines for certification programs (Chalak et al. 2013).

Sweet potato little leaf phytoplasma (SPLL) pathogen "*Candidatus* Phytoplasma aurantifolia" causes the devastating disease of sweet potato (*Ipomoea batatas*) in several countries. As the crop is clonally propagated, typically using shoot cuttings and also storage roots, the efficiency of cryotherapy for production of phytoplasma-free plants from sweet potato stocks infected with SPLL disease was assessed. Shoot tips (3–4-leaf primordia) were excised from in vitro grown sweet potato plants. Survival (88%) and regrowth (85%) of the shoot tips after cryotherapy was slightly less when compared to control (untreated) treatments. However, no phytoplasma was detected in any of the regenerated shoots. PCR amplification products (c. 1.8 kb) of the expected size for SPLL pathogen was not observed in these regenerated plants (Fig. 10.3). In contrast, phytoplasmal bodies were readily observed in the control untreated plants and all the shoot tips of stock cultures. The phytoplasma-free plants obtained by cryotherapy and grown in the greenhouse had a normal appearance, good growth, and large storage roots, as for the original pathogen-free clone (199004-2). Histological and ultrastructural studies with light and transmission electron microscopy (TEM) indicated that cryotherapy was lethal to all cells except those in the apical dome of the meristem and the two youngest

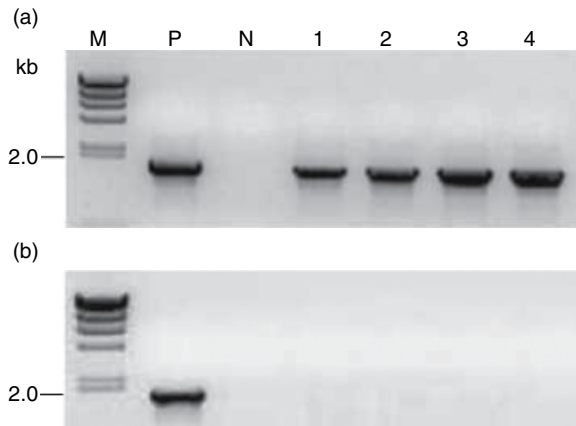


Figure 10.3 Detection of sweet potato little leaf phytoplasma in *in vitro* shoot cultures by PCR assay after cryotherapy. (a, b) Lane M: DNA ladder; lane P: positive control; N: negative control; lanes 1–4: presence of c. 1.8 kb product in (a) and absence of this product from (b) indicating the effective elimination of the pathogen in cryotherapy-treated shoot cultures. Source: Wang and Valkonen (2008a). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

leaf primordia. The surviving parts of the shoot tip contained vascular tissue and sieve elements, but TEM observations did not reveal any phytoplasmal bodies in them. In contrast, abundant phytoplasmal cells could be located at the lower nonsurviving parts of shoot tip 1.5–2.5 mm from the apical dome (Fig. 10.4). In the greenhouse, the plants treated with cryotherapy had vigorous growth that was distinctly different from that of the plants from untreated shoot tips. Cryotherapy provided an alternative tool for elimination of phytoplasmas from infected planting materials. In addition, this procedure could be of use for long-term storage of sweet potato germplasm, facilitating safe international movement of germplasm as cryopreserved stocks (Wang and Valkonen 2008b; Section 10.4.1).

10.4 Appendix

10.4.1 Elimination of Sweet Potato Little Leaf Phytoplasma from Sweet Potato by Cryotherapy of Shoot Tips (Wang and Valkonen 2008b)

10.4.1.1 Preparation of Plant Materials

- i) Surface-sterilize young shoots from plants grown under greenhouse conditions with sodium hypochlorite solution (1%) for 10 min; wash three times with sterile distilled water and blot on filter paper to remove excess water.
- ii) Excise single buds (0.5 cm in length); grow in 9 cm petridishes containing solid survival medium (SM) consisting of basic medium (BM) supplemented with 2.2 μ M benzylaminopurine (BAP); the basic medium composed of Murashige-Skoog medium (MS) inorganic medium supplemented with 88 mM sucrose, 0.62 mM calcium nitrate, 1.1 mM ascorbic acid, 0.4 mM calcium pantothenate, 0.12 mM putrescine dihydrochloride, 0.57 mM L-arginine and 0.24 Gelrite (Merck), and adjust the pH to 6.0 with NaOH prior to autoclaving at 121°C for 20 min.

(a)

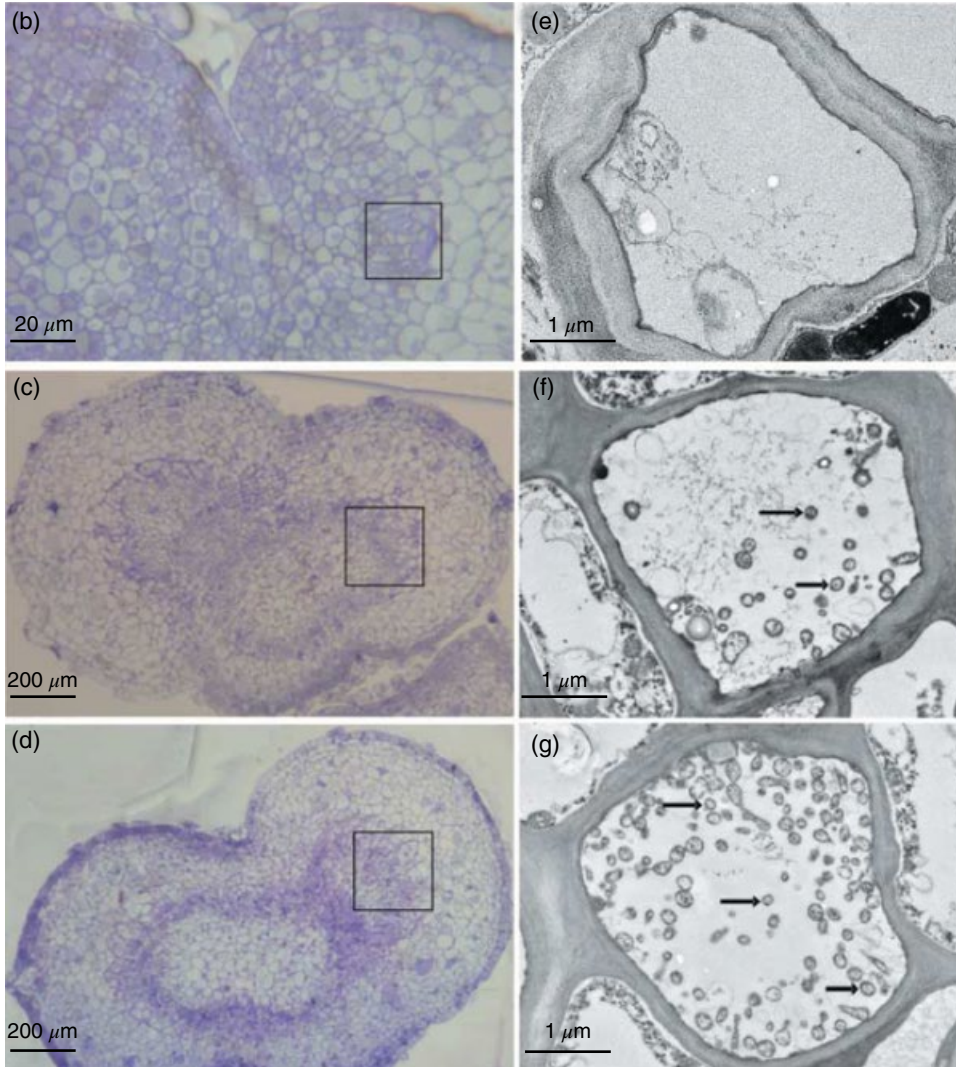
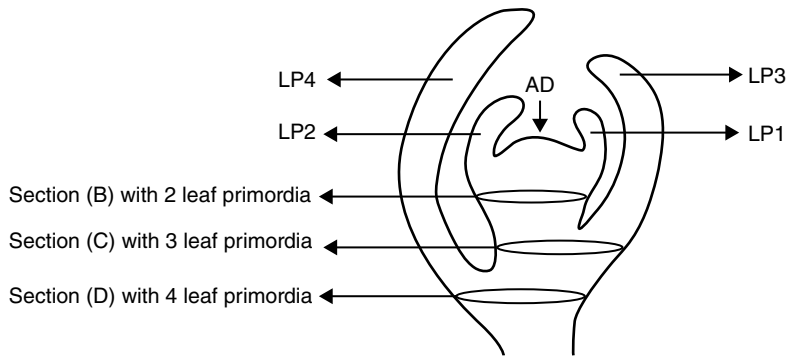


Figure 10.4 (a) Visualization of sweet potato little leaf phytoplasma in TEM cross-sections of shoot tips taken at different positions of leaf primordia of plantlets after cryotherapy. (b, e) Cross-sections of leaf primordia closer to the apical dome (AD) show the elimination of the phytoplasma; (c, d, f) cross-sections of leaf primordia away from AD show partial or lack of effectiveness of cryotherapy; and (c, d, f, g) phytoplasma cells are indicated by arrows. Source: Wang and Valkonen (2008a). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

- iii) Grow the developing tissue culture at $22 \pm 2^\circ\text{C}$ under a 16h photoperiod by using cool-white fluorescent tubes.
- iv) Micropropagate plantlets by growing on shoot multiplication medium (SMM) composed of BM containing $29\mu\text{M}$ gibberellic acid and subculture the stock cultures once every 3 weeks.

10.4.1.2 Cryotherapy of In-Vitro-Grown Shoot Tips

- i) Excise shoot tips (1 mm long) containing 3–4-leaf primordia (LP) from 3 week old in vitro stock cultures and freeze in liquid nitrogen for 1 h; warm the beads that form by dropping shoot tips in sodium alginate solutions into calcium chloride solution in a water bath at 40°C for 3 min and wash with liquid ammonium-free MS supplement with 1.2 M sucrose for 20 min.
- ii) Surface dry the shoot tips by blotting on cellulose tissue; post-culture the beads for survival on ammonium-free SM in the dark for 3 days; transfer to fresh SM and culture under light conditions as provided for stock cultures.
- iii) After 2 weeks of post-culture, transfer the surviving shoot tips to SMM for shoot regrowth and express the regrowth as the percentage of shoot tips regenerating to normal shoots at 2 months after post-culture.
- iv) Subculture regenerated shoots once every 3 weeks; after 4–5 subcultures on SMM, observe for the formation of whole plantlets with well-developed roots and transfer the cryotherapy-treated in vitro plantlets to soil and establish under greenhouse conditions.
- v) Test the plantlets for the presence of the phytoplasma using polymerase chain reaction (PCR) to assess the extent of elimination of the pathogen.

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11

Crop Disease Management

Reduction of Pathogen Inoculum

Plant disease management systems include reduction of pathogen inoculum through cultural practices and physical and chemical techniques as the initial strategy for restricting disease incidence and spread. Successful and profitable cultivation of crops depends on several factors, among which use of disease-free seeds and planting materials is the basic requirement. This aspect of disease management was discussed earlier in Chapter 10, where the various methods of producing disease-free seeds and propagules that may be useful for certification and quarantine programs were described. In addition to using disease-free seeds and propagules, field sanitation is important for reducing/eradicating the pathogen population available in the geographical location.

11.1 Reduction of Pathogen Inoculum by Cultural Practices

Cultural practices have been followed with the primary purpose of increasing the yields of crops. These practices, however, have significant influence on the incidence and spread of diseases under glasshouse and field conditions. The effects of cultural practices may vary widely from region to region and may be difficult to assess, since the beneficial effects only become slowly apparent over a period of several years or seasons. The effectiveness of cultural practices in reducing pathogen inoculum with consequent reduction in disease incidence and subsequent spread and on plant growth conditions has been assessed in some of the pathosystems.

11.1.1 Elimination of Infected Plants and Debris

Proper disposal of infected plant materials is essential for preventing buildup of inoculum capable of infecting subsequent crops. Sorghum downy mildew caused by *Peronosclerospora sorghi* causes extensive leaf shredding of infected plants at the time of oospore formation. Oospores in millions are added to the soil if infected plants and debris are not properly disposed of before oospore formation. Infected plants left in the fields, volunteer (self-sown) plants growing from infected seeds, and additional hosts of the pathogen form important sources of inoculum. Removal of all infected plants and plant parts reduces the amount of inoculum, resulting in considerable reduction in disease incidence in several crops, especially in crops such as banana, stone fruits, citrus,

and sugarcane (Kommedhal and Todd 1991). Volunteer plants growing from infected potatoes left in the field after harvest may serve as sources of inoculum for bacterial and virus diseases. Roguing of infected plants in the early stages of crop growth has been suggested as a practical measure to reduce the spread of viral diseases from the internal sources of infection. In perennials crops, even when the disease spread is slow as in the case of cocoa swollen shoot disease, roguing out infected plants and replanting with virus-free plants may result in relatively greater productive stands (Matthews, 1991). Regular roguing of infected plants was effective in reducing the spread of *African cassava mosaic virus* in tropical Africa (Robertson, 1987) and *Banana bunchy top virus* in eastern Australia (Dale, 1987).

Spread of sugarcane diseases such as smut (*Ustilago scitaminea*), red rot (*Colletotrichum falcatum*), and red stripe (*Xanthomonas rubrilineans*) could be reduced by roguing infected stools or shoots (Palti, 1981). Eradication of all weed plant species present in and adjacent to the main field to reduce the sources of inoculum is recommended to reduce the incidence and subsequent spread of diseases (Narayanasamy, 2002). Fusarium head blight (FHB) disease, affecting small grain crops, has a worldwide distribution, significantly reducing the production and quality of grains. In addition, the pathogens of FHB, *Fusarium graminearum* and *F. culmorum*, produce the mycotoxins in grains which are harmful to human beings and animals consuming the contaminated grains. The residues of maize left on the soil surface and in the top 10 cm of soil in tilled and untilled (no tillage) fields were quantified and their relative influence on *Fusarium* infection and deoxynivalenol (DON) contamination was evaluated. The total amount of residues in the first layer of soil (10 cm) was correlated with DON contamination ($R^2 = 0.848$). The results indicated that the residues lying on the surface of the soil had a major role in infection by *Fusarium* spp. and DON contamination of grains ($P < 0.05$) and that the proper disposal of previous crop residues left on the soil surface should be carried out to reduce the incidence of FHB disease in the subsequent crops (Maiorano et al. 2008).

Eradication of sources of virus infection is an important strategy to reduce pathogen inoculum to restrict the incidence and spread of the virus diseases. Management of *Citrus tristeza virus* (CTV) has been shown to be effective by planting virus-free rootstocks together with eradication of CTV-infected trees by the Central California Tristeza Eradication Agency (CCTEA) (Dodds and Gumpf, 1991). The growers in the West Fresno Pest Control District and the Tulare County Pest Control District, representing 45% (44,100 ha) of the acreage in Central California, withdrew from the eradication program between 1995 and 1996 and did not remove CTV-infected trees from their orchards. In contrast, growers in the Pest Control District of Central Valley, South Tulare County, and Kern County continued to participate in the CTV eradication program. The impact of leaving infected trees on the rate of CTV spread was determined by comparing temporal incidence of CTV in plots in Strathmore, Tulare County without eradication with incidence in a plot in McFarland, Kern County with eradication. CTV incidence in the Strathmore plots ranged over 6–42% with annual spread rates of 1.6–3.6% during 1997–2003. On the other hand, CTV incidence in McFarland plot increased over 0–5% between 2001 and 2003 before infected trees were removed. Using a subplot hierarchical bulk sampling method, virus incidence over a 3 year period in a 6.5 km² area near McFarland was estimated to range over 0.09–0.69%, indicating that CTV suppression was still effective in this area even in the presence of aphid vector (*Aphis gossypii*) that could transmit CTV isolates efficiently.

The CTV levels were high in some noneradicated areas in central California. Molecular analysis using PCR assay and sequence-specific primers showed that all isolates (36) tested had a genotype identical to the T30 mild isolate from Florida (Yokomi and De Borde, 2005).

11.1.2 Effects of Tillage Practices

In order to preserve the soil status to provide optimum conditions for plant growth and to maintain long-term productivity of soils, tillage is an important operation followed in crop management systems. Different implements are employed to turn, mix, loosen, or compact the soil. Changes in water retention, aeration, and soil temperatures therefore occur to varying degrees. These changes exert significant influence on the survival and dissemination of phytopathogens in the soils and the residues of crops left in the soil after harvest. Most of the soilborne fungal and bacterial pathogens, in addition to antagonistic microorganisms, complete a part or entire life cycle in the soil or on the plant residues left on the soil. The soil conditions altered by tillage can be expected to influence both pathogens and the antagonists to a different extent (Cook and Baker, 1983). Tillage operations reduce the disease risk primarily by reducing pathogen inoculum by hastening the decomposition of infested residues, physically separating the pathogens from the host plants by burying them in deep soil layers or by changing the soil conditions (Yang, 2002). In recent years, reduced tillage and conservation tillage practices have been followed due to a reduction in cost, improvement of soil structure, and water infiltration ensuring reduction of soil erosion. However, these tillage practices lead to accumulation of plant residues in the top layer of soil, forming potential sources of inoculum of a range of pathogens ((Jirak-Peterson and Esker, 2011); Váňová et al. 2011).

Tillage in general offers the beneficial effects of destroying the weeds and volunteer plants that may serve as sources of inoculum of pathogens. Deep ploughing may bury the pathogens for inactivation by soil microflora, or the pathogens may be exposed to sunlight resulting in inactivation by solar radiation and heat as in the case of *Rhizoctonia solani* and *Verticillium dahlia*, capable of infecting several crop plant species ((Papavizas and Lewis, 1979); Butterfield in (Fry, 1982)). In the presence of the stubbles from a fallow-sown, no-till wheat cover crop, the dispersal of soil inoculum of *Phytophthora capsici*, incitant of Phytophthora blight of bell pepper, was suppressed resulting in considerable reduction in disease incidence. The final disease incidence level was significantly low (2.5–43.0%) in no-till plots, whereas the high incidence (71–72%) was recorded in pepper planted in bare field where all dispersal mechanisms of *P. capsici* operated without hindrance (Ristaino et al. 1997). The effectiveness of moldboard plough, chisel plough, and no-till in reducing Fusarium head blight incidence in wheat was assessed. The lower incidence and severity of FHB in plots ploughed with moldboard plough than in chisel plough and no-till treatments were apparent. In addition, yields of wheat were greater by 10% in moldboard-ploughed plots than in either chisel-ploughed or no-till treatments. The results suggested that the use of conservation tillage and reduced till systems might have contributed to the FHB epidemics in the Upper Midwest in the USA (Dill-Macky and Jones, 2000).

The effects of conventional and minimum tillage treatments in combination with 2 and 3 year crop rotations on the development of soil suppressiveness to potato canker and black scurf disease caused by *Rhizoctonia solani*, dry rot caused by *Fusarium* spp., and silver scurf disease induced by *Helminthosporium solani* were assessed. Analysis of root zone bacteria recovered from the rhizosphere (exoroot) and potato root tissue

(endoroot) showed that antibiosis activity inhibiting the growth of soilborne pathogens was at a maximum level in bacterial isolates recovered from the endoroot tissues of 3 year rotation crop (barley, red clover, and potato) under the minimum tillage system. The results suggested that the soil agroecosystems could be modified through rotation and conservation tillage practices to improve disease suppression by enhancing the antibiosis of endophytic and root zone bacteria (Peters et al. 2003). Various methods of tillage have been followed to loosen the soil and improve the soil aeration. In addition, pathogen propagules in the deeper soil layers may be brought to the soil surface and activated or killed due to the exposure to sunlight and higher temperature on the soil surface. The influence of tillage practices on the incidence of wheat Karnal bunt disease caused by *Tilletia indica* in the Indo-Gangetic Plains of India was investigated. Samples of wheat 365, 171, and 370 from fields adopting zero- (no-) tillage (ZT), furrow-irrigated raised bed system (FIRBS), and conventional till (CT), respectively, were collected. Incidence of Karnal bunt disease incidence index and percent-infected samples were calculated, and data were analyzed statistically. Lowest mean incidence (9%) of the disease was observed in zero-tillage fields, whereas disease incidence levels were 18.1% and 16.2% in FIRBS and CT fields, respectively. Disease incidence index also followed the same trend as disease incidence. The results suggested that zero tillage might be adopted for a few years to reduce the soil inoculum levels, resulting in the reduction of Karnal bunt disease in this region (Sharma et al. 2007).

The different long-term conventional and conservation tillage treatments were evaluated for their efficacy in reducing the incidence and diversity of *Fusarium graminearum* and other *Fusarium* spp. causing Fusarium head blight (FHB) disease. The isolation frequency of *Fusarium* spp. and the total number of colony-forming units (CFUs) were affected by sampling years (2000 and 2001) and the cultivated crop (winter wheat, mustard, or maize), and showed significant differences between tillage treatments (moldboard plough, chisel plough, or rotary tiller). Diversity levels of *Fusarium* spp. in moldboard-ploughed plots were lower compared with those under chisel plough and rotary tiller treatments. The number of total CFU g⁻¹ of soil and *Fusarium* spp. was higher in 2000 when wheat was cultivated than in 2001 when maize was grown, and showed differences between treatments. The tillage depth also seemed to influence the *Fusarium* populations. The deeper the tillage, the lower the number of *Fusarium* spp. isolated. In conservation tillage treatment, a higher diversity of *Fusarium* spp. was found than that in moldboard-ploughed plots. Further, lower populations of pathogenic *F. graminearum* and *F. culmorum* were found in plots ploughed by moldboard. The results indicated that incidence of *Fusarium* spp. in soil might be increased by conservation tillage due to changes in soil conditions in these plots (Steinkellner and Langer, 2004). Fumonisin contamination in corn was assessed in conventional till and no-tillage systems. No-till corn following oats showed the highest mean fumonisin levels and differed significantly from conventional till corn, following either oat or fallow. Fumonisin levels ranged over 0.13–19.52 µg g⁻¹ (mean 9.97 µg g⁻¹) and 3.70–7.75 µg g⁻¹ (mean 6.29 µg g⁻¹) in no-till corn following oat in two growing seasons of 2006 and 2007. Although no-till is advantageous from a soil conservation standpoint, it may enhance the risk of higher levels of fumonisin accumulation in grains (Ono et al. 2011).

The effects of various wheat cropping systems on Fusarium head blight (FHB) disease severity and contamination levels of mycotoxins (deoxynivalenol, nivalenol, and zearalenone) were investigated. Four wheat cropping systems, namely a conventional system, integrated system, a direct drilling system, and an organic system in fields with

natural pathogen infection, were included in the investigation conducted during 1999–2002. The severity of FHB disease and mycotoxin levels differed considerably between years, reflecting climatic effects. The severity of FHB differed between cropping system in all three years were considered separately. The most severe disease incidence was recorded in direct drilling and conventional cropping system treatments in 2000. The levels of grain contamination by mycotoxins depended on both climatic conditions and cropping systems. Mycotoxin levels and disease severity reached a peak in 2000. Mycotoxin levels were highest in the direct drilling system. No clear relationship could be seen between disease severity and mycotoxin contamination levels of deoxynivalenol, nivalenol, or zearalenone under the natural conditions which existed during the period of experimentation (1999–2002) (Champeil et al. 2004).

The influence of conventional and no-tillage systems on the incidence of *Fusarium* head blight (FHB) disease, in combination with other agronomic practices such as nitrogen fertilization, and the influence of the environmental conditions on different wheat cultivars for three consecutive years was assessed. Maize was the previous crop in the first year and wheat preceded the experimental wheat crop in the latter two years. Tillage treatments – conventional tillage and no-till – were the main plot treatments in the split plot design. The FHB disease was observed in all the years. Differences in symptom expression were associated with the environmental factors prevailing during the different seasons. In the first year, the only interactions for FHB incidence were between no-till and urea application in split doses. Seedling emergence, seed health, 1000-kernel weight, and deoxynivalenol (DON) concentration did not differ, suggesting that the fertilizers and genotypes did not affect the susceptibility to FHB disease. Severity of FHB in the second year was greater because of the favorable environmental conditions. Tillage and fertilization did not affect the variables analyzed. Cultivars showed significant differences and DON values were high. In the third year, no significant differences were seen due to unfavorable climatic conditions. The results indicated that weather conditions exerted greater influence than tillage practices on FHB disease incidence (Lori et al. 2009).

11.1.3 Effects of Sowing Time and Planting Density

Date of planting/sowing of crop depends on several factors such as crop duration (long or short), availability of irrigation facility/rainfall, and other requirements for good growth of crop plants to realize maximum yield potential. From the crop disease management point of view, date of sowing/planting may be determined with the primary objective of reducing the period for which the crop may be at a vulnerable stage or viable pathogen propagules are available. In the case of viruses and other vector-borne pathogens, the date of sowing has to be so adjusted that the crop is not exposed to the period when the vector population is high with active movements. The pattern of incidence of different diseases on a crop in a given location over several years has to be studied to plan a realistic proposal to avoid the disease by suitable manipulation of sowing date. Although this approach may appear to be simple, some problems imposed by crop requirements and seasons have to be overcome (Narayanasamy, 2002).

Early planting of sugar beets, when other conditions favor rapid crop development, reduced the severity of sugar beet yellows and increased the yield in Germany (Heitefuss, 1989). Early sowing of sugar beet permitted its germination and growth when temperatures were too low for infection of roots by the fungal vector *Polymyxa betae*.

On the other hand, late sowing resulted in infection soon after sowing and development of the pathogen was also rapid. Early sowing had the additional advantage of avoiding rhizomania disease caused by *Beet necrotic yellow vein virus* transmitted by *P. betae* (Blunt et al. 1992; (Rush and Heidel, 1995)). Field experiments were conducted for three years (1995, 1997, and 2001) to determine the effects of date of planting (DOP) of soybean on the incidence of *Phomopsis longicolla* (PL) under natural conditions at Stoneville, USA. There was a significant effect of DOP on the incidence of *P. longicolla* and seed germination. Early planting of maturity group IV varieties resulted in higher level of disease incidence, lower seed health rating, and seed germination (Mengistu and Heatherly, 2006).

Planting density (spacing between plants and rows) as determined by rate of sowing (seed rate) or spacing adopted has a distinct effect on the amount of foliage canopy and consequently on the microclimate around the crop. High rates of seeds sown in nurseries or seedlings planted closely favor the diseases caused by *Rhizoctonia* and *Fusarium*. Maize stalk rots caused by *Gibberella fujikuroi* was favored by close planting (Nyvall, 1979). The severity of potato silver scurf disease caused by *Helminthosporium solani* and black scurf disease induced by *Rhizoctonia solani* were increased with an increase in plant density/unit area (Firman and Allen, 1995). Seed decay caused by *Phomopsis* spp. is endemic in all soybean-growing states in the USA. *Phomopsis* seed decay reduced the yield, quality, and germination of seeds. The effects of planting date and cultivars were determined by planting the susceptible soybean cultivar Asgrow 3834 and resistant lines SS93-6012 and SS93-618 in mid-April, mid-May, and mid-June. Generally yields averaged over years (1998–2000) were significantly greater for mid-April than mid-June plantings, and yields of cultivars were similar within a planting date. The percentage of Asgrow 3834 seed infected with *Phomopsis* spp. was significantly greater than for lines SS93-6012 and SS93-6181 seed for all planting dates. The percentage of Asgrow 3834 seed infected with *Phomopsis* spp. was significantly greater for mid-April than mid-May and significantly greater for mid-May than for mid-June plantings. The germination of seed from mid-June plantings was significantly greater than seed from mid-April plantings and significantly greater for resistant lines than for Asgrow 3834 planted in mid-April and mid-May. The percentage of Asgrow 3834 seed infected with *Phomopsis* spp. was significantly greater than lines SS-93-601 and SS93-618 for 1998 and 2000 (Wrather et al. 2003).

The effects of the date of sowing of maize (corn) on the susceptibility of maize hybrids to ear rot caused by *Fusarium* spp. and mycotoxin contamination of grains were investigated under natural conditions. Three sowing dates (March, April, or May) were compared using two hybrids with different maturity classes. The ears were scored for disease incidence and severity of symptoms at harvest and the harvested kernels were analyzed for mycotoxins, namely fumonisin B1, zearalenone, deoxynivalenol (DON), aflatoxin B1, and ochratoxin A. Sowing maize in May resulted in higher levels of infestation by European corn borer (39%) (considered to predispose plants to infection by the pathogen) *Fusarium* kernel infection and ear rot severity by a factor of 4 and 3, respectively, than in plots sown in March. The sowing date did not affect the nature of mycotoxin present in the kernels which depended on the climatic conditions and on their influence on the infection and development of *Fusarium* spp. The late sowing appeared to increase the contents of fumonisin B1 and DON to levels significantly higher than in crops sown in March or April. Late sowing (after May 10) multiplied the risk of production of fumonisins and DON in grains at harvest by a factor of 11.2 and 1.9, respectively. On the other hand, production of zearalenone and aflatoxin B1 in maize grains was not altered by dates of sowing (Blandino et al. 2009a).

The influence of plant density on the susceptibility of medium and medium-late maturity maize hybrids to ear rot and to mycotoxin contamination in natural infection conditions was investigated. Plant density significantly influenced the percentage of kernels infected by *Fusarium* infection (+24%) and higher fungal ear rot severity (+43%) than plots with lower plant densities, but the type of mycotoxin produced by the pathogen in the kernels did not show variation due to planting density. However, the climatic conditions prevailing during the season influenced infection and the development of different fungal genera and species associated with maize ear rot disease. The natural occurrence of mycotoxins found in each year (2001–2004) was always significantly higher in crops with higher plant density. Cultivation of a medium-late maturity hybrid instead of a medium maturity hybrid could lead to higher contamination of mycotoxins. Growing maize crops in temperate areas with high plant populations might result in production of mycotoxins in larger amounts and consequent higher mycotoxin risk in kernels (Blandino et al. 2008a). In a later investigation, the effects of the date of sowing, planting density, and nitrogen fertilization in kernel infection by *Fusarium verticillioides* and *F. graminearum* were assessed in maize under natural conditions. The levels of contamination of maize kernels with fumonisin and deoxynivalenol were determined in different treatments. The fumonisin concentrations were less affected by plant density, hybrid maturity, and nitrogen levels. The presence of deoxynivalenol (DON) was related to late sowing, high plant density, and nitrogen fertilization (Blandino et al. 2009b).

High plant density has been reported to reduce the incidence and spread of virus diseases of annual crops transmitted by vectors. This phenomenon of reduction in the incidence of viral diseases by adopting high-density planting (closer spacing) has been used as a practical measure of disease management in narrow-leaved lupin (*Lupinus angustifolius*). Adopting a narrow spacing (17.5 cm) resulted in the reduction of *Bean yellow mosaic virus* incidence in narrow-leaved lupine (Jones, 1994). Plant density and cowpea variety had significant effect on the incidence of *Cowpea aphidborne mosaic virus*, *Cowpea mild mottle virus*, *Cowpea mosaic virus*, *Cowpea chlorotic mottle virus*, and *Cowpea golden mosaic virus*. The lowest mean disease incidence was the lowest in plot with a spacing of 25×75 cm and the disease incidence was greater in plots with a spacing of 50×75 cm. However, the cultivar IT89KD-288 at a spacing of 50×75 cm had the lowest disease incidence and highest yield compared to other combinations (Aliyu & Balogun, 2011).

11.1.4 Effects of Irrigation Practices

Among the different agricultural practices, irrigation probably has a greater influence on the development of both host plant and microbial plant pathogens. Irrigation is essential to maintain required soil moisture levels for the absorption of plant nutrients from the soil and for maintaining turgidity of plant tissues. Both high soil moisture and water stress may predispose plants to infection by different kinds of pathogens. Four kinds of irrigation systems are generally used for various crops, depending on the quantum of water available. Flooding is carried out when the quantity of water available is abundant, but this system may spread the pathogen inoculum throughout the field. However, flooding the soil (flood fallowing) was found to be effective for the eradication of *Fusarium oxysporum* f.sp. *cubense*, the causal agent of banana Panama (Fusarium) wilt disease. This strategy was successful because flooding drastically restricted the oxygen supply to the soil, rendering conditions very unfavorable for pathogen survival. The treatment was effective for 4–5 years, after which the pathogen

reappeared (Kelman and Cook, 1977). Furrow irrigation saves some water, but spreads the pathogen inoculum along the furrows. The sprinkling system restricts the amount of water used further, but causes enormous splash dissemination of inoculum while surface dissemination is limited. The trickling system conserves water to the maximum extent, but aids in the pathogen dissemination. As irrigation is an indispensable component of field crop cultivation, suitable modification/changes in the other cultural practices have to be considered for application under field conditions existing in a geographical location.

Many fungal and bacterial pathogens are carried from one part of the infested field to other parts through irrigation water. Fungal pathogens such as *Verticillium dahliae*, *Thanatephorus cucumeris*, *Phytophthora infestans*, *Fusarium oxysporum* f.sp. *cubense*, and others are known to be spread through irrigation water. Likewise, *Xanthomonas oryzae* pv. *oryzae* (Xoo) exuded from rice leaves falls into irrigation water and is carried to adjacent fields which are irrigated from one source of water. High populations of bacteria all year around were present in irrigation water in canals and in rice fields (Ou, 1972). In these cases, if water from an infested field is prevented by suitably modifying the irrigation system, the incidence of the disease(s) may be appreciably reduced. Water released into the sewage system by sugar refineries processing sugar beets was found to carry the fungal pathogen *Polymyxa betae*, which is the vector of the rhizomania disease (Sutic and Milovanovic, 1980). The effect of three irrigation methods – daily drip, 3 day drip, and alternate-row furrow irrigation – on the incidence of *Phytophthora* root rot disease caused by *Phytophthora capsici* in pepper was compared. Drip irrigation increased the yield of marketable green fruits by providing either favorable soil moisture conditions for pepper or unfavorable conditions for the pathogen development (Xie et al. 1999). *P. capsici* produces zoospores that initiate infection of roots. Conditions of wet and dry cycles in soil are primarily required for completion of different stages in its life cycle. Rainfall and periodic furrow irrigation provide the wet–dry cycle in the soil, favoring sporangia production during dry period and zoospore release during wet period. Disease percentage was higher (78.39%) in drip irrigation system than in basin (56.27%) and furrow irrigation (59.92%) methods (Sağır et al. 2005).

The effects of irrigation on the incidence of *Phomopsis longicolla* (PL), seed germination, and seed health rating (SHR), a measure of diseased seeds and germinated seedlings in the early soybean production system, were determined along with those of date of planting (DOP). A positive relationship between disease incidence and SHR was observed for 1996, 1997, and 2001. Irrigated treatments in drier years (1995 and 1997) produced a higher level of PL incidence than in nonirrigated treatments, suggesting that soil moisture could have increased relative humidity in the canopy that provided favorable conditions for pathogen development. The incidence of PL disease was associated with total rainfall and rainfall frequency for each year. Disease incidence was higher in 1996 and 2001 than in 1995 and 1997, when a conducive moist environment overrode the effects of irrigation. The results indicated that seed health rating (SHR) and seed germination percentages could be a reliable basis for evaluation of *P. longicolla* on seed quality and that the existence of conducive environmental conditions was necessary to assess the significant treatment effects (Mengistu and Heatherly, 2006).

The beneficial effect of supplemental irrigation under drought stress conditions in improving the growth and yield of potatoes has been observed, but its influence on the incidence of tuber diseases was also indicated. Surface sprinkler irrigation was applied based on tensiometer or moisture block readings deployed in field plots. The severity of

black scurf disease (*Rhizoctonia solani*), black dot (*Colletotrichum coccodes*), silver scurf (*Helminthosporium solani*), and common scab (*Streptomyces scabiei*) was quantified on potato tubers randomly sampled at harvest and kept at storage temperature of 7.2°C before visual assessment. The mean incidence of black scurf, silver scurf, and black dot disease ranged over 3–18%, 2–33%, and 4–9%, respectively, in best, unirrigated, and reduction irrigation treatments. The results suggested that irrigation might increase disease incidence. However, other cultural practices such as rotation and application of amendments might be useful to reduce the disease incidence selectively to some extent (Olanya et al. 2010). *Colletotrichum coccodes*, causal agent of black dot disease of potato tubers, is spread through infected progeny tubers. Introduced into the previously uncontaminated soil through infected tubers, the pathogen could survive in the soil for at least 8 years (Dillard and Cobb, 1998); the contaminated soil could therefore be a potential source of inoculum for *C. coccodes*, leading to infection of subsequently planted potato crops for several years. The effects of cultural practices such as use of cultivar resistance, irrigation, and crop duration were studied by performing field trials during 2004–2007. Development of black dot disease on underground plant parts was monitored in two field trials conducted in England and Scotland. First symptoms of infection on stems were seen at 90 days after planting (dap) and the disease incidence increased rapidly thereafter, reaching 99% at 132 dap in an English trial. The incidence of the disease on stems, stolons, and roots progressed at a slower pace in the Scottish trial. The differences in disease incidence on stems, stolons, and roots did not reflect the levels of resistance of potato cultivars Maris Piper (least resistant) and Sante and Saxo (more resistant). The final disease incidence did not show significant variations across the cultivars (Fig. 11.1). Irrigation coupled with application of the fungicide azoxystrobin significantly reduced black dot disease on stem and stolons compared with other treatments (cultivar resistance and crop duration). The black dot disease on stolons and roots progressed at a similar rate at English and Scottish sites with irrigation (with or without azoxystrobin) reducing stolon and root infection by the pathogen. The results suggested that development of black dot on stems, stolons, and roots could be reduced by combining irrigation and azoxystrobin application. However, azoxystrobin had little effect on disease incidence at the English site, whereas effectiveness of irrigation in reducing disease incidence could be clearly observed (Brierley et al. 2015).

11.1.5 Effects of Crop Nutrition

Several factors such as soil fertility (productivity), water availability, weather, cost/benefit ratio, and marketability of the harvested produce have to be considered while choosing a crop in a particular location. Crop nutrition exerts a decisive influence on the growth and yield of crop plants, as well as on the development of diseases caused by microbial pathogens which have to derive nutrition from the susceptible plant hosts. As the human population increases, there is no matching increase in cultivable land area which is progressively shrinking because of industrial development and the increasing demand for housing. Crop production strategies therefore have to be modified, resulting in enhancement of the application of chemical fertilizers to increase yield and synthetic chemicals to protect crops against diseases and insects. However, early success achieved in controlling crop diseases created the wrong impression that chemical control could offer a lasting solution to the disease problems confronting the growers. The development of resistance in plant pathogens to chemicals and a growing awareness

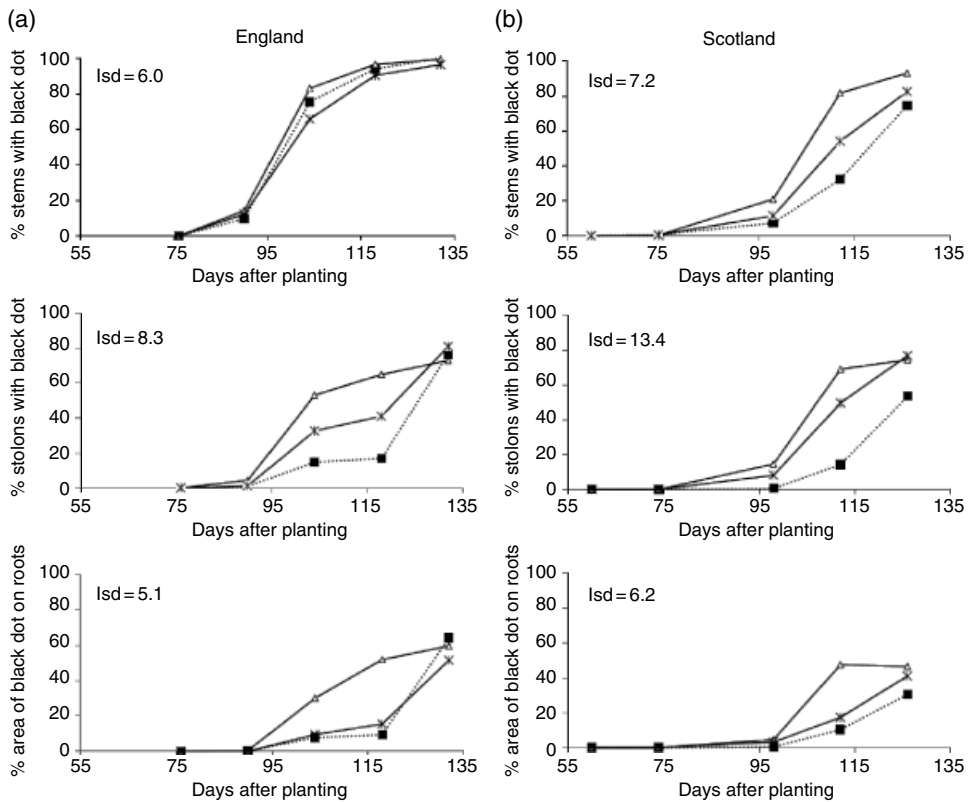


Figure 11.1 Development of potato black dot disease on stems, stolons, and roots of three potato cultivars with different levels of resistance in (a) England and (b) Scotland. Disease severity ratings measured on potato cultivar Maris Piper (■), Sante (Δ), and Saxo (X). Source: Brierley et al. (2015). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

for the protection of the environment dramatically changed the approaches for solving plant disease problems from chemical methods to alternative methods aimed at restricting or replacing the use of fertilizers and chemicals applied against plant pathogens. The availability of sufficient nutrients to the plants is essential for satisfactory plant growth and desired level of yield. Many cultural practices such as the application of organic amendments and crop sequence (rotation) have been shown to influence disease incidence, severity, and function through altered biological activity in the soil and crop environment. It is known that plant response to a particular nutrient may be different when its level moves from deficiency to sufficiency than from sufficiency to nutrient excess. It is necessary to maintain a nutritional balance for optimum crop response, since each nutrient functions as part of a delicately balanced interdependent system influenced by plant genetic constitution and environment. Interactions between specific nutrients and pathogen development have to be clearly understood to effectively modify the nutrient requirements of the chosen crop to increase crop yield and quality and restrict the development of diseases to the required levels (Huber and Haneklaus, 2007; Narayanasamy, 2013).

11.1.5.1 Use of Organic Matter

Soil fertility may be increased by the application of organic matter, rather than through provision of nutrients to the plant density. Further, many microorganisms antagonistic to plant pathogens are activated due to addition of organic matter to the soil, and this may lead to suppression of the development of diseases transmitted by soilborne pathogens that may eventually become seedborne. Organic matter incorporated into the soil is called amendment, while organic matter spread on the soil surface may be referred to as mulch. Organic amendments may promote biological destruction of pathogen inoculum through germination-lysis mechanism and intensification of microbiological activity, leading to degradation of propagules of phytopathogens. Organic amendments may exert stimulatory or inhibitory effects on the microbial plant pathogen populations and disease development. They may either prevent infection by activating the soil microflora, potentially competitive with or antagonistic to plant pathogens present in the soil, or inhibit plant pathogen development by producing toxic metabolites in the soil environment when the organic matter decomposes in the soil. Proper composting of the organic matter before incorporation into the soil is essential, since some pathogens may survive in the organic amendments and are likely to be introduced into the soil inadvertently.

A range of soil amendments have been evaluated for their efficacy in reducing the pathogen population and disease development. Incorporation of barley straw in the topsoil reduced the populations of *Verticillium dahliae* infecting potato. Reduction in disease incidence was attributed to the direct quantitative reduction in pathogen population, since there was no change in host resistance or fungal pathogenicity (Harrison, 1996). Verticillium wilt disease of potato could be effectively contained by application of Sudan grass or corn (maize) as green manures (Davis et al. 1996). Application of perennial peanut and chitosan reduced the incidence of crown and root rot of bell pepper caused by *Phytophthora capsici* and also disease severity (Kim et al. 1997). Potato common scab (PCS) disease, one of the major economically important diseases, is caused by *Streptomyces scabies*, *S. turgidiscabies*, and *S. audiscabies*. Green manures provide substantial benefits to the soil by improving soil structure and suppressing soilborne diseases. Lopsided oat cultivar (*Avena strigosa*) used as a green manure crop in Japan was evaluated for its efficacy in reducing the incidence of PCS disease and increasing the healthy marketable potato tuber yield. Lopsided oat or woolly pod vetch (*Vicia villosa*) were more effective in suppressing PCS disease severity than oat and continuous potato cultivation ($P < 0.0001$). The marketable tuber ratio was more significantly increased (65.3% and 74.0%, respectively) compared with oat and continuous potato cultivation. The relationship between *S. turgidiscabies* density and disease incidence was positively correlated. In all field experiments, densities of *S. turgidiscabies* in the lopsided oat treatments were lower than for other treatments. The lopsided oat treatments produced higher marketable tuber ratio than sugar beet treatment. In field experiments conducted during 2000–2001 lopsided oat cultivation alone, and with the application of Ferosand (to decrease soil pH) at 1.8 t ha^{-1} or a resistant potato cultivar treatment, was significantly more effective at suppressing the disease severity and incidence than sugar beet cultivation, even under high disease pressure. The results suggested that lopsided oat used as a fallow green manure crop could reduce the severity of potato common scab disease and increase marketable potato yield (Sakuma et al. 2011).

Ralstonia solanacearum race 3 biovar 2, the incitant of potato brown rot (bacterial wilt) disease, is an economically important bacterial pathogen occurring in tropical, subtropical, and temperate regions of the world. The effects of conventional and organic

management and various amendments on brown rot development in potato tubers in different soil types, namely sand or clay from Egypt desert and Dutch (Netherlands) soils, were determined. Brown rot infection was only slightly reduced in organically raised crops compared to conventionally managed potato crops in sandy soils from Egypt. However, organic management significantly increased disease incidence and pathogen survival in Dutch sandy and clay soils, which correlated with the high dissolved organic carbon (DOC) contents in the organic Dutch soils. There was no correlation between disease incidence or severity and bacterial diversity in the potato rhizosphere in differently managed soils. Cow manure amendment significantly reduced disease incidence in organic Dutch sandy soils, but did not affect the bacterial populations. However, cow manure reduced densities of *Ralstonia solanacearum* in Egyptian sandy soils. A clear shift in population, most probably by microbial competition, was detected with denaturing gradient gel electrophoresis (DGGE) in these and Dutch sandy soils after manure amendment. The amendment with compost did not have a suppressive effect in any of the soil type investigated. It is possible that the absence of disease suppressive effect of mineral and organic nutrients in these soils could be responsible. The suppressive effects of manure added to the organic sandy soils from Netherlands and Egypt could have been due to the immediate release of ammonia, which is toxic to microorganisms, or a shift in the microbial community. Amendment of soils with wood chip compost was not effective in potato brown rot suppression. Likewise, compost from tree bark or coffee residues did not suppress, but actually increased, bacterial wilt in tomato. Amendment of conventional soils with NPK provided remarkable disease suppression only in Egyptian sandy soil and a reduction in the population of *R. solanacearum* in soils and rhizosphere of potato plants in Egyptian clay soil. When Ca and K contents of soils were high potato brown rot incidence or severity was generally low, both in soils naturally high in these elements and in soils amended with manure. The results indicated that mechanisms of potato brown rot disease suppression might vary strongly depending on the soil type (Messiha et al. 2007).

11.1.5.2 Use of Inorganic Fertilizers

Intensive agriculture systems involving the use of inorganic fertilizers, high-yielding cultivars (with susceptibility to diseases), and enhanced plant density/unit area had to be followed because of the rapid increase in human populations. Adequate organic matter was not available to meet the requirements of various crops, necessitating the application of fertilizers in large quantities. Fertilizers may act on the host plants indirectly, leading to an increase or reduction in disease incidence and/or severity. They may also have some effect on the microclimate and the microorganisms that favor plant growth or act as antagonists of plant pathogens.

Nitrogen favors vegetative growth of plants, which is required in the early growth stage of crops. However, an excessive supply of nitrogen is known to increase plant susceptibility to several diseases affecting different crops. Heavy doses of nitrogen increased the susceptibility of rice plants to blast disease caused by *Pyricularia grisea*. Delaying or splitting the application of nitrogen may therefore reduce rice blast incidence (Ou, 1972). Potato tuber rot induced by *Phytophthora infestans* was significantly increased at higher levels of nitrogen (Heitefuss, 1989).

The form of nitrogen that is applied may have some influence on the development of crop diseases. Development of cabbage club root disease caused by *Plasmodiophora brassicae* could be effectively suppressed by combined application of nitrate fertilizer

and lime to increase soil pH (Smiley, 1975). The effects of nitrogen fertilization on the development of *Fusarium* head blight (FHB) disease in wheat and the resulting deoxynivalenol (DON) contamination in kernels were investigated. The FHB disease incidence was recorded in two locations under natural conditions. Five different types of nitrogen fertilizer (both organic and mineral) were applied at five input rates of 0–160 kg ha⁻¹. A significant increase in disease severity was observed with increasing N supply, regardless of the type of N applied. Depending on the type of N fertilizer applied, the percentage of diseased spikelets increased from 2.2% at zero N rate up to 6.6% at 160 kg ha⁻¹. Under in vitro greenhouse conditions, spring wheat varieties including Durum wheat were inoculated with *Fusarium graminearum* and *F. culmorum* strain, known producers of DON. Significant increases in FHB intensity and DON contamination in the grain were observed with increasing N from 0 to 80 kg ha⁻¹. At higher input, levels of FHB intensity and DON contamination were at constant levels. The application of nitrogen fertilizers may not be useful for the management of FHB disease (Lemmens et al. 2004).

Field experiments were conducted in northwest Italy to determine the effects of the type (urea and a slow-release fertilizer) and the application rate of nitrogen (N) fertilizer (0, 100, 200, 300, or 400 kg ha⁻¹) on the susceptibility of maize hybrids to ear rot caused by *Fusarium* spp. and to mycotoxin contamination under natural infection conditions. Disease incidence and severity at harvest were scored and the contents of the mycotoxins, fumonisin B1, zearalenone, deoxynivalenol, aflatoxin B1, and ochratoxin A were determined. No significant differences were observed between the two types of N fertilizer in any of the years (2000–2004) for zearalenone, fumonisin B1, or ochratoxin A. Ear rot incidence and severity were generally higher in ears from plants fertilized with insufficient N, but high N fertilizer doses (>300 kg ha⁻¹) significantly increased zearalenone content while for fumonisins the highest contaminations were related to nitrogen deficiencies (+80%). Deoxynivalenol (DON) and ochratoxin A were not detected in all treatments, but when present in detectable concentrations they did not show a clear relationship to N fertilization rate. On the other hand, a negative correlation was found between N rate and aflatoxin B1 contamination when the climatic conditions during the ripening period favored accumulation of aflatoxin B1. The results suggested that a balanced N fertilizer application (200 kg ha⁻¹) might lower mycotoxin contamination, and this practice might be adopted without compromising yield and quality (Blandino et al. 2008b). The effects of nitrogen levels on *Fusarium* spp.-induced fumonisin levels in corn were investigated. Plots treated with 0 and 22.5 kg ha⁻¹ nitrogen showed highest mean fumonisin levels, differing significantly from those plots treated with 45.0 and 90.0 kg ha⁻¹ nitrogen. Fumonisin levels correlated negatively with nitrogen fertilization rates ($P < 0.05$) (Ono et al. 2011).

Since adequate nitrogen fertilization was considered to be one of the factors for achieving high yields in wheat (*Triticum aestivum*), the influence of nitrogen supply on the development of speckled leaf spot disease caused by *Septoria tritici* was studied under field conditions during 1996 and 1997 seasons. Six cultivars were supplied with nitrogen at 0 and 100–150 kg ha⁻¹ levels in a split-plot design. Percentages of necrosis (severity) of the two upper leaves of plants in each treatment were recorded three times during the growing season. With weather conditions being conducive for disease development in 1996, areas under the disease progress curve (AUDPC) were higher in fertilized treatment. On the other hand, with insufficient rains in 1997, the disease progressed faster in plots fertilized with N in the flag leaf which was exposed to conducive environmental conditions from its appearance. The cultivar characteristics,

climatic, and agronomic conditions had a distinct influence on the effect of nitrogen application. The results indicated that the effect of nitrogen fertilization in favoring the development of *S. tritici* might be useful for developing disease management system for different wheat cultivars and for optimizing conditions for the selection of disease tolerant/resistant lines (Simón et al. 2003).

Fusarium graminearum is the major component of Fusarium head blight (FHB) disease of wheat in Serbia. The influence of nitrogen (N) fertilization on FHB intensity (disease index), spike weight (SW), grain weight/spike (GWS), disease incidence, and accumulation of deoxynivalenol (DON) and zearalenone (ZON) was investigated using two cultivars Pobeda and Renesansa under field conditions during two growing seasons of 2010–2012. Three nitrogen rates (0, 75, and 150 kg ha⁻¹) were applied. The levels of N applied did not significantly affect FHB index or mycotoxin accumulation in wheat grains in 2011. In 2012 however, higher DON and ZON levels were recorded at the highest rate of N (150 kg ha⁻¹). It is likely that in 2012 weather factors for Fusarium kernel infection were more favorable compared to the effect of N rates. Likewise, in both cultivars harvested kernels under conditions of artificial inoculation had higher disease incidence and increased DON contents which, in most cases, exceeded tolerance limits adopted in the European Union countries. This situation indicated that the cultivation of susceptible cultivar was at risk of mycotoxin A contamination which is of a serious concern in the production of quality wheat grains. Individual management strategies could reduce the incidence of FHB and the risk of mycotoxin contamination. Nevertheless, integration of two or more strategies is likely to be more effective in reducing FHB incidence and mycotoxin contamination of wheat grains (Krnjaja et al. 2015).

The need for rational use of fertilizers and other agricultural practices that do not favor lodging of cereal crops before harvest has been revealed by experiments conducted during 2002 to 2006. The effects of lodging on the level of deoxynivalenol (DON) and nivalenol (NIV) contamination in wheat, barley, and rice infected with *Fusarium graminearum* species complex were assessed. A total of 66 grain samples collected from each field in 12 Japanese prefectures was analyzed using each sample set consisting of grains from lodged and nonlodged plants. All samples of wheat and barley were contaminated with DON and NIV. On the other hand, NIV was the only contaminant of rice grains. In intervention trials to investigate the effects of lodging duration, a small area of wheat inoculated with *F. graminearum* was completely lodged by trampling. The levels of DON + NIV increased by 27–51% in wheat within 5 days of lodging compared to nonlodged control plots. In the case of rice, half of each plot area was lodged completely by trampling at 20 days prior to harvest. A significant increase in the level of NIV was observed due to lodging over the nonlodged rice at optimum and delayed harvest, indicating that lodging significantly increased the level of mycotoxin in three crops. The results showed that lodging of wheat, barley, and rice should be avoided to prevent mycotoxin contamination in grains (Nakajima et al. 2008).

The effects of agronomic practices such as nitrogen fertilizer rates, irrigation frequencies, and previous crops in rotations on the incidence of powdery scab disease of potato caused by *Spongiospora subterranea* and potato yields were assessed. Nitrogen application (400 kg ha⁻¹) increased tuber weight/plant by 38%. However, incidence and/or severity of powdery scab were increased by nitrogen applications. Measurements of *S. subterranea* DNA in soil samples taken at harvest greatly reflected the severity of powdery scab in harvested tubers. The amounts of DNA were greater after nitrogen application than without nitrogen fertilizer, less after “optimum” irrigation than “low”

irrigation, and greater after a potato/wheat rotation than after potato/pea. The field plots were used for two subsequent potato growing seasons, without the agronomic practices applied earlier, to determine whether the preplanting measurements of *Spongospora* DNA in soil could predict powdery scab in harvested potatoes. The disease generally decreased during the following two growing seasons and the effects of different agronomic treatments on powdery scab had disappeared by the second season. However, the greater amounts of pathogen DNA in soil in plots where nitrogen had been applied compared to where no fertilizer was used continued for the two following growing seasons. Relationships between amounts of preplanting *S. subterranea* DNA content in soil and powdery scab in subsequently harvested tubers were weak in the second growing season and nonexistent in the third season. The results showed that agronomic practices, especially nitrogen application, might increase severity of powdery scab in harvested tubers. In addition, preplanting measurements of pathogen DNA in the soil did not provide reliable predictions of the incidence/severity of powdery scab in harvested potatoes (Shah et al. 2014).

The application of phosphorus favors plant growth by promoting rapid root development. Enhancement of resistance to diseases in crops receiving adequate phosphoric fertilizers has also been indicated. Higher levels of phosphorus were reported to reduce the incidence and lesion morphology of potato scab disease caused by *Streptomyces scabies* (Davis et al. 1976), potato late blight induced by *Phytophthora infestans* (Heitefuss, 1989), and cowpea anthracnose disease caused by *Colletotrichum lindemuthianum* (Adebitan, 1996). Addition of phosphorus in the deficient soil restricted the development of wheat leaf blotch disease due to *Septoria nodorum* (Cunfer and Touchton, 1979) and reduced the incidence of *Cucumber mosaic virus* in spinach (Cheo et al. 1952). Phosphates are generally applied to correct deficiency in soil, but their effect on disease incidence has not been investigated adequately enough to allow valid conclusions to be drawn. Potassium has a key role in the osmoregulation in the cell and maintenance of electrical balance across the plasma membrane. The deficiency of potassium, phosphorus, magnesium, and minor nutrients leads to impairment of processes involved in the maintenance of cell structure and function. The formation of cellulose and sclerenchymatous structures is accelerated by a supply of adequate concentrations of potassium, resulting in enhancement of resistance to fungal diseases of cereal, counteracting the adverse effects of excess nitrogen which lead to lodging of plants (Heitefuss, 1989). Potassium supply in the absence of nitrogen and phosphorus increased the incidence of *Soybean mosaic virus* (SMV) disease, but plants receiving complete fertilizer (20:20:20 kg NPK ha⁻¹) showed lower incidence of the virus disease and the highest increase in the yield of soybean (Pacumbaba et al. 1997).

The stimulation of passive and active mechanisms of disease control may be possible by appropriate nutrient management tactics. Mineral nutrients are components of plants and regulate metabolic activity associated with resistance of a plant species and virulence of a pathogen capable of infecting the plant species concerned. Nutrients such as calcium may suppress the development of diseases caused by *Erwinia* spp. (bacterial soft rots of potato) and *Rhizoctonia solani* (potato black scurf) by increasing the structural integrity and resistance of the middle lamella, cell-wall components, and cell membranes to counteract the activity of extracellular macerating enzymes produced by these pathogens ((Bateman and Basham, 1976); Kelman et al. 1989). Immobilization of manganese (Mn) at infection sites of *Magnaporthe grisea* predisposed rice to blast disease. The ability to oxidize Mn from the reduced, plant-available form to the

oxidized nonavailable form was identified as a virulence mechanism of fungal pathogens. However, some isolates of *M. grisea* that could not oxidize Mn were also found to be virulent. Many cultural disease control practices may function through their effect on mineral availability. Transformation from insoluble Mn^{+3} or Mn^{+4} oxides to plant-available soluble Mn^{+2} is highly dependent on environmental conditions; many factors predisposing plants to diseases may act through their effect on Mn availability (Huber and Haneklaus, 2007). The effect of magnesium on the development of sheath blight disease caused by *Rhizoctonia solani* on rice plants of two cultivars grown in nutrient solution was assessed. Sheath blight disease progress on inoculated sheath was evaluated by measuring lesion expansion (in millimeters) at 24, 48, 72, and 96 h after inoculation. The magnesium (Mg) concentration on leaf sheath tissue was the highest at Mg rates of 0.389 and 0.40 mM for cultivars BR-IRGA409 and Labelle, respectively. A linear model revealed the relationship between area under lesion expansion progress curve (AULEPC) and the Mg rates applied. The AULEPC decreased by 48.7% and 26.2% for cultivars BR-IRGA409 and Labelle, respectively, as the Mg rates in the nutrient solution increased from 0.062 to 0.5 mM. The results indicated that the high foliar Mg concentration played a pivotal role in decreasing sheath blight lesion expansion (Schurt et al. 2014).

11.1.6 Effects of Other Crops

Land use patterns may vary in different seasons and locations, depending on the water availability, rainfall, and other environmental conditions. Many crops may be cultivated in the same farm in rotation with the primary crop or as intercrop along with the primary crop. Different crops in adjacent fields in the same farm may be raised. The incidence of diseases may be influenced by other crops, depending on their susceptibility/resistance levels to the pathogen(s) infecting the primary crop. These crops may exert either a positive or negative effects, depending on whether they are susceptible or resistant to the pathogens of primary crop.

11.1.6.1 Crop Sequence

Crop sequence or crop rotation involves the use of appropriate crops in sequence for a definite duration of time (2–3 years in most cases). The effectiveness of crop sequence in reducing disease incidence depends on several factors such as duration, environment, the nature of the pathogen(s) to be managed, and crop requirements. Use of nonhost(s) in rotation for a sufficient period may allow enough time for the decomposition of infected crop residues and/or a reduction in the viability of the pathogen survival structures and the ability of the pathogen to produce inoculum, thus eliminating the potential source(s) of the disease. Crop rotation may be effective for certain diseases under a set of conditions if the sources of inoculum are present only in the field concerned or the movement of the pathogen from adjacent or other fields is limited. Crop rotation may be effective for the diseases caused by the pathogens that are soil-borne or plant-residue-borne. The inclusion of legumes in crop rotation may provide an additional benefit in reducing disease incidence because of their ability to fix atmospheric nitrogen that can be used by the succeeding crops. Crop rotation may not be effective in the case of seedborne pathogens, pathogens that have a wide host range, or pathogens that can survive for long time (several years by producing resistant structures) in the soil. Further, crop rotation may not be an effective option for the management of pathogens that have additional mode(s) of dispersal, such as air or irrigation water.

The incidence of crown rot disease of wheat caused by *Fusarium graminearum* was reduced by chickpea-wheat rotation compared with a wheat-wheat monocropping system. The mean incidence of the disease was 12% for chickpea-wheat rotation, as against 30% infection in wheat-wheat system (Felton et al. 1998). In a later investigation, the effects of crop rotation including canola, lupine, medic, and medic-clover mixture on the incidence of wheat crown rot disease of wheat were assessed. Wheat and canola in rotation significantly increased the incidence of the disease. The disease incidence and severity were the highest in this treatment. Medic-clover mixture, as the rotation component, reduced the disease incidence to the maximum extent and enhanced the yield of wheat grains (Lamprecht et al. 2006). The effects of the previous and adjacent crops on *Fusarium* head blight (FHB) disease incidence were determined. The number of viable airborne propagules of *Fusarium graminearum* (teleomorph *Gibberella zeae*) trapped at anthesis, FHB index, percentage of seeds infected with *F. graminearum*, and deoxynivalenol (DON) accumulation in seed of winter wheat from commercial fields across southeastern Ontario were recorded. More viable airborne propagules of the pathogen were trapped in wheat fields that were planted on corn or wheat stubble than in wheat fields in which previous crops were nonhosts. Previous crop, field size, and tillage interacted affecting FHB index, DON accumulation, and the percentage of seeds infected with *F. graminearum*. Large fields where corn was planted one year previous to wheat with minimum or no tillage had the highest values. Adjacent crops (nonhost, corn, and wheat) affected the number of viable airborne propagules trapped, FHB index, and percentage of seeds infected with *F. graminearum*. The number of viable airborne propagules trapped at wheat anthesis was not predictive of FHB symptoms or DON accumulation in grain (Schaafsma et al. 2005).

The influence of different cropping systems and cropping successions (sequence) was investigated on natural *Fusarium* sp. contamination and fumonisin levels in corn. In addition, the effect of different levels of nitrogen fertilizer (0, 22.5, 45, and 90.0 kg ha⁻¹ nitrogen supplemented with potassium oxide) on fumonisin contamination was assessed. The cropping system, consisting of a conventional and no-till corn following oats, showed the highest mean fumonisin levels and differed significantly ($P < 0.005$) from all other treatments in 2006. Fumonisin levels ranged over 0.13–19.52 µg g⁻¹ (mean 6.97 µg g⁻¹) and over 3.70–7.75 µg g⁻¹ (mean 6.29 µg g⁻¹) in no-till corn following oats during the 2006 and 2007 growing seasons respectively (Ono et al. 2011). *Fusarium* head blight (FHB) disease, infecting small grain cereals, is the result of complex interactions between weather conditions and agricultural practices including crop rotation, tillage, fungicide application, and host resistance. The influence of maize-wheat rotation on the development of disease symptoms of FHB and deoxynivalenol (DON) content in winter wheat was investigated under field conditions using randomized complete block design during 2009–2010 and 2011–2012 in Belgium. The susceptibility of maize cultivar to *Fusarium* spp. and maize harvest method had only a minor effect on the FHB incidence and DON content of wheat grains during the subsequent growing season. The tillage method and resistance of wheat cultivars were more important and capable of exerting significant influence on FHB incidence and DON content. Further, the quantitative effect of these factors depended on the disease pressure. Repeated maize-wheat rotation in combination with favorable weather conditions for FHB might have resulted in accumulation of pathogen inoculums, which led to DON contents of up to 9.90 mg kg⁻¹ in 2012 (Landschoot et al. 2013).

The effect of crop rotation on the incidence of *Phytophthora* blight in pepper (*Capsicum annuum*) was determined. Rotation of pepper with peanut (groundnut) reduced the disease incidence more effectively (by 39%) than with sesame (by 11%) (Kim, 1989). Inclusion of nonhosts in the rotation and/or fallowing may reduce the selection pressure for a given soilborne pathogen and “starve” it out, preventing the large pathogen population buildup and consequently resulting in the reduction in infection of primary crop. Incidence and development of soilborne diseases affecting potato crops are significantly influenced by the frequency and composition of rotation with other crops. The potato pink rot pathogen *Phytophthora erythroseptica* may survive as oospores in the soil for many years and may be endemic in soils where potatoes are cultivated repeatedly; however, it might be possible to reduce the incidence of pink rot disease by adopting crop rotation (Lambert and Salas, 2001). The effect of 2 and 3 year crop rotations and conservation tillage (reduced or no-till) practices on the incidence of potato pink rot disease caused by *Phytophthora erythroseptica* was investigated in Prince Edward Island, Canada. Spring barley and potato cultivar Russet Burbank were included in the 2 year rotation, whereas the 3 year rotation comprised barley (undersown with red clover), red clover, and potato. the development of pink rot disease was significantly less pronounced in potatoes from 3 year rotational soils than from 2 year rotational soils. Potato plants grown in a greenhouse using soils from field plots had less disease in the 3 year rotation than in the 2 year rotation ($P=0.05$). The results suggested that potatoes grown in soils managed under a 3 year rotation appeared to be intrinsically more resistant to infection by *P. erythroseptica* than those managed under the 2 year rotation. Inclusion of red clover in the 3 year rotation might contribute to the disease-suppressive effect in the succeeding potato crop because of its ability to fix atmospheric nitrogen and consequent provision of additional nutrients (Peters et al. 2005).

The effects of the origin of seed potatoes and the cropping history on the phenotypic structure of late blight pathogen *Phytophthora infestans* populations were investigated in Germany during 2000–2002. Populations originating from fields with a history of potato cropping with only a short or no rotation (old fields) were compared with populations from new fields (not cropped to potato for at least 30 years). Seed potatoes were either obtained from breeders or produced on-farm (certified). The importance of seed potato infection in the establishment of new *P. infestans* populations was assessed. Isolates were characterized for mating type, virulence, and rep (repetitive extragenic palindromic) -PCR fingerprints. Among a total of 639 isolates examined from 31 sites, mating types A1 and A2 coexisted in all 3 years in 60–92% of the sites. Over all 3 years, 53 pathotypes were detected in a subsample of 272 isolates. The isolates originating from new fields had significantly higher frequencies of the virulences v1, v2, v6, and v7, indicating the general effects of seed introduction into a new region. Thirty-six fingerprints were detected in a subsample of 281 isolates of which 22 were unique, while 4 occurred in all 3 years and at many sites. Pathogen populations from potato fields that were grown from seed tubers of geographically different origin differed significantly based on χ^2 tests. The genetic distances were less than 0.1 among local populations. On the other hand, distances of US lineages US-1, US-6, US-7, and US-8 ranged over 0.22–0.47. Populations from old fields were more diverse and 14 of the 22 rep-PCR types occurred there among 132 isolates tested, in comparison to 6 in the new fields ($n=140$ isolates) and 2 among 6 isolates from volunteers. The results suggested that both sexual and asexual reproduction cycles play a role in pathogen establishment (Bouws and Frinckh, 2007).

Rotation crops may help conserve, maintain, or replenish soil resources and improve the physical and chemical properties of soil, whereas cover crops are primarily grown to cover and protect the soil from erosion and nutrient losses between periods of crop production. Crops used in rotation with potato, canola, millet (*Panicum milliaceum*), soybean, sweet corn, barley, and red clover and continuous potato (nonrotation) were evaluated for their ability to reduce the incidence/severity of *Rhizoctonia* canker (black scurf), silver scurf, and powdery scab diseases in potato tubers. Overall, canola, rapeseed, sweet corn, and barley/clover as rotation crops reduced the severity of *Rhizoctonia* canker relative to the control ($P=0.001$). Canola and rapeseed rotations reduced the severity to the maximum extent (15–45%) in individual years during 2000–2006 and averaging 20–23% overall during the 7 years. Canola, rapeseed, and bean rotations were also the most effective in reducing black scurf on potato tubers. These rotations had lower scurf severity than most other rotations, and reductions of 32–38% relative to the continuous potato control ($P=0.0002$) were observed (Fig. 11.2). Other rotation crops did not have a significant effect on common scab severity. Reductions in pathogens associated with the use of cover crops were generally modest (5–20%). However, the combined effects of using a cover crop in conjunction with an effective rotation crop were substantial and greater than using either strategy separately. The combination of winter rye cover crop with canola or rapeseed rotations reduced black scurf and common scab by 25–41% compared to control and 21–37% over barley/clover standard rotation. *Verticillium* wilt became a prominent disease only after four full rotation cycles. The incidence of *Verticillium* wilt was the lowest in barley rotations. Barley/clover and rapeseed rotations resulted in the highest soil bacterial populations and microbial activity. All rotations had distinct effect on soil microbial community characteristics. Addition of a cover crop increased the soil bacterial populations and microbial activity, in addition to improving tuber yield by 34%. The results suggested that a 3 year or longer rotation might be more effective, as the 2 year rotation adopted in this investigation had a limited effect on severity of the diseases of potato (Larkin et al. 2010).

Various practices adopted at pre- and postharvest stages may result in different levels of infection of maize (corn) kernels by *Aspergillus flavus* or *Fusarium* spp. Samples from 300 farmers' stores from four agroecological zones in West Africa were collected at the beginning of storage (sample A) and at six months later (sample B). *A. flavus* was predominantly present in samples A (27–47%) than in samples B (8–26%). *Fusarium* spp. were detected in 38–58% of the A kernels samples in both years, but decreased slightly to 29.5% in B samples. Aflatoxin was detected in 38.8% of 744 samples tested with an average of 105 ppb, and 60% of the aflatoxin-positive samples had contamination level of 20 ppb (the intervention levels recommended by the World Health Organization or WHO). Planting local maize varieties, intercropping with cowpea, groundnut, or cassava, use of urea as nitrogen source, damage to grains in the field, prolonged harvesting, long drying periods in the field, and winnowing were the factors associated with increased aflatoxin contamination. On the other hand, planting improved maize cultivars, mixed cropping with vegetables, use of NPK fertilizer, drying of harvested cobs for 60–90 days, drying ears without husk, and the removal of poor-quality ears contributed to a reduction in aflatoxin accumulation in maize kernels (Hell et al. 2003). Aflaroot disease of groundnut (peanut) caused by *Aspergillus flavus* affects peanut production quantitatively and qualitatively, since the mycotoxin aflatoxin produced in kernels poses potential danger to the health of human beings and animals. The efficacy of crop rotation to reduce *A. flavus* populations was assessed, in addition

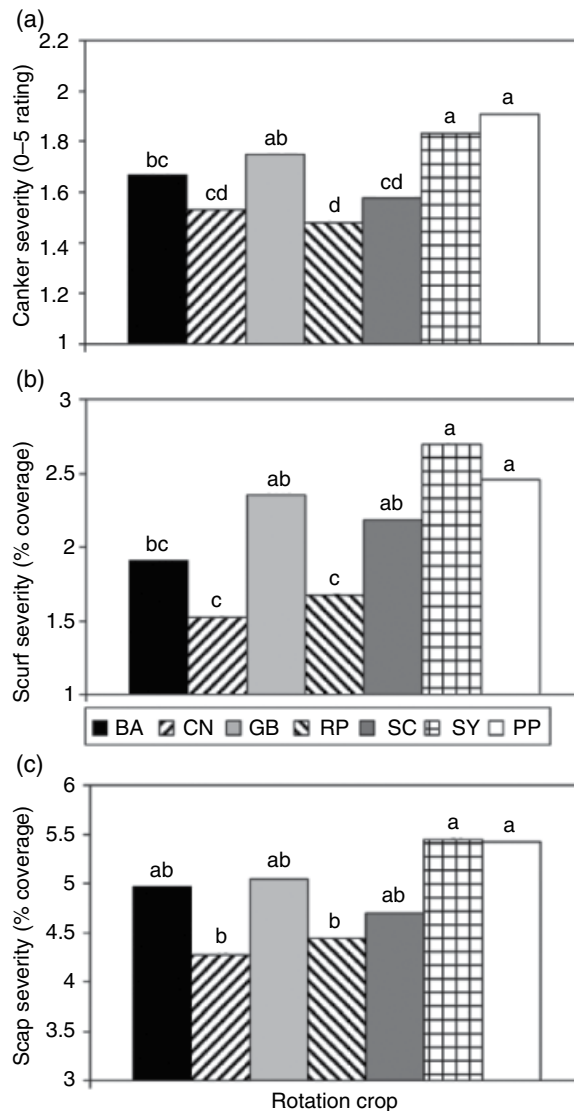


Figure 11.2 Effects of different rotation crops during 2000–2006 on incidence of *Rhizoctonia* canker, black scurf, and common scab diseases in potato. (a) *Rhizoctonia* canker; (b) black scurf; and (c) common scab. Two-year rotational crops include barley (BA), canola (CN), millet/rapeseed (RP), green bean (GB), sweet corn (SC), soybean (SY), and potato (PP, control without rotation). Bars with the same letter are not significantly different as per Fischer's protected least significant difference test ($P < 0.05$). Source: Larkin et al. (2010). Reproduced with permission of the American Phytopathological Society, MN, USA.

to the identification of microorganisms that could reduce population levels of aflatoxin-producing fungi in soil. Peanut crop sequences, continuous peanut, four years of continuous bahia grass followed by peanut, and peanut-corn-cotton were evaluated for their efficacy in reducing the disease incidence and pathogen populations. Enumeration of soil microorganisms indicated that using bahia grass in rotation with peanut reduced

A. flavus population levels, compared with other cropping sequences. As the minimum population density of 2.6×10^6 CFU g⁻¹ soil was required for detection of *A. flavus* in the soil, molecular techniques such as ribosomal intergenic spacer analysis (RISA) had to be employed to determine microbial community structure with emphasis on aflatoxin-producing fungi (Sudini et al. 2011).

The possibility of using crop rotation to reduce the incidence of lettuce drop disease caused by *Sclerotinia minor* was examined. The efficacy of broccoli as a rotation crop was assessed. When lettuce was grown continuously, there was no increase in the incidence of lettuce drop disease for at least two years although soilborne sclerotia were more numerous. Rotation with broccoli reduced disease incidence to some extent only in the first year. The sclerotial density was the lowest in plots with lettuce followed by a fallow period (LFL) and highest in plots with continuous lettuce (LLL). At another location, the reduction of disease was significant in plots with broccoli in rotation. The number of broccoli crops rather than the sequence of lettuce rotations with broccoli was critical for reducing the numbers of sclerotia, possibly because of volatiles formed during the breakdown of glucosinolates present in broccoli. The results indicated that broccoli could be a desirable candidate to effectively reduce the incidence of lettuce drop disease, because it is a nonhost for *S. minor* and contains glucosinolates in the tissues inhibitory to the pathogen (Hao et al. 2003). The effectiveness of the use of *Brassica* crops as green manure or cover crop in reducing the incidence of potato stem rot disease caused by *Sclerotinia sclerotiorum* was investigated. *Brassica napus*, *B. juncea*, and *B. campestris* were grown in three naturally infested potato fields during three cropping seasons over 2008–2010. Disease incidence and mean percentage of dead plants were significantly reduced by all three *Brassica* spp. Growing *B. juncea* was the most effective practice, reducing the disease incidence by 55.6% (average over all years and sites). On the other hand, the average disease reduction percentage by *B. napus* and *B. campestris* were 31.6% and 45.8%, respectively. Cultivation of *Brassica* crops showed potential for inclusion in crop rotation with potato to contain potato stem rot disease (Ojaghian et al. 2012).

Canola (*Brassica napus* and *B. rapa*), a major oilseed crop grown worldwide, is severely infected by blackleg disease caused by *Leptosphaeria maculans* (anamorph *Phoma lingam*). The effects of a 4 year crop rotation and two tillage systems on blackleg disease incidence were investigated during 1999–2002 in Canada. Wheat (W) and flax (F) as rotation crops with canola (C) and conventional tillage (T) or zero-till (Z) were included in the study. Tillage and rotation had significant effects in reducing disease in 2001. Growing canola in rotation with wheat significantly reduced stem infection by 18% in zero-till plots and by 8% in tilled plots, compared with continuous canola. The disease incidence and severity were the least in tilled plots when canola was rotated with wheat. Zero-till plots showed greater levels of disease incidence and severity, compared with tilled plots with or without rotation. Tillage reduced stem infection and severity in all rotation plots, except in the canola-wheat-flax-canola rotation ($P < 0.05$). Tillage could play an important role in reducing the pathogen inoculum since *L. maculans* might survive in stubbles of previous canola crop and ploughing might accelerate the decomposition of canola residues, resulting in the elimination/reduction of pathogen inoculum and consequent lower level of blackleg disease (Guo et al. 2005). Canola was traditionally grown as a rotation crop once in 4 years but market opportunities led to canola being intensively cultivated, resulting in increased incidence of blackleg disease caused by *Leptosphaeria maculans*. A field experiment with split-plot

design was conducted, including blackleg-resistant cultivars in combination with pea, wheat, and flax and fungicide application. Blackleg incidence and severity was increased in rotations comprising more than one canola crop every year, regardless of cultivar, although the blackleg-resistant cultivar was much less affected than the susceptible cultivar. The yield of the resistant canola cultivar was similar among rotations that included canola every 2, 3, and 4 years. Fungicide application was of limited value to maintain canola yield. The results suggested that cultivar resistant to blackleg disease might be preferable for growing canola more intensively than once every 4 years with limited yield reduction. Intensification of canola rotations might increase the risk of inoculum carryover, resistance breakdown, and consequent yield loss. It would therefore be desirable to adhere to less intensive inclusion of canola rotations (once every 4 years) as an effective blackleg disease management strategy (Kutcher et al. 2013).

11.1.6.2 Monoculture

Continuous monoculture of annual crops may provide conditions for rapid multiplication of pathogens such as *Verticillium dahlia*, with a high level of saprophytic survival in the soil. In contrast, some pathogens have a low level of saprophytic survival in the soil and, after reaching certain levels, disease incidence begins to decline. In the case of potato scab pathogen *Streptomyces scabies*, the disease incidence showed a reversible pattern in that there was a rise in disease incidence up to some years after which the soils became more and more suppressive to the pathogen, resulting in a progressive reduction in disease incidence. Such soil that does not support development of the pathogen in subsequent years is designated suppressive soils (Shipton, 1977). Use of varietal mixtures has been demonstrated as an effective method of disease suppression in some pathosystems. Infection by wheat leaf blotch pathogen *Septoria nodorum* was significantly reduced when a mixture of wheat cultivars was grown (Jeger et al. 1981). Rice cultivar mixtures were tested for their effectiveness in controlling brown spot disease caused by *Helminthosporium oryzae* and rice blast disease caused by *Magnaporthe grisea*. The spread of brown spot disease was significantly reduced by planting in alternate rows of two rice cultivars with different levels of resistance to different strains of *H. oryzae* (Grummer and Roy, 1966). A mixture consisting of three upland rice cultivars was tested along with pure stands of individual cultivars in the Philippines. Leaf blast severity was less in the rice mixture than in the pure stand of the most susceptible component, whereas no difference in the blast disease severity on the other cultivars, either in the mixed or pure stands, could be observed (IRRI 1984).

11.1.6.3 Multiple Cropping

The system of growing two or more crops as intercrop, mixed crop, or barrier crops has been followed traditionally in several countries. Two or more crops may be planted simultaneously or in a staggered manner so that the growth periods of the two crops overlap. Crops that are not hosts for the same pathogen(s) are selected for a mixed cropping system. Crops which are planted as intercrop or mixed crop may (i) modify the microclimate of another crop; (ii) intercept or filter out the spores of the pathogen; (iii) restrict the movements of vectors of the viruses or pathogens; and (iv) reduce the infectivity of nonpersistent (stylet-borne) viruses which are lost by their vectors when they feed on nonhosts of the viruses. Intercropping or mixed cropping has been reported to be effective in reducing the incidence of some diseases.

Planting sesame (*Sesamum indicum*) or peanut as intercrops significantly reduced the Phytophthora blight of pepper caused by *Phytophthora capsici* in Korea (Kim, 1989). Extracts of soils on which peanut or sesame was grown and the root exudates of these intercrops inhibited the mycelial growth, sporangium formation, and release of zoospores by the pathogen. In soils amended with the extracts, incidence of Phytophthora blight disease was considerably reduced (Lee et al. 1990, 1991). The influence of intercropping in three consecutive cropping seasons on the incidence of barley diseases was assessed by including dual-grain legume (pea, faba bean, and lupine) -barley intercropping (IC), as compared to the respective sole crops (SC). Reduction in disease incidence was achieved in all IC systems compared to the corresponding sole crops with a general reduction in the range of 20–40%. Barley net blotch disease caused by *Pyrenophora teres* was the most serious disease during the three years of experimentation (Hauggaard-Nielsen et al. 2008). The reduction of disease caused by bacterial pathogen could be achieved by intercropping practice. The effect of intercropping cowpea (*Vigna unguiculata*) with cassava or maize was investigated in five cropping systems, namely cowpea monoculture at high density and at low density, cowpea-maize within row, and cowpea-maize in alternate rows, on the incidence of *Xanthomonas axonopodis* pv. *vignicola*, causal agent of bacterial blight disease. Cowpea monoculture at high density showed a higher level of disease incidence than cowpea-cassava in alternate rows in 1996 and cowpea-maize in alternate rows in 1997. The disease severity index was reduced by 50% in cowpea-cassava in alternate rows in 1997, but not in 1996. The yield loss of cowpea could be compensated by the additional yield of intercrops (Sikirou and Wydra, 2008).

Plant diversification in any form such as mixed cropping, cover crops, border plants, intercrops, and trap crops known as barrier cropping may be useful for protecting the primary crop plants against vector-transmitted virus diseases. The incidence of *Watermelon mosaic virus* could be effectively reduced by growing oats as barrier crop (Chalfont et al. 1977). Likewise, cultivation of different crops as inter-, mixed-, or barrier crops was shown to be effective in reducing the incidence of some virus diseases. *Potato virus Y* (PVY) infection of pepper (*Capsicum* spp.) was reduced by more than 50% when sunflower was grown as a border crop to protect pepper plants (Simmons, 1957) and in potato with borders of sorghum, soybean, or wheat (Di Fonzo et al. 1996). The incidence of PVY and *Cucumber mosaic virus* (CMV) in pepper was significantly reduced by planting maize or sunflower as barrier crops. It was suggested that reduced infection by viruses might be due to a decrease in infectivity of vectors that landed on maize or sunflower which are nonhosts of the virus, before reaching the susceptible pepper plants (Avilla et al. 1996). In a later study, sorghum, corn, or vetch as border crops were evaluated for their effectiveness in protecting pepper crops against infection by PVY and CMV. Sorghum and vetch borders were effective only in two of four experiments conducted. The level of reduction in virus disease incidence was less than 15% (Feres, 2000).

The effectiveness of intercropping with grain sorghum, peanut, soybean, or corn to reduce the incidence of *Watermelon mosaic virus* (WMV) and *Papaya ringspot virus-W* in pumpkin was determined in five field experiments conducted over a 3 year period. Disease incidence was reduced by 43–96% ($P < 0.05$) in plots with grain sorghum as intercrop. On the other hand, no significant reduction in disease incidence could be observed in pumpkin in plots with borders of peanut, soybean, or corn. Borders of grain sorghum were able to reduce the virus disease incidence to a lower

level compared to grain sorghum as intercrop. Intercropping soybean and peanut with pumpkin reduced disease incidence to a lesser extent than intercropping with grain sorghum (Damiconi et al. 2007).

The effectiveness of the intercropping models such as cassava-pepper, plantain-pepper, and maize-pepper was assessed for the extent of protection provided by them to pepper against *Pepper vein mottle virus* during 2004–2006. The effectiveness of the intercrops was assessed based on the extent of reduction in the number of plants infected, enhancement of plant vigor, and yield and quality of pepper fruits. Based on the averages of disease incidence for three years, virus disease incidence and severity were significantly reduced by all three intercrops tested. The incidence of virus disease in sole crop of pepper was relatively higher (42%). Plantain-pepper combination showed the maximum level of disease incidence of 58.3%. The yield was increased to the maximum extent (5.98 t ha^{-1}) in plots with maize intercropped with pepper as against pepper as sole crop yield (1.54 t ha^{-1}) which was far low (Fajinmi and Fajinmi, 2010). Barrier crops may have different mechanisms for reducing the incidence of virus diseases transmitted by aphid vectors in a nonpersistent manner. During the process of feeding, the aphids may probe the nonhosts as they cannot differentiate the hosts from nonhosts. Virus particles present in the stylets/mouth parts may be lost, reducing the virus charge in the probing aphids. The aphids may therefore become either noninfective or carry fewer virions that may not be able to initiate infection in the primary host (crop plant). Barrier plants may therefore function as a virus-sink for nonpersistent viruses. Alternatively, barrier plants may act as physical barriers, resulting in a reduction in the number of aphids having access to the primary crop host and consequent reduction in virus infection. Another possibility is the ability of the barrier crops to protect the primary crop by camouflaging them from vector insects. In yet another way, the protector plants may attract the aphids and function as a “decoy.” Although the barrier crop may provide protection to the primary crop, the competition for nutrients and space should not be a limiting factor for its use as protector crop (Hooks et al. 2007).

11.2 Reduction of Pathogen Inoculum by Physical Techniques

Crop diseases can be effectively managed by dovetailing physical and chemical techniques with conventional cultural practices to reduce the size of the initial pathogen population present in and/or on the seed and propagules, and to reduce the pathogenic potential (virulence) of the pathogen(s). Physical and chemical methods may be applied to whole plants, planting materials, and field soils to restrict the disease incidence.

11.2.1 Treatment of Seeds and Propagules

Seeds and propagules such as tubers, setts, corms, and bulbs may be exposed to different physical and chemical agents to reduce the pathogen inoculum by inactivation. As an initial step, infected seeds that are small in size, malformed or mixed with fungal structures such as ergot, smut sori, or sclerotia may be separated by winnowing or sieving because of the differences in seed size or weight. Alternatively, the seeds may be immersed in 20% sodium chloride (common salt) solution. The lighter, infected seed and fungal structures will float on the surface of saline solution and can be easily

removed. The seeds are then dried and stored after treatment with recommended plant protection chemicals until they are sown. Various physical techniques based on heat, radiation, and microwaves have been applied to eliminate the pathogens present in the seeds and propagules with different degrees of effectiveness.

11.2.1.1 Heat Treatments

The effectiveness of heat as a disinfectant was first demonstrated to eliminate the potato late blight disease caused by *Phytophthora infestans* from seed tubers by exposing them to a current of hot air at 40°C for four hours. Later, hot water was used for control of smuts of barley and oats (Jensen, 1888). Application of heat for the elimination of *Ustilago tritici* and *U. nuda*, causal agents of loose smut of wheat and barley respectively, was found to be effective. The seeds were immersed in water at 20–30°C to stimulate the dormant mycelium of the pathogens present in them, and the pathogens were killed when the seeds were immersed in hot water at 50–52°C (Martin and Woodcock, 1983). A useful modification of this procedure that could be adopted in tropical countries was developed by Luthra (1953) in the Punjab state in India. The wheat seeds are soaked in cold water for about four hours and then spread as a thin layer under the sun (temperatures around 50°C) for another four hours. The seeds are stored under dry conditions. This method was cumbersome but simple, and adopted widely by growers for the control of loose smut disease of wheat. The bacterial pathogen *Xanthomonas campestris* pv *vignicola* could be eliminated by solar treatment. The cowpea seeds were soaked in tap water for four hours (38°C), then for four hours under bright sun (08:00–12:00 noon) and dried for 5 hours under sunlight. This method was effective in areas where day temperatures reached 45°C or more (Jindal et al. 1989). A similar approach was found to be effective for the control of pineapple heart rot disease caused by *Phytophthora cinnamomi* by exposing pineapple crown buds, lateral buds, suckering buds, and rhizomes to sunlight for 4–8 h at a temperature of 33°C (Yang and Chang, 1998). The efficacy of treatment of winter wheat seeds with electron treatment was compared with hot water treatment (52°C for 10 min) for the control of bunt disease caused by *Tilletia caries*. There was no adverse effect on seed germination due to electron treatment, whereas hot water treatment reduced seed germination (Winter et al. 1998).

The effects of heating seeds at 60°C or 70°C for up to 14 days on loads of *Fusarium graminearum* (Fg) and seed viability of two samples each of barley (*Hordeum vulgare* B1, B2), Canadian western red spring wheat (*Triticum aestivum*, RS1 and RS2), and Canadian western amber durum wheat (AD1, AD2) were assessed. The samples RS2 and B2 with an incidence of 23 and 84% of Fg respectively were also heated at 60°C for 24 days and at 80°C for 10 days. Pathogen populations and seed viability were assessed by plating seed onto potato dextrose agar (PDA) and filter paper, respectively. Fg was eliminated from RS2 after 15 days at 60°C, 5 days at 70°C, or 2 days at 80°C. In B2, Fg was eliminated after 21 days at 60°C, 9 days at 70°C, or 5 days at 80°C. After heating at 50°C or 70°C, the frequency of *Cochliobolus sativus* in B1 declined slightly but significantly over time, whereas it substantially increased in B2. A significant reduction in the incidence of *C. sativus* was observed in B2 after heating at 80°C. Germination rates of seeds in most samples were not affected by the treatment times and temperatures that were sufficient to eradicate *F. graminearum*, but significant decline in viability was noted for AD2 and B1 heated at 70°C. The seed germination of B2 increased when heated at 70°C, but declined when heated at 80°C. The results showed that thermotherapy

might be useful for restricting national and international movements of *F. graminearum* and other heat-sensitive pathogens in germplasms used for research and breeding purposes (Patrick et al. 2002).

In a later investigation during 2001–2002, seed samples of spring wheat infested with *F. graminearum* or uninfested seeds were heat treated at 30, 50, 70, or 90°C or kept at room temperature (20–25°C) for 5 days. Seed germination (%) and field assessments including seedling emergence, plant height and grain yield, 1000-grain weight, and test weight were recorded. Treatments at 30°C and 50°C were ineffective and treatment at 90°C killed both the seed and the pathogen. At 70°C, recovery of *F. graminearum* was less than 1%. Emergence from seed infested with *F. graminearum* was significantly lower than emergence from seed free from pathogen in each year. Based on the results, heat treatment at 70°C for 5 days was recommended as a means of essentially eradicating *F. graminearum* from wheat seed prior to sowing and preventing the movement of *F. graminearum* to other areas that were free of the pathogen (Gilbert et al. 2005).

Application of hot air at 85°C for five days to pepper (chilli) seeds effectively reduced seedborne infection by fruit rot pathogen *Colletotrichum capsici*. The hot air treatment reduced seed germination to some extent (Kobayashi, 1990). Drying tomato seeds with discoloration in an air oven at 29.5–37.5°C for 6 h resulted in elimination of *Phytophthora infestans*, incitant of late blight disease (Vartanian and Endo, 1985). Similar dry heat treatment of tomato seeds at 78°C for 2 days resulted in the eradication of *Tomato mosaic virus*. Heat treatment did not show any adverse effect on seed germination and was found to be more effective when compared to soaking the seeds in trisodium phosphate recommended for seed disinfection (Green et al. 1987). Soaking onion seeds in hot water at 50°C for 20 min was found to be more efficient in reducing seedborne infection by *Alternaria porri* and *Stemphylium vesicarium*. However, seed germination and seedling emergence were affected (Aveling et al. 1993).

Aerated steam treatment was found to be effective in eliminating the seedborne pathogens. *Alternaria carthami* in sunflower seeds and *Fusarium moniliforme* in corn seeds were eliminated by treating the seeds at 54°C and 62°C for 30 min, respectively, but seed viability was affected slightly (Navaratnam and Shuttleworth, 1983). Eradication of *Septoria apiicola* completely from celery was achieved after treatment of seeds with aerated steam at 64°C for 30 min. Likewise, the bacterial pathogens *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds and *Xanthomonas campestris* pv. *campestris* in cabbage seeds were eliminated after treatment at 56°C for 30 min and 54°C for 30 min, respectively (Navaratnam et al. 1980). Hot water treatment of rice seeds at 60°C for 10 min effectively suppressed colonization of seeds by *Drechslera oryzae*, causal agent of brown spot disease, which is internally seedborne (Krishnamurthy et al. 2001).

A high-precision method involving the use of warm air in moisture equilibrium with the wheat seeds to be treated was employed for the elimination of seedborne fungal pathogens *Tilletia caries* and *Microdochium nivale*. The warm air treatment of wheat seeds was as effective as chemical seed treatment. In addition, seed germination and plant development were not impaired due to warm air treatment. This method may be useful for large-scale applications for pathogen elimination at a low cost (Forsberg, 2001). Canadian western red spring wheat and barley seeds with high levels of infection (84% and 23%) by *Fusarium graminearum*, causing Fusarium head blight (FHB) disease, were subjected to different dry heat treatments at 60°C for 15 days, at 70°C for 5 days, or at 80°C for 2 days. These treatments effectively eradicated the pathogen from wheat seeds. A similar approach for successful elimination of the pathogen from barley seeds

by treatment at 60°C for 21 days, 70°C for 9 days, or at 80°C for 5 days. Seed germination in most of the samples was not significantly affected (Clear et al. 2003).

The effectiveness of the application of dry heat treatment for the eradication of the bacterial pathogen *Acidovorax axonopoides* subsp. *citrulli* (*Aac*) causing bacterial fruit blotch (BFB) disease in melon, watermelon, and wax gourd from the seeds was evaluated since the Japanese Government legally restricted entry of this pathogen into Japan. The seeds of melon, cucumber, squash, wax gourd, and bottle gourd infested with *Aac* were treated at different temperatures and were sown in the soil. Complete disinfection of seeds of melon and cucumber and small-seeded squash was achieved by dry treatment at 85°C for 3–5 days. The large-seeded squash, wax gourd, and bottle gourd were sensitive to dry heat, indicating that disinfection of seeds for elimination of BFB pathogen seeds by dry heat treatment may not be feasible in crops with large seeds (Kubota et al. 2012).

Thermotherapy has been shown to be effective in eliminating some plant viruses from seeds and propagules. However, heat treatment of seeds carrying a virus internally has not been effective in eradicating the virus in a high percentage of treated seeds, and heat treatments generally impair seed viability. The effectiveness of heat treatments for the eradication of *Lettuce mosaic virus* (Walkey and Dance, 1979), *Cowpea mosaic virus* (Verma, 1971), and *Capsicum mosaic virus* (Stijger and Rast, 1988) from seeds has been reported. Dry heat treatment of tomato seeds at 70°C for 3 days or at 80°C for 1 day was effective in eliminating *Tomato mosaic virus* (TMoV) in less deeply seated seeds extracted freshly (Broadbent, 1976). Heat treatment of tomato seeds at 85°C for 24 h eliminated TMoV without impairing seed germination (Noor-Hassan et al. 1988). *Cucumber green mottle mosaic virus*, a member of the genus *Tobamovirus*, is easily transmitted through sap inoculation and is also seed-transmitted. The CGMMV could be eliminated by heat treatment of seeds. In order to determine the effect of heat treatment on CGMMV, virus-contaminated oriental melon (*Cucumis melo*) seeds from field-grown plants were gradually dried at 35°C and 50°C for 24 h each and then incubated at the different temperatures of 72, 75, 79, or 82°C for 72 h. Heat treatment at 75°C for 72 h completely inactivated CGMMV on contaminated seeds, as indicated by bioassay of the heat-treated seeds on *Nicotiana benthamiana*. Heat treatment reduced the amount of coat protein (CP) of CGMMV in the preparations purified from heat-treated seeds. However, there was no clear relationship with temperature. Electron microscope observations of virus particles purified from heat-treated seeds showed increased breakdown of virions into a range of fragments as the temperatures increased. Results of RT-PCR confirmed the heat inactivation of virus in melon seeds using primers for a heat-sensitive region. The results indicated that elimination of CGMMV from melon seeds could be due to inactivation of virions present on seed surface (Kim et al. 2003).

Thermotherapy through use of hot water has been shown to be effective in eliminating pathogens from propagules. *Sugarcane mosaic virus* and its strains (A, B, D, H, and I) were eliminated from sugarcane setts by serial hot water treatment on four successive days at 54.8, 57.3, 57.3, and 57.3°C for 7 min on each day (Benda, 1972; Benda and Ricaud, 1978). For the control of sugarcane grassy shoot disease caused by phytoplasma, treatment of sugarcane setts in aerated steam was demonstrated to be effective in eliminating the pathogen (Edison and Ramakrishnan, 1972). The ratoon stunt disease caused by *Clavibacter xyli* subsp. *xyli* was efficiently eliminated by treating the setts in hot water at 50°C for 2–3 h (Steindl, 1961). Hot air (Steindl, 1961) and moist-hot air treatment (Shukla et al. 1974) were also reported to be effective against sugarcane

ratoon stunt disease. The adverse effects of hot water/hot air/aerated steam treatment on germination of setts are considered a limiting factor. However, large-scale use of heat therapy as a disease control measure is advocated in several sugarcane-growing countries.

Agrobacterium (tumefaciens biovar 3) vitis, causal agent of crown gall disease of grapevine, destroys infected plants in due course causing serious production losses. Hot water treatment of dormant cuttings of grapevine cultivar Thompson seedless and rootstock NAZ3 (*Vitis vinifera* x *V. rupestris*) at 50°C for 30 min effectively eradicated or reduced the pathogen population below detection level. *A. vitis* strains differed in their sensitivity to heat, but they showed greater levels of sensitivity to heat compared with strains of *A. tumefaciens* biovar 1 and 2. Treatment for 30 min killed a population of about 10^3 CFU mL⁻¹ in broth culture. Nontumorigenic biovar 1 strains could be recovered from hot-water-treated cuttings. The effects of hot water treatment of dormant cuttings were assessed in a field nursery for a period of 9 months. The viability and growth of different cultivars/rootstocks were affected by hot water treatment to different levels. The number and length of canes, root dry weight and length of canes, dry weight of roots, and length and diameter of trunks increased in most cultivars/rootstocks following hot water treatment. Treatment time generally affected the bud survival and, in most cases, increased the level of callus formation at the base of the cuttings. Hot water treatment of dormant grapevine cuttings provided a simple, effective, economical, and environmentally safe means of eradicating *A. vitis* (Mahmoodzadeh et al. 2003).

The effectiveness of hot water treatment (HWT) for the elimination of elm yellows phytoplasma from dormant cuttings and branches of elm plants was assessed. The selected planting materials were treated in hot water at $50 \pm 0.5^\circ\text{C}$ for 45 min. The cuttings were then processed for rooting and the rooted plants were grown in the greenhouse for two years. The plants were closely checked for the development of disease symptoms and also indexed by nested PCR assay. None of the 105 plants growing from HWT-treated cuttings showed disease symptoms, and they were also PCR negative. Plants from untreated cuttings developed symptoms and the presence of EY phytoplasma was also detected by PCR assay (Boudon-Padieu et al. 2004). The efficacy of hot water treatment alone or in combination with the application of tetracycline hydrochloride at a concentration of 500 ppm was assessed for the elimination of sugarcane white leaf phytoplasma. Soaking cane stalks in hot water at 50°C for 2 h, followed by soaking the stalks in tetracycline HCl for 2 h, reduced the phytoplasma incidence up to 28.57% more than using hot water treatment alone. Further, the phytoplasma population was reduced by the combined treatment to the maximum extent as revealed by PCR assay, in addition to enhanced germination of setts (Kaewmanec and Hanboonsong, 2011).

Verticillium sp. and *Fusarium* sp. are involved in the internal discoloration of horseradish roots. Horseradish propagative rootstocks (sets) 0.5–3.0 cm in diameter and 20–25 cm long were treated by immersing them in water at 44–50°C at 1°C interval for 30 min. Treated and untreated sets were cultured on acidified potato dextrose agar to detect the presence of the fungal pathogens in the sets. Treated and untreated control sets were planted in the greenhouse and fields to determine the effects of hot water treatment on germination of sets and vigor of the plants. Treatments at 46°C and below were ineffective at eradicating the sett-borne inoculum. The most effective hot water treatment was 47°C for 20 min, which did not affect set germination or plant vigor but eliminated pathogens to the maximum extent. Temperatures of 48–50°C either delayed or reduced set germination as well as plant vigor (Eranthodi et al. 2010).

Azalea web blight caused by binucleate *Rhizoctonia* species seriously affects the production of Rhododendron cultivar Gumpo White. The effectiveness of submerging terminal leafy cuttings in water at 50°C for 21 min for the elimination of the pathogen was assessed. In addition, the rooting response and tissue sensitivity of evergreen azalea cultivars (12) to submersion of leafy cuttings in water at 50°C for 20, 40, 60, and 80 min were determined. The cuttings showed difference in their thermosensitivity when exposed to 50°C for 60–80 min, resulting in differing responses in root development and final leaf count. Incremental increase in temperature of water resulted in increased leaf damage. However, submerging azalea cuttings of all 12 cultivars in hot water at 50°C eliminated *Rhizoctonia* spp. from stem and leaf tissue with only a low likelihood of sustaining detrimental damage to cuttings (Copes and Blythe, 2011).

11.2.1.2 Forced-Air Treatment

The effect of fan-forced wind on the incidence of rice blast disease caused by *Magnaporthe grisea* was assessed under field conditions in two successive seasons in Japan. Electric fans (5 kW, 110 cm blade diameter) set on the ridge of paddy fields at a height of 5 m above ground level were used to artificially generate wind. The fan operated twice daily for 30 min periods at 23:00 h and 04:00 h from June 15 to September 1, and the wind speed ranged over 2.0–7.3 m s⁻¹. In season 1, the incidence of both rice leaf blast and panicle blast was significantly lower in zones receiving wind at speeds of 2.6–7.2 m s⁻¹. However, the zone receiving a wind velocity of >7.3 m s⁻¹ was severely affected by leaf and panicle blast. The zone that received a wind speed of 2.6 m s⁻¹ or lower showed inefficient reduction of leaf and panicle blast disease, but it was better than the central zones. In season 2, a fan-forced wind was applied from June 16 to September 9 with the velocity adjusted to 3.0–6.0 m s⁻¹. The incidence and severity of leaf and panicle blasts (chaff, rachis-branch, and neck blast) were efficiently reduced in wind-treated fields. The fan-forced wind was more effective in reducing the rice blast disease severity than conventional fungicides. The results indicated the potential of fan-forced wind for managing rice blast disease effectively (Taguchi et al. 2014).

11.2.1.3 Radiation and Microwave Treatments

Ionizing radiation, particularly gamma rays, has been used extensively for the preservation of food products and also for extending the shelf life of fruits and vegetables. Gamma irradiation was shown to be effective in eliminating fungi associated with maize (corn) seeds without affecting seed germination (Cuero et al. 1986). Elimination of the endophytic fungus *Acremonium coenophialum* from tall red fescue seeds by applying gamma radiation at noninjurious doses was achievable (Bagegni et al. 1990). Following dry treatment of barley seeds with gamma irradiation up to 10,000 roentgens, the incidence of *Barley stripe mosaic virus* (BSMV) in plants growing from treated seeds was significantly reduced. However, BSMV was not eliminated when seeds pre-imbibed in water were irradiated in a similar manner (Halliwell and Langston, 1965).

Seed transmission of *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) was reduced by exposing *Prunus* seeds to irradiation treatment (Megahed and Moore, 1969). Effectiveness of laser treatment for the elimination of four seedborne pathogens *Ustilago segetum*, *Pyrenophora graminea*, *Cochliobolus sativus*, and *Fusarium* spp. was evaluated. Barley seeds exposed to laser beams were sown in the field. Seed transmission of the fungal pathogens to developing seedlings was reduced significantly (Bel'skii and Mazulenko, 1984). *Erwinia carotovora* subsp. *carotovora* (Ecc) could be

eliminated from artificially contaminated tobacco seeds which were subjected to microwave treatment at 625 W for 20 min. Seed germination was not impaired by the microwave treatment. In contrast, microwave treatment of larger seeds of cabbage and bean reduced seed germination, possibly because of their inability to radiate the heat away from the seed during the treatment (Hankin and Sands, 1977).

The efficacy of infrared irradiation as a sterilant of seeds was evaluated for eliminating bacteria from wheat seeds. Bacterial population was significantly reduced from 5.7×10^4 CFU to 0.73×10^3 CFU by exposing the seeds to 2.0 kW range for 60 sec. Intermittent irradiation was more effective in maintaining the internal quality of wheat. A negative correlation between irradiation doses and extent or reduction of bacterial population on the seeds was observed (Hamanaka et al. 2000). Different doses of ultraviolet-C (UV-C) were applied to cabbage seeds to assess its effect on the development of black rot disease caused by *Xanthomonas campestris* pv. *campestris* (Xcc). A dose of 3.6 kJ m^{-2} was optimum and effective in reducing black rot disease incidence and the pathogen population density in infected cabbage leaves. Seeds exposed to UV-C at 3.6 kJ m^{-2} produced plants with the most desirable color, highest weight, largest head diameter, and delayed maturity. Seeds treated with a low hermetic UV-dose of 3.6 kJ m^{-2} showed 90%, 40%, 60%, and 6% reduction in black rot in plants when stored at room temperature for 2 days, 1, 5, and 8 months respectively. Eight-week-storage at room temperature for two days after UV-C treatment was the most effective (Brown et al. 2001).

The effects of microwave treatments applied for 0, 15, 30, and 45 s on fungal pathogens present in and on winter wheat seed were determined. Agar plate isolation and DNA analysis were carried out to assess the pathogen populations and seed germination and seedling emergence and seed viability were determined by tetraolium tests. For all durations tested, microwave treatment significantly reduced levels of seed contamination; it was especially effective in controlling *Fusarium* spp. and *Microdochium nivale* in wheat. Conventional seed survived microwave treatment better than organic seed. Seed moisture content was inherently linked to losses in vigor upon exposure to microwaves. The death of fungal pathogens was attributed to heat and desiccation rather than denaturation of pathogen DNA, since fungal DNA concentration in seeds did not decrease with the observed loss of culturable fungal propagules. The results indicated that microwave treatment of wheat seed could be applied to reduce fungal populations significantly on both conventional and organic wheat seed (Knox et al. 2013).

11.2.1.4 Use of Inorganic Mulches

Plastic foils have been used as inorganic mulch for effective management of some pathogens which are transmitted via seeds/plant propagules and become soilborne in the absence of the primary host (crop) plants. Soil solarization involves the use of thin clear polyethylene sheets to cover the soil after irrigation during summer or seasons with high temperatures. The solar energy is trapped by plastic sheets which prevent loss of heat by evaporation and convection currents. Solarization results in a layer of pasteurized or disinfested soil, but not sterilized soil. The effect of solarization is similar to that obtained by application of aerated steam. It is important that high soil moisture levels are maintained during solarization. The main benefit of soil solarization appears to be its long-term benefits, as it is a physical treatment which does not have a phytotoxic effect or leave any harmful residue as in the chemical treatments. Further, solarization selectively eliminates pathogens while maintaining high biological activity. Another (probably the most important) advantage of employing solarization for elimination of

pathogens is that the method is simple and safe, with none of the hazards associated with chemical treatments. Despite its several advantages, use of solarization is limited to areas where hot conditions exist and to crops which have a high economic value (Narayanasamy, 2002).

The effectiveness of soil solarization using plastic foil mulch for the control of several soilborne pathogens has been reported. Effective elimination of *Verticillium dahliae* and *Rhizoctonia solani* was achieved by covering the soil with polyethylene sheets (Pullman et al. 1981). Soil solarization using photoselective low-density polyethylene sheet for a period of 32–49 days prior to planting tomatoes significantly reduced the population of *Burkholderia solanacearum* up to a depth of 15 cm. The increase in temperature in solarized soils was 5.7, 7.1, and 5.0°C over nonsolarized soil at depths of 5, 15, and 25 cm, respectively. The sensitivity of the pathogen to higher temperatures developed in the soil covered with plastic foil might have a bearing on the effectiveness of soil solarization in the elimination of the pathogen present at different soil depths (Chellemi et al. 1994).

Soil solarization using transparent polyethylene sheets was carried out in commercial tomato production in plastic houses for the control of tomato bacterial canker disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). Soil mulching with polyethylene film was done for about six weeks in plastic houses. Symptoms induced by *Cmm* were drastically reduced throughout the tomato growing season. In contrast, the bacteria belonging to the genera *Pseudomonas*, *Bacillus*, and *Streptomyces*, present in the tomato rhizosphere, were able to survive the enhanced temperatures attained following solarization. The fluorescent pseudomonads isolated from the rhizosphere soil of tomato plants raised in solarized soil induced resistance to the bacterial canker disease when applied to tomato seeds. The beneficial effects of soil solarization were expected to convince the growers in favor of adopting the soil solarization practice using polyethylene sheets to reduce incidence of tomato bacterial canker disease (Antoniou et al. 1995).

The environmental conditions in the soil and above are significantly altered when the soil is covered by polyethylene mulch. The magnitude of effects depends on the characteristics of the polyethylene sheet used and how the covering of the soil is fixed (attached to the soil or not). Different kinds of polyethylene mulches, including a water-permeable agricultural textile treated with copper hydroxide, were evaluated for their efficacy in suppressing or preventing the development of potato tuber blight disease caused by *Phytophthora infestans* under field conditions. Black polyethylene film and copper hydroxide-coated agricultural textile reduced the incidence of tuber blight relative to the appropriate controls, while other mulches did not have any effect on the disease development. Black polyethylene film might block water, resulting in the prevention of infiltration of inoculum through potato hill and alteration of the soil environment within the hill. This might markedly restrict the movement of inoculum and infection capacity of *P. infestans*. The disease suppression by agricultural textile was attributed to the copper hydroxide coating (6 g m^{-2}) which could kill the sporangia and zoospores of *P. infestans* in the soil. The protection offered to tubers by the mulches was only partial, so their use may not be acceptable to potato growers (Glass et al. 2001).

In a later investigation, the effectiveness of covering the soil with polyethylene before planting, with or without application of fungicides commonly applied when growing organic tomato crop to suppress the development of late blight disease of tomato caused by *Phytophthora infestans*, was evaluated. Polyethylene mulch provided consistent,

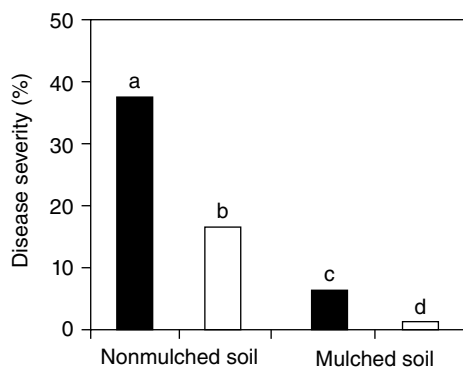


Figure 11.3 Effectiveness of mulching with bicolor aluminized polyethylene before planting and application of chemicals provide additive effects by reducing disease severity. Unfilled bars: with chemical (Kocide 2000 + Neemguard) application and filled bars: without chemical application. Source: Shtienberg et al. (2010). Reproduced with permission of the American Phytopathological Society, MN, USA.

effective, and highly significant suppression of the late blight disease, the control efficacy being $83.6 \pm 5.5\%$. However, the fungicides were less effective with a control efficacy of $34.5 \pm 14.3\%$. The combination of mulch and fungicides demonstrated an additive effect, reducing the disease severity by 96% (Fig. 11.3). All kinds of polyethylene mulches, namely bicolor aluminized, clear, or black, were effective in suppressing tomato late blight disease development. Other benefits of using mulches could be reduction in weed infestations and saving of irrigation water requirement by 30% (Shtienberg et al. 2010).

The possibility of reducing the incidence of viral diseases by using reflective plastic mulches has been investigated. These mulches reflect short-wave light which confuses the incoming alate aphids, resulting in a reduction of the number of aphids alighting on plants. Consequently, the incidence of viruses transmitted by them is reduced. Plastic foils of different colors have been tested for their effect on the vectors of viruses (attraction/repellence) and consequently on the incidence of virus diseases. Significant reduction in the incidence of *Cucumber mosaic virus* (CMV) and *Potato virus Y* (PVY) was achieved by placing a yellow polyethylene sheet spread with a sticky substance to trap the aphids, which were attracted by the yellow color (Loebenstein and Raccach, 1980). The repellent action of the reflective surfaces was shown as the principal reason for their use in the control of aphid-borne nonpersistent viruses. Infection of bell pepper by CMV and PVY was significantly reduced by using gray plastic mulch, and the winged aphids trapped in the mulched plots were only 6–12% of that in the unmulched plots (Loebenstein et al. 1975).

Use of silver spray mulch and silver plastic mulch reduced the incidence of *Watermelon mosaic virus*, *Zucchini yellow mosaic virus*, and *Cucumber mosaic virus* on zucchini. In the crop raised in fall 100% of plants grown in unmulched plots with and without insecticide application were infected at first harvest, whereas only 10% of plants grown over silver mulch exhibited infection. Silver-pigmented mulches were more effective in repelling aphids and delaying disease incidence than white-pigmented mulches. Yields of marketable fruits increased by 75% and 80% due to silver polyester film and silver spray mulches, respectively ((Loebenstein and Raccach, 1980); Summers et al. 1995).

Symptom development in squash following infection by ZYMV, *Watermelon mosaic virus 2*, and CMV was delayed due to the use of aluminum foil mulch. The number of marketable fruits increased significantly in mulched plots. Infectivity by these viruses on courgette was reduced by reflective mulches (Ansanelli et al. 1997).

Aphid-transmitted viruses, namely *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV), severely infect cantaloupes, resulting in heavy losses. Reflective, metalized plastic mulch with a thin coat of aluminum ions reduced the incidence of the diseases caused by these viruses. The number of winged aphids/leaf was significantly higher on plants grown over bare soil (control) than those grown over plastic mulch. The lower aphid counts on plants grown over plastic mulch resulted in lower virus disease incidence. The virus disease incidence in plots covered with plastic mulch was below 10% through mid-September 2002. The incidence of virus diseases was related to the number of winged aphids/leaf, with the highest percentage of infected plants present in the control plots (Summers et al. 2005). The effect of integration of UV-reflective plastic mulch and commercially available resistance-inducing compound BioYieldTM on the incidence of *Watermelon mosaic virus* (WMV) infecting summer squash (*Cucurbita pepo* var *melo*) was assessed. Incidence of WMV was significantly reduced in plants grown on silver-on-black (UV-reflective) mulch compared with plants grown on black mulch (control). In the fall trial, highly UV-reflective silver mulch was also included for testing. Incidence of WMV and ELISA detection data showed that WMV infection was significantly lower in squash plants in the silver mulch treatment compared to silver-on-black and black mulch treatments. However, yields of squash plants in the silver-on-black mulch treatment were higher than those from plants in the silver or black mulch treatments. The treatments with BioYieldTM neither reduced WMV incidence nor increased the squash fruit yields in both spring and fall trials (Murphy et al. 2008).

11.3 Reduction of Pathogen Inoculum by Chemical Techniques

11.3.1 Treatment of Seeds

11.3.1.1 Chemical Application

Treatment of seeds with certain chemicals facilitates the application of fungicides and the seed surface may be more uniformly covered by the seed-treating chemicals. For example, cotton seeds are routinely treated with concentrated sulfuric acid (added at the rate of 1 kg/10 kg of seeds) to delint them. The seeds are then washed with water to remove the acid and shade-dried before the application of fungicides or antibiotics to protect them against seedborne and soilborne pathogens. Immersion of seeds in hot liquid carbon tetrachloride solution was found to be less damaging than hot water. Seeds of flax were exposed to carbon tetrachloride at a range of temperatures for the control of two seedborne pathogens, *Mycosphaerella linicola* and *Aureobasidium lini*. Treatment of seeds at 70°C for 15 min or at 65°C for 20 min significantly reduced the seedborne inoculum of the pathogens. Heat transference through carbon tetrachloride did not reduce seed germination, but affected the seedborne pathogens (Cruickshank, 1954). Treatment of larger seeds such as peas with carbon tetrachloride at 70°C for 2 h reduced infection by the internally seedborne pathogen

Mycosphaerella pinodes, but failed to eradicate the pathogen without causing phytotoxicity (Maude, 1960). The use of hot refined soybean oil as heat transference medium was evaluated for controlling the internal infections of *Phomopsis* spp. in soybean seeds. Immersion of soybean seeds at 70°C for 5 min or at 140°C for 10 s was effective in eliminating the fungal pathogen from seeds (Zinnen and Sinclair, 1982).

11.3.1.2 Fermentation

Fermentation of seeds in water or hot juice has been shown to be effective in reducing seed transmission of some pathogens. Transmission of *Tobacco mosaic virus* through seed of tomato was reduced by seed fermentation. The bacterial fruit blotch (BFB) of watermelon caused by *Acidovorax avenae* subsp. *citrulli* could be reduced by fermentation of seeds in watermelon juice or debris prior to washing and drying. Seed transmission of the bacterial pathogen was reduced from 61% to less than 1%. Fermentation of seeds was as effective as treatment with hydrochloric acid (1%) (Hopkins et al. 1996).

11.3.2 Treatment of Soils

Application of elemental sulfur lowers soil pH, making the conditions unsuitable for the development of soilborne pathogens. Some of the bacterial diseases may be managed by incorporation of sulfur in the soil. Infection of potatoes by *Burkholderia* (*Pseudomonas*) *solanacearum* could be reduced by treating the soil with sulfur to lower the soil pH to 3.8 in June, and the pH was maintained below 4.0 until November. Excess acidity was neutralized by the addition of limestone and the pH was increased to 5 or more. Bacterial pathogen population was significantly reduced under such conditions in Florida, USA (Stevens and Stevens, 1952). Potato scab caused by *Streptomyces scabies* was also contained by similar soil treatment with sulfur followed by liming the soil (Smiley, 1975; Fry, 1982). Soil treatment with lime to raise the soil pH was reported to be effective in reducing the club root disease of crucifers. This disease caused by *Plasmodiophora brassicae* affects cabbage, cauliflower, and broccoli, reducing the production significantly. The addition of lime to raise the soil pH to 7.2 arrested the development of the club root formation in the affected plants. The higher pH reduced spore germination, restricting the ability of the pathogen to induce the disease (Walker, 1957). The extent of disease suppression due to lime application was found to be proportional to how thoroughly the lime was mixed with soil (Dobson et al. 1983). Acid soils are generally favorable to many fusarial wilts which may be suppressed by liming the soil to raise the pH to 7.0 (Cook and Baker, 1983).

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12

Crop Disease Management

Enhancement of Genetic Resistance of Crop Plants

The management of crop diseases has been attempted by adopting various methods either singly or in combination, resulting in varying levels of reduction in disease incidence and/or severity. These methods are directed toward the host and/or pathogen(s). Host management has primarily nongenetic and genetic components. The nongenetic component consists of cultural methods and physical and chemical techniques, and their effectiveness and feasibility of adoption have already been described (Chapter 11). The genetic component involving identification of sources of resistance to disease(s), and the utilization of selected sources of resistance in breeding programs to develop cultivars with resistance to seed- and propagule-transmitted diseases of various crops caused by microbial pathogens, is discussed in this chapter. Host management by incorporating genes for disease resistance is less expensive and environmentally safe. The farmers can grow the resistant cultivars without any need for additional cost and expertise, since the usual agronomic practices already followed would be enough in most cases. Since the buildup of the pathogen population is reduced by host resistance, management of crop diseases using resistant cultivars may be expected to be more effective and economical when considered from the benefits that may accumulate over a period of time and the area in which resistant cultivars are grown. In general, cultivation of resistant varieties is compatible with other methods of crop disease management (Narayanasamy 2002, 2008).

12.1 Types of Disease Resistance

A crop plant species has several varieties or genotypes, varying in several characteristics such as size, height, duration, yield potential, and levels of resistance to different biotic and abiotic stresses. Crop cultivars vary in their levels of resistance to a disease, one among several types of biotic stresses. Likewise, plant pathogens causing diseases exist in the form of several *formae speciales*, varieties, races, or biotypes with variations in their pathogenic potential (virulence/aggressiveness) inducing mild or severe symptoms on host plant species. Two broad kinds of resistance have been distinguished: vertical resistance and horizontal resistance, as proposed by Vanderplank (1963).

12.1.1 Vertical and Horizontal Resistances

A pathodeme may be defined as a host population consisting of individuals with a recognizable character of resistance in common. Likewise, a pathotype may be defined as a pathogen population consisting of individuals with a recognizable character of virulence in common (Robinson 1969). Two kinds of disease resistance may be differentiated using Potato-*Phytophthora infestans* pathosystem. One potato variety has the R1 gene conferring resistance to one of the races (pathotypes) of *P. infestans*, causing late blight disease of potato. This potato cultivar is resistant to pathogen race (0) that does not have a matching virulence gene, but it is susceptible to any other race of the pathogen with matching virulence gene. The unmatched resistance gene (R1) triggers the resistance mechanism in this potato cultivar. Similarly, the potato cultivar with resistance gene R2 shows resistance to infection by the race that does not possess matching virulence gene. Potato genotype with R2 shows resistance to infection by the race that does not possess matching virulence gene 2, and shows susceptible reaction to races with virulence gene 2. As the differences in the level of resistance to different races of the pathogen are parallel to the vertical axis when plotted in a graph, this kind of resistance was designated vertical resistance (Vanderplank 1963). *P. infestans* was able to overcome the effects of resistance conferred by the newly identified resistance genes, hand in hand, by producing new pathotypes (races) capable of colonizing plants incorporated with new genes for vertical resistance. It appeared that the pathogen was very highly adaptable and capable of forming new populations armed with virulence gene(s) to match the R gene(s) of the host, making the attempts to employ R genes to protect potatoes unsuccessful. The pathogen produced new races at a faster rate than the plant pathologists and breeders could produce cultivars with unmatchable resistance gene(s). However, in areas where late blight was severe, the R gene cultivars showed practical use.

The value of vertical resistance for breeding for disease resistance has been considered doubtful in the case of pathogens such as *Pyricularia oryzae*, causing rice blast disease. In the Philippines, 262 races of *P. oryzae* were identified using 12 Philippine differentials. It would be very difficult to develop cultivars with durable vertical resistance as the pathogen is pathogenically highly variable and adjustable. An international cooperative test was conducted at 60 test locations in 30 countries for identification of a rice cultivar with a broad spectrum of vertical resistance, but none of the test entries was found to be resistant or susceptible at all locations. The cultivar Tetep showed resistant reaction in the maximum percentage of tests (98%). Only a few races could infect Tetep, which showed the highest level of vertical resistance (Ou 1979).

Horizontal resistance may be exemplified by considering two cultivars of potato (A, B) which may not show distinct variations in the level of resistance to races of *Phytophthora infestans*, but a moderate level of resistance may be shown to all races of the pathogen. Between cultivars, one cultivar (A) may be more resistant than the other cultivar to all races of *P. infestans*. Differences in this kind of resistance between cultivars will be parallel to the horizontal axis of the graph. This type of resistance is therefore named horizontal resistance (Vanderplank 1963). Horizontal resistance is not due to the presence of an unmatched gene R in the host, as in the case of vertical resistance. Horizontal resistance is independent of the virulence gene of the pathogen. Horizontal resistance may be recognized by exposing the cultivars to infection by all available races of the pathogen under field conditions. The resistant plants that remain after elimination of plants with vertical resistance possess horizontal resistance. Horizontal resistance is

essentially due to polygenic action and quantitative inheritance. The grades of resistance are not distinctly defined and vary continuously. Horizontal resistance has been variously referred to as nonspecific, field, general, or polygenic resistance. Horizontal resistance may be due to a combination of many mechanisms, resulting in reduction in rates of infection, invasion of the host, and/or reproduction of the pathogen. The severity of both epidemics and endemics may be significantly reduced by growing cultivars with horizontal resistance. The level of horizontal resistance is proportional to the concentration (frequency) of polygenes in the test cultivar, since horizontal resistance is governed by polygenes. The complete set of polygenes required for the expression of horizontal resistance may be located in the different individual plants in a genetically mixed population. It may be very difficult to achieve a high level of horizontal resistance since the traditional genetic transfer techniques cannot be employed to incorporate them in a single cultivar, as in the case of vertical resistance which is governed by single gene. A number of parents with low levels of resistance to the target pathogen may be crossed in all combinations. The genes for resistance may be gradually accumulated by repeatedly crossing selected progenies over a number of generations. This diallele selective mating system was proposed by Jensen (1970).

12.1.2 Durable Resistance

The rapid loss of protection provided by single resistance genes necessitated the use of a combination of effective resistance genes; these could offer protection to the cultivars for longer periods since the pathogen would have to make multiple changes simultaneously to become compatible with the combination of resistance genes. Johnson (1981, 1983) introduced the concept of durable resistance that remains effective in a cultivar, when used over a wide area for a considerable period of time, in an environment that is favorable to the development of disease. Systems of disease management aimed at prolonging the useful life of resistance gene(s) require knowledge of the genetics of host resistance, population genetics, evolutionary biology of the pathogen, and the interaction of crop management practices with host resistance. Durability of vertical resistance may be markedly affected by the composition of pathogen population and environmental factors.

Resistance conferred by major genes in the rice-*Pyricularia* (= *Magnaporthe*) *grisea* pathosystem was rapidly broken down since the pathogen was highly adaptable to the introduced resistance genes. However, the rice cultivar IR 36 showed durable resistance which was associated with quantitative/partial resistance based on minor genes (Parlevliet 1988). This type of resistance could be recognized because of the reduction in size and number of lesions formed on leaves of IR 36 cultivar. This partial resistance was inherited through a number of minor genes (Yeh and Bonman 1986; Wang et al. 1989). The partial resistance exhibited by a rice cultivar may be durable under an irrigated tropical production system, but it may not be effective in more blast-conducive temperate environments. This differential effectiveness was observed when the Japanese rice cultivar Reimei was introduced in Korea. This cultivar exhibited an adequate level of partial resistance in northern China, but was susceptible in Korea where environmental conditions were more conducive for blast development (Lee et al. 1989). Another example to indicate the usefulness of durable resistance for tackling the crop disease problem is that of the rice bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae*. The Chinese rice cultivar Nongken 58 showing durable resistance to the pathogen was grown on four million hectares in the Yangtze River Valley and Shandong

Province for over 20 years. The resistance gene *Xa-3* (from *japonica* rice) and *Xa-4* (from *indica* rice) provided effective protection for at least 15 years (Lee et al. 1989). Rice cultivars with *Xa-4* gene could be grown for avoiding bacterial blight epidemics successfully for 20 years in the Philippines (Bonman et al. 1992). Likewise, potato cultivar with quantitative resistance to one of the viruses (*Potato leafroll virus A*, *virus X*, and *virus Y*) showed susceptible reaction to other viruses infecting potato (Joosten 1988).

12.2 Identification of Sources of Resistance to Crop Diseases

Crop cultivars may be affected adversely to different degrees depending on their levels of resistance/susceptibility to the target pathogen(s) when the environmental conditions are favorable for disease development. Development of an efficient and reliable screening procedure to identify the source(s) of resistance to diseases in the cultivars or wild relatives is therefore the basic requirement for any breeding program aimed at developing cultivars resistant to the disease(s) concerned. All available cultivars and genotypes and wild relatives have to be screened under greenhouse and field conditions to select donors of resistance for transfer of resistance gene(s) to susceptible cultivars. Various methods have been applied to assess the levels of resistance/susceptibility, depending on the host–pathogen combinations. Visual assessment of pathogen infection and disease severity is the most common method of screening of the cultivars and genotypes to score intensity of symptoms and to categorize the entries into different groups based on the level of resistance on a standard scale (0–9) representing increase in intensity of symptoms. Although the visual assessment is simple and easy to perform, it lacks accuracy and specificity and is subject to variation due to assessors' skill and experience. In addition, it may not be possible to assess resistance to individual pathogens involved in a disease complex. Dependence on visual assessment alone may result in unreliable conclusions on the resistance of genotypes when symptoms are not distinct, nonspecific, and likely to be confused with abiotic stress-related symptoms, as in the case of wheat leaf blotch disease complex caused by *Septoria tritici* and *Stagonospora nodorum*. The coalescing lesions and pale pycnidia associated with infection by *S. nodorum* may be difficult to differentiate from natural senescence of leaves. Diagnostic techniques such as immunoassays and nucleic acid-based techniques, which are not dependent on symptom development, may be employed to detect, differentiate, and quantify the pathogen population in various plant tissues infected by microbial pathogens. These techniques are useful especially in detecting pathogen infection in plants remaining asymptomatic (Narayanasamy 2001, 2011).

12.2.1 Screening for Disease Resistance

12.2.1.1 Assessment of Resistance by Visual Examination

Screening of germplasm collections and cultivars to assess their levels of resistance/susceptibility to disease(s) caused by microbial pathogens has been performed under in vitro, greenhouse, and field conditions. An in vitro detached-leaf assay involving inoculation of detached leaves with *Microdochium nivale*, a component of Fusarium head blight (FHB) complex affecting wheat, was employed and compared with whole-plant resistance ratings to FHB of 22 commercial cultivars and 21 wheat genotypes identified

as potential sources of FHB resistance. An incubation temperature of 10°C and isolates of *M. nivale* var. *majus* of intermediate pathogenicity were found to be the most suitable for differential expression of several components of partial disease resistance (PDR), namely incubation period, latent period, and lesion length in wheat genotypes used in the detached-leaf assay. Significant differences were recorded between each component of PDR within commercial cultivars and the genotypes tested. Commercial cultivars except cultivar Tanker, identified with high resistances across all three PDR components in detached-leaf assay also had high whole-plant FHB resistance ratings. Agreement between resistances found in the detached-leaf assay and resistance to FHB suggested that resistances detected in detached leaves were under the same genetic control as much of the resistance expressed in the wheat head of the commercial cultivars tested. In contrast, the genotypes E2 and E12 together with Summai #3 known to have high levels of whole-plant FHB resistance showed less resistance in each PDR component in the detached-leaf assay. Such whole-plant resistances, which were highly effective and not detected by detached-leaf assay, did not appear to be present in Irish and UK commercial cultivars (Browne and Cooke 2004).

The relative resistances of 15 winter barley, 3 winter wheat, and 3 winter oat cultivars and two spring wheat cultivars were evaluated using *Microdochium nivale* in detached-leaf assays. Barley cultivars showed incubation periods comparable to and latent periods longer than the most FHB-resistant Irish and UK wheat cultivars evaluated. Incubation periods were longer for oats compared to barley or wheat cultivars. Oat cultivars differed from both wheat and barley in that mycelial growth was observed before obvious tissue damage was detected under macroscopic examination, indicating tolerance of infection rather than inhibition of pathogen development. The results suggested that longer periods of incubation in barley and oat cultivars than in wheat might play a role in overall FHB resistance, if under the same genetic control as PDR components expressed in the head (Browne and Cook 2005b).

Partial disease resistance (PDR) to Fusarium head blight (FHB) has been identified in diverse germplasm sources that differ with respect to the chromosomal regions governing partial disease resistance (Liu and Anderson 2003; Paillard et al. 2004). Partial resistance or quantitative resistance is characterized by a reduced rate of epidemic development in a host population and may be attributed to various components, including lower infection frequency and longer latent (incubation) period. Resistance determined in vitro using a seed-germination assay correlated with whole-plant assay FHB ratings among European wheat cultivars was hypothesized to relate to resistance expressed in the developing grain. Resistance in the seed-germination assay was not correlated with PDR components detected in the detached-leaf assay, suggesting that it was a genetically distinct PDR component from those detected using the detached-leaf assay (Browne and Cooke 2005a).

The in vivo evaluation of PDR components against the potential of the detached-leaf and seed-germination assays might be useful to distinguish between specific sources/mechanisms of FHB resistance when combined with data on whole-plant FHB reaction and pedigree information. Components of PDR to FHB detected in seed-germination assay were compared with whole-plant FHB resistance of 30 USA soft red winter wheat entries in the 2002 Uniform Southern FHB Nursery. Highly significant differences ($P < 0.001$) observed between cultivars in the in vitro seed-germination assay inoculated with *Microdochium majus* were correlated to FHB disease incidence, severity, FHB index, Fusarium-damaged kernels (FDK), deoxynivalenol (DON) concentration in grain,

and incidence/severity/kernel damage index (ISK) caused by *Fusarium graminearum*. Multiple regression analysis explained a greater percentage of variation in FHB resistance as explanatory factors using the seed-germination assay and the detached-leaf assay (reported earlier) PDR components. Shorter lesion lengths in detached-leaf assay and higher germination rates in the seed-germination assay were related to greater FHB resistance across all disease variables, collectively explaining 62% variation for incidence, 49% for severity, 56% for FDK, 39% of DON, and 59% for ISK index. The incubation period was most strongly related to disease incidence and early stages of infection, while resistance detected in the seed-germination assay and latent period were related to a greater decline in the level of FDK and a smaller reduction in DON concentration. Resistance detected in the seed-germination assay interacted with the relationship between FHB and DON accumulation. Higher resistance detected in the seed-germination assay was associated with greater reduction in FDK than FHB symptom (Browne 2007).

The vast majority of isolates of *Fusarium graminearum*, the principal cause of Fusarium head blight (FHB) disease in North America, produced 3- or 15-acetyldeoxynivalenol (3-ADON/15-ADON) in addition to deoxynivalenol (DON). In order to understand clearly the effect of population shift from 15-ADON to 3-ADON producers on relative aggressiveness of isolates and mycotoxin accumulation, a total of 58 isolates for spring wheat cultivars Robin (susceptible) and 5602 HR (moderately resistant) was tested. In the first experiment three isolates, each of which produced either 15-ADON or 3-ADON, were tested using spray inoculation. In the second experiment, 20 isolates which produced 15-ADON and 20 isolates producing 3-ADON were tested using point inoculation. No significant difference in aggressiveness among isolates could be detected, based either on geographic origin or mycotoxin type. Analysis of seeds from inoculated heads by gas chromatography/mass spectrometry indicated that 3-ADON-producing isolates had significantly higher DON levels than the 15-ADON isolates in cultivar Robin after both spray and point inoculation and in cultivar 5602 HR after point inoculation. DON levels following point inoculation by 15-ADON isolates were similar in both cultivars. The 15-ADON isolates from Alberta produced less DON than 15-ADON isolates from Manitoba and Saskatchewan consistently. More ADON was produced by 15-ADON isolates than by 3-ADON isolates (Gilbert et al. 2010).

Resistance to Fusarium head blight (FHB) in wheat was categorized into two types: resistance to initial infection (later referred to as type I resistance); and resistance to spread of blight symptoms within a spike (later referred to as type II resistance) by Schroeder and Christensen (1963). Five types of active resistance to FHB in wheat were defined by Mesterházy (1995) as follows: resistance to initial infection (type I); resistance to spread of the pathogen within a spike (type II); resistance to kernel infection (type III); tolerance (ability to produce marketable grain in the presence of FHB (type IV); and resistance to mycotoxin accumulation in grain (type V). Type I and II have been widely differentiated and adopted in other investigations, whereas type III, IV, and V do not appear to be well defined and are often used differently by researchers. Resistance to spread of FHB pathogen(s) within a spike has been studied intensively in wheat. The Chinese cultivar Sumai 3 was more frequently used as a source of this type of FHB resistance in most countries (Dubin et al. 1997). Type II resistance may be determined by inoculating a single floret near the middle of the spike and counting the spikelets blighted due to disease during 8–21 days after inoculation (dai) (Bushnell et al. 2003). Resistance to spread of the disease in a spike (type II) was found to be easier to quantify

than resistance to infection (type I). The single floret inoculation technique employed for evaluating type II resistance was shown to be more reliable and accurate and less sensitive to environmental factors, and has been applied as a standard greenhouse screening procedure in many investigations (Bai et al. 2003b; Bushnell et al. 2003; Engle et al. 2003). Using this procedure, the type II resistance from Sumai 3 and other lines was transferred to spring wheat cultivars that were grown commercially (Mergoum et al. 2008).

Analysis of the relationship between components of partial disease resistance (PDR) detected using in vitro detached-leaf assay and seed-germination assays inoculated with *Microdochium majus* and FHB resistance to *Fusarium graminearum* was performed. FHB resistance was assessed using point inoculation of individual spikelet (Type II) and by inoculation of wheat spike uniformly (Type I resistance). Shorter incubation periods, longer latent periods, shorter lesion lengths in detached-leaf assay, and higher germination rates in seed-germination assay were related to greater FHB resistance measured by single point inoculation (Type II), collectively explaining 54% of the variations. Overall, the relationships observed for Type II FHB resistance were similar to Type I resistance. However, the relative magnitude of effects of the individual PDR components determined in vitro varied between FHB disease resistance parameters. Resistance in seed germination and latent period in the detached-leaf assay were more strongly related to resistance assessed by point inoculation (Type II) and severity. Type I, as opposed to incubation period, was most strongly related to disease incidence. The results revealed that individual components of partial disease resistance differentially affected the aspect of FHB disease progression in wheat spike (Browne 2009).

The in vitro detached-leaf assay was employed to evaluate barley genotypes with known field reactions to Fusarium head blight (FHB) disease for partial disease resistance (PDR) components. The detached leaves were inoculated with *Fusarium graminearum* or *F. culmorum* and incubated at room temperature ($21 \pm 2^\circ\text{C}$) or low temperature ($10 \pm 1^\circ\text{C}$). Both pathogens had a short incubation and produced larger lesions at room temperature. *F. culmorum* produced less well-defined necrotic lesions, compared with those from inoculation using *F. graminearum* at both temperatures tested. The PDR components for *F. graminearum*, including latent period, lesion size, and macroconidial production, were correlated significantly. A negative correlation between incubation period and field ratings in one of three tests, latent period and field ratings in two of three tests, and a positive correlation between lesion size and field disease severity ratings for FHB in only one of three tests were noted. On the other hand, few PDR components of *F. culmorum* showed any correlation with each other. Overall, the most distinct differentiation between resistant and susceptible barley genotypes resulted from inoculation with *F. graminearum* at room temperature, including significant correlation between incubation and latent periods with field ratings. The results indicated that measurement of PDR components may not be a dependable basis for routine use to complement field-based disease scores, although the duration of latent period might be useful for differentiating highly susceptible and resistant genotypes (Kumar et al. 2008).

Artificial inoculation of different sets of wheat genotypes under field conditions was carried out by spraying suspensions of isolates of *Fusarium graminearum*, *F. culmorum*, *F. avenaceum*, *F. sporotrichoides*, *F. poae*, *F. verticillioides*, *F. sambucinum*, and *Microdochium nivale* during 1998–2002. The severity of Fusarium head blight (FHB) disease, *Fusarium*-damaged kernels (FDK), reduction in yield, and deoxynivalenol (DON) contamination were also measured to determine the nature of resistance.

The severity ratings of FHB in wheat genotypes inoculated with different *Fusarium* spp. and *M. nivale* were similar. This trend was observed for all other parameters measured. The extent of DON contamination of wheat grains by DON producers of *F. graminearum* and *F. culmorum* was determined. Highly significant correlations were observed between FHB, FDK, yield loss, and DON contamination. Resistance components such as resistance to kernel infection, resistance to DON contamination, and tolerance were identified in more susceptible genotypes. The Western European genotypes produced up to 700 mg DON/kg seed, whereas the Hungarian genotypes produced only 100 mg DON/kg seed at a similar FDK level. The results indicated that the resistance of genotypes was related not only to any particular isolate of *F. graminearum*, but also to isolates of other *Fusarium* spp. Further, measuring both FDK and DON contamination levels was also shown to be an important factor to be included in programs for the selection of resistant germplasm and cultivars (Mesterházy et al. 2005).

Fusarium head blight (FHB) disease of wheat is caused by different species of *Fusarium*, *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* (mycotoxin producers) and also by *Microdochium majus* and *M. nivale* (nonmycotoxigenic pathogens). Resistance of wheat to FHB appeared to be horizontal and nonspecies-specific. Two components of host resistance to FHB have been widely recognized as resistance to infection (type I) and resistance to spread within the spike (type II). Single spikelet (point) inoculation assessed type II resistance only, while spraying conidial suspension on spike assessed the combined effect of type I and type II resistances (Miedaner et al. 2003). Type I resistance may be inferred following assessment by both point (type II) and spray (type I plus II) inoculation. The genetic basis of resistance to FHB for identifying and mapping type II resistance was investigated more frequently because of the relative simplicity of assessing this type of resistance. Evaluating type I resistance was more challenging and usually carried out in field trials using spray or natural inoculation, and estimating disease as the percentage of infected spikes in plots or as disease incidence (Steiner et al. 2004). Using *Tri5* gene disrupted transformants of *F. graminearum*, DON was shown to be not essential to facilitate the spread of disease from the point of infection (Proctor et al. 1995; Bai et al. 2002). The relationship between parameters of resistance to FBH and the in vitro tolerance to DON of the winter wheat cultivar WEK 0609, along with susceptible cultivar Hobbit, was investigated using double haploid progeny lines (DHLs) developed from a cross between these cultivars. Analysis indicated that WEK 0609 resistance significantly reduced symptom development, yield loss, and the fungal pathogen DNA (FDNA) and DON content of grain relative to Hobbit. Although both DON and FDNA contents were greater in susceptible than in resistant progeny lines, the ratio of DON to FDNA decreased with increasing susceptibility. The resistance of WEK 0609 appeared to have a greater effect on colonization of the grain by the pathogen than on the accumulation of DON within the grain. In vitro tolerance of WEK 0609 to DON did not seem to be related to FHB resistance in this cultivar, so the in vitro assessment of tolerance to DON was not considered to be a reliable basis for selecting the progenies for FHB resistance derived from this cultivar (Gosman et al. 2005).

The usefulness of species or isolates that produce little or no toxin and infect wheat, but cannot spread within spike, as tools to identify type I resistance was investigated. Following spray inoculation, the average disease severity caused by *Fusarium culmorum* (52.44%) was significantly higher ($P < 0.001$) than that caused by *Microdochium majus* (14.17%). Disease following point inoculation differed dramatically between

pathogens, while *F. culmorum* caused necrosis and bleaching symptoms both above and below point of inoculation. On the other hand, *M. majus* was unable to spread beyond the spikelet into which it was inoculated. Following point inoculation with *F. culmorum*, the number of damaged spikelets per spike ranged from 12.17 (in cultivar Goodwood) to 1.25 (in cultivar Dekan), with an average of 5.98. In contrast, *M. majus* was unable to spread beyond the inoculated spikelet, infecting an average of 0.97 and 0.36 spikelets in two trials. The isolates of *F. graminearum* producing deoxynivalenol (DON) and nivale-nol (NIV) induced similar disease levels in the wheat cultivar Mercia at 12 and 18 days postinoculation after spray inoculation. In contrast, following point inoculation disease levels were significantly different for the DON- and NIV-producing isolates of *F. graminearum* at all three scoring dates (Fig. 12.1). The results indicated that spray inoculation with *Microdochium* spp. might be used to assess type I resistance directly in the absence of any confounding effects caused by differing levels of type II resistance. The cultivar Spark (UK winter wheat) exhibited the highest level of overall resistance and appeared to possess both type I and type II resistance to FHB disease. On the other hand, cultivars Grief, Nirvana, Dekan, and Renan had highly contrasting responses to point inoculation with *F. culmorum* and spray inoculation with *M. majus* with relatively high levels of resistance against *M. majus*, indicating that they possessed predominantly type I resistance with very little type II resistance. The results indicated that the use of appropriate non-DON-producing FHB pathogen species or isolates in spray inoculation experiments, combined with point inoculation using DON-producing isolates, may be useful for identifying and characterizing FHB resistance in wheat (Gosman et al. 2010).

Floral structural characteristics have been reported to influence the level of resistance of wheat cultivars to Fusarium head blight (FHB) disease caused by *Fusarium graminearum*. The closed-flowering (no anther extrusion) characteristic was found to be effective for increasing resistance to FHB infection. The relationship between the degree of anther extrusion (AE) and FHB damage was investigated using double haploid lines (DHLs) derived from F1 plants from crosses between closed-flowering and open-flowering cultivars. These DHLs exhibited various degrees of AE significantly different among DHLs regardless of the year and environment (pot- or field-grown). FHB severity was the lowest in closed-flowering DHLs, and DHLs with partially extruded anthers showed significantly more severe FHB symptoms than those with closed-flowering phenotypes. In general, DHLs with partially extruded anthers also had relatively severe FHB symptoms compared with those exhibiting full anther extrusion. FHB severity was significantly correlated with Fusarium-damaged kernels (FDKs) and deoxynivalenol (DON) concentration. The results showed that partially extruded anthers were sources of infection, whereas the closed-flowering phenotype had improved resistance to FHB infection. The phenotypes with rapid anther extrusion and ejection could also contribute to the avoidance of FHB infection (Kubo et al. 2013).

The influence of inoculum sources (conidial suspension, infested barley, and corn kernels) and inoculation frequency on the development of Fusarium head blight (FHB) and the deoxynivalenol (DON) content was investigated. Three spring wheat genotypes were used in a disease nursery in Ottawa, Canada. Development of FHB was monitored by visually estimating disease severity on a scale of 0–9, six times during each growing season. The severity of FHB over time was represented as the area under the disease progress curve (AUDPC). The intensity of symptoms was rated at a critical development stage (soft dough), as initial-time disease severity (DS), percentage of infected spikelets (IS), and FHB index. Inoculated plants showed greater DS compared with

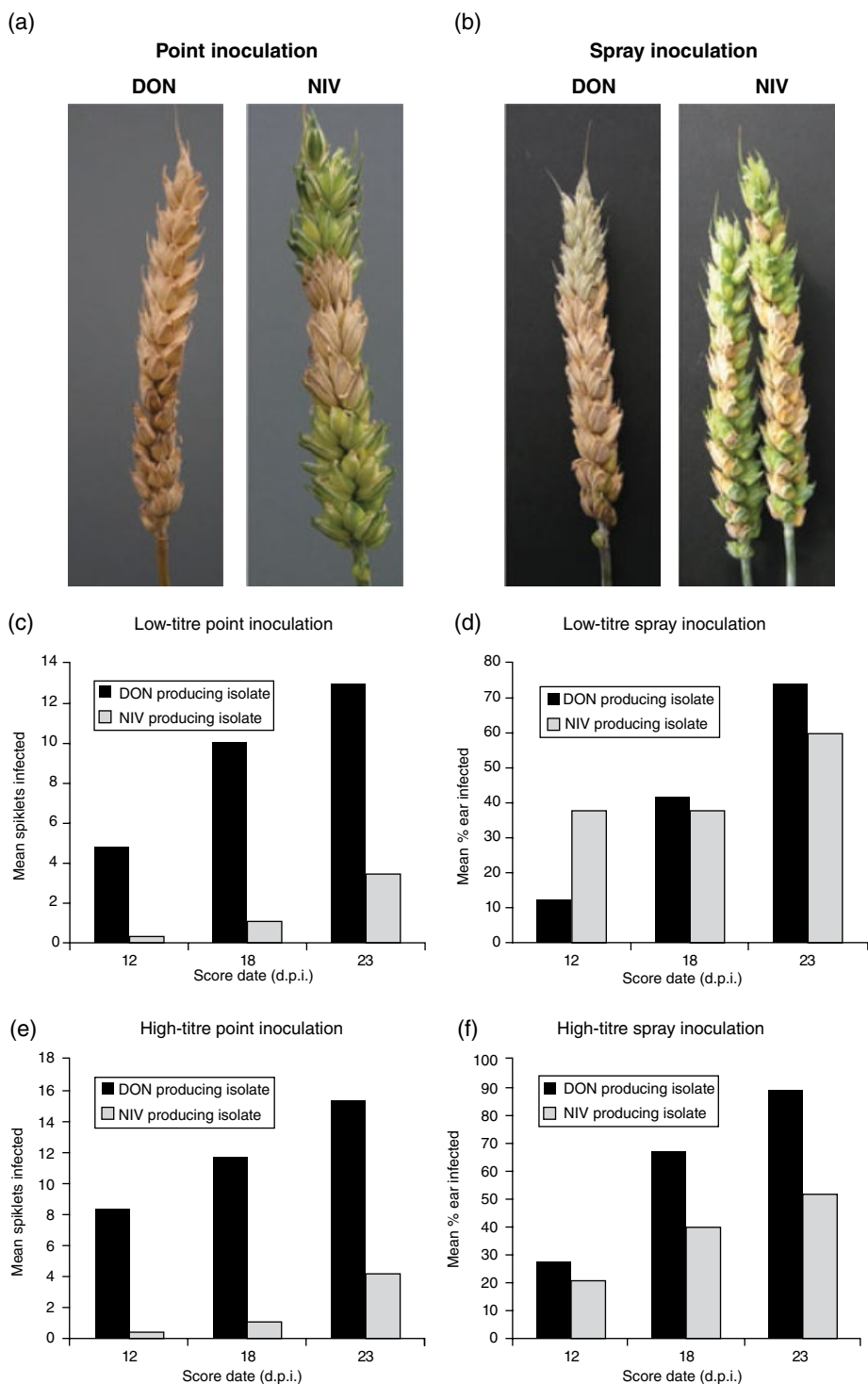


Figure 12.1 Effects of low- and high-titer of *Fusarium graminearum* conidia in combination with (a) point- and (b) spray-inoculation with the pathogen on (c–d) means of spikes and ears infected by DON- and NIV-producing isolates in spring wheat cultivar Mercia. (e–f) High-titer inoculum produced greater disease severity; DON-producing isolates spread rapidly, while NIV-producing isolate was restricted to inoculated spikelets within the spike. Source: Gosman et al. (2010). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

uninoculated control plants. Regardless of inoculum source, two inoculations produced as much FHB, FDK, and DON as three inoculations. The conidial suspension or infested kernels as a source of inoculum yielded significantly greater AUDPC, DS, FHB index, IS, and DON contents than infested debris. Conidial suspension was a more effective inoculum source than the infested kernels or infested debris which showed similar levels of infections. The results indicated that conidial suspension and infested kernels could be used as inoculants for assessing FHB in nurseries (Xue et al. 2006).

Fusarium head blight (FHB) pathogen causes yield losses and unacceptable quality of grains because of accumulation of mycotoxins. In addition, the end-use quality such as alteration of baking characteristics was seriously affected by the disease. The ability of kernels to prevent penetration of the pathogen and mycotoxin accumulation has been investigated to select the wheat genotypes with resistance not only to FHB incidence but also to restrict accumulation of mycotoxins in grains. Eight modern Swiss spring wheat cultivars were grown in small irrigated field plots (3×1.5 m) inoculated at anthesis with a mixture of *F. culmorum* isolates. Disease ratings from 7 to 28 days postinoculation were compared with postharvest analyses for the accumulation of the mycotoxin deoxynivalenol (DON) and different baking quality parameters. Results indicated that the accumulation of DON in the kernels was correlated with visible symptoms on the ear before harvest. In terms of baking quality parameters, water absorption, dough softening, and dough resistance were impaired in susceptible varieties after FBH infection, while these parameters were not affected in resistant cultivars. Further, based on the results obtained, kernel resistance to FHB was defined by low DON accumulation in kernels and by the stability of several baking quality parameters under conditions of high FHB infection pressure (Gärtner et al. 2008).

The use of wheat cultivars resistant to Fusarium head blight (FHB) disease was considered to be the most effective option for the management of the disease. Deoxynivalenol (DON) accumulation might occur at unacceptable levels in kernels in the cultivars which have improved levels of resistance to infection and spread of *Fusarium graminearum*. The role of genetic variation for resistance to kernel infection (RKI) and resistance to toxin accumulation (RTA) in providing protection against DON was investigated. Spikes with different levels of visual symptoms from each of 32 genotypes and at four environments were collected, and DON content and fungal biomass (FB) from each sample were estimated. Significant genetic effects were recorded for RKI and RTA. The difference among genotypes was more striking for RTA than for RKI, and might have a greater impact on controlling DON levels in the grain. The average r^2 values for RTA regression were 0.39 as against 0.24 for RKI. Some genotypes consistently had low FB in the kernels, despite increasing visual symptoms suggesting a higher RTA in those genotypes. Although RKI and RTA were significantly correlated ($r = 0.58$; $P = 0.05$), RTA was predictive of DON accumulation because it modeled 32–44% of the genotype sum of square for DON among genotypes with acceptable levels of resistance to pathogen infection and spread. The results suggested that RKI and RTA in grains were independent of type I and II resistance in the wheat population and all mechanisms could be combined to produce low DON levels (Sneller et al. 2012).

Fusarium verticillioides (= *F. moniliforme*) and *F. proliferatum*, causing Fusarium ear and kernel rot of corn (maize), produces a group of homologous mycotoxins known as fumonisin which is implicated as a contributing factor in outbreaks of oesophageal cancer and neural tube birth defects in humans. Cleaned and dry milled corn meant for human consumption should not contain more than $2\text{--}4\text{ }\mu\text{g}$ of fumonisin g^{-1} of kernels,

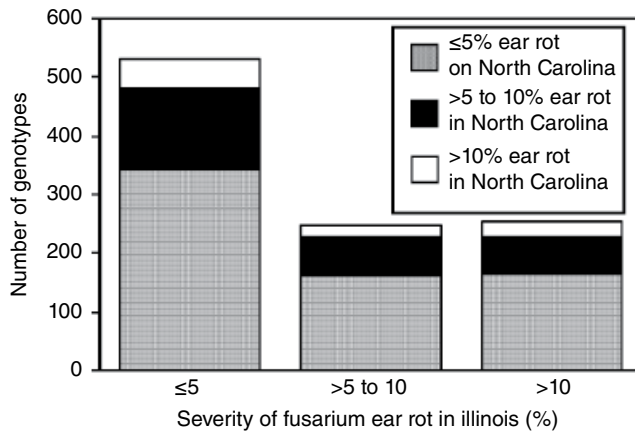


Figure 12.2 Evaluation of corn hybrids under inoculated conditions for resistance to Fusarium ear kernel rot disease. Assessment of disease severity by visual rating based on percentage (0–100%) of ears with symptoms in different corn hybrids. Source: Clements et al. (2004). Reproduced with permission of the American Phytopathological Society, MN, USA.

as per the US Food and Drug Administration (CFR 2001). Local infection of kernels was found to be the most important pathway of infection, although systemic movement of *F. verticillioides* from infected seed and stalks to developing kernels was also indicated (Munkvold et al. 1997). Corn inbreds with high levels of resistance to fumonisin production and accumulation in grains were identified. A genetically diverse collection of inbreds were evaluated to identify potential sources of resistance to fumonisin production and accumulation in grain and Fusarium ear and kernel rot. The F1 hybrids with the inbred FR1064 and 1589 and 1030 inbreds were evaluated in inoculated and naturally infected trials, respectively, in 2000. Inoculum was prepared by blending an equal number of cultures of six isolates of *F. verticillioides* and spray inoculated using backpack sprayers. Primary ears from all plants were harvested and dried with forced air at 32°C to reduce the grain moisture to 14%, and visually rated for severity of Fusarium ear and kernel rot as a percentage (0–100%) of ear with symptoms. The severity of corn ear and kernel rot was affected by trials, hybrids, and trial-hybrid interactions ($P < 0.0001$). Treatment means for ear rot severity ranged from 0 to 59% in Illinois and from 0 to 33% in North Carolina (Fig. 12.2) (Clements et al. 2004).

Wheat lines which were adapted to the southern USA, possessing diverse sources of FHB resistance, were evaluated under greenhouse and growth chamber conditions. Wheat lines with resistance to DON chemotype isolates were even more resistant to NIV chemotype isolates. The chemotypes had similar abilities to initiate infections across the diverse resistance sources used in this investigation. The DON chemotype isolates caused more disease than NIV chemotype isolates. Differences between the DON and NIV chemotypes were most pronounced for the percentage of spikes with premature apical death. Averaged across all wheat lines and both isolates within a chemotype, isolates of the DON and NIV chemotypes caused premature apical death on 25.1 and 7.0%, respectively. DON chemotypes of *F. graminearum* were more aggressive than NIV chemotype. Selecting lines for resistance to the DON chemotype could also select for resistance to NIV chemotype. Florets at the tip of spikes that were affected by premature apical death rather than direct fungal infection were not included in the

count of florets blighted by the pathogen. Significant levels of resistance to both initial infection and spread within a spike were found among lines. Quantifying resistance to initial infection was improved by standardization of the inoculum and environmental conditions. Additional information related to resistance to spread within a spike was obtained by calculating the area under the disease progress curve (AUDPC) from 7 to 21 days after inoculation. The number of initial infection for 15 resistant lines was reduced by 58–91% compared with susceptible check, while the number of blighted florets within a spike following single-floret inoculation was reduced by 48–86% and 50–87% for 15 resistant lines compared with the number of blighted florets on Cocker 9835 for DON and NIV chemotype isolates, respectively, indicating significant effect on FHB severity and mycotoxin accumulation (Horevay et al. 2011).

Fusarium head blight (FHB) of wheat in the USA is primarily caused by the deoxynivalenol (DON-) producing chemotype *Fusarium graminearum*. The nivalenol (NIV) chemotype of *F. graminearum* was also involved in FHB disease in Louisiana and Arkansas states however, necessitating the need to develop wheat cultivars with resistance to both DON-producing and NIV-producing chemotypes. Isolates of *F. graminearum* and *F. culmorum* with DON chemotype appeared to be more phytotoxic or aggressive than isolates with NIV chemotype. Wheat cultivars (8) and rye cultivars (12) were inoculated in the field at three locations using spray inoculation with one DON chemotype isolate and one NIV chemotype isolate of *F. culmorum*, predominantly causing FHB in Europe. Based on disease severity, yield, and toxin content in grain, all tested wheat cultivars and rye lines were found to be more resistant to the NIV chemotype than to DON chemotype of *F. culmorum* (Miedaner and Reinbrecht 2001).

As genetically inherited resistance was considered the most effective option for the management of Fusarium head blight (FHB) disease of barley, a total of 266 barley cultivars and breeding lines originating from China were evaluated for FHB resistance and concentration of DON in grain. Barley plants were inoculated with isolates of *Fusarium graminearum* under field conditions by injecting conidial suspension into a single spikelet of each spike. FHB symptoms were determined by visual examination and DON content was analyzed by high-performance liquid-chromatography (HPLC) technique. Significant differences in FHB ratings and DON levels were observed among cultivars. Visual symptoms of FHB varied over 4.88–71.75% of spikelets at 21 days after inoculation and over 7.86–113.33 units of area under disease progress curve (AUDPC). Twenty-seven lines were more resistant to FHB, with fewer than 12% infected spikelets than the control resistant cultivar Zhedar 2. Twenty-one of the lines mentioned above originated from the area in the middle–low valley of the Yangtze River where FHB epidemics occurred frequently. DON levels in grains ranged over 0.05–24.39 mg kg⁻¹ among tested barley lines. Correlation coefficients were significant between FHB symptom ratings and DON levels. However, no significant correlation was observed between symptom severity and plant height or between symptom severity and heading date (Ma et al. 2009).

The occurrence of epidemics of Fusarium head blight (FHB) disease of barley caused by *Fusarium graminearum* (teleomorph *Gibberella zeae*) in the USA strongly indicated the need to develop commercial barley varieties with resistance to FHB and the ability to allow low levels of DON accumulation. FHB incidence, FHB index, and DON levels of Virginia hulled and hulless barley genotypes were evaluated for over 5 years (2006–2010) in FHB nurseries in Virginia. Barley genotypes were sprayed at 50% anthesis with aqueous suspensions of macroconidia (50,000 conidia mL⁻¹) to create sufficient

disease pressure under field conditions. Susceptibility of barley genotypes to FHB was similar at cell stages of grain development. The mean DON concentration was the lowest in 2008, whereas FHB incidence and index were highest in the same year. Significant differences in FHB and DON resistance were observed among genotypes that were selected to evaluate resistance stability across years. FHB incidence was positively correlated with DON accumulation. Early spike emergence resulted in high FHB incidence and index in 2007, 2008, and 2010. The genotypes (hulless) and Thoroughbred (hulled) ranked among the most FHB-resistant genotypes (Khalibi et al. 2012).

The wheat “Frontana”/“Remus” double haploid population ($n = 210$ lines) were investigated to map and validate the “Frontana” resistance quantitative trait loci (QTL) focusing on *Fusarium*-damaged kernels (FDK). The plant materials were evaluated for FHB resistance under six epidemic conditions created by artificially inoculating plants with either *F. graminearum* or *F. culmorum*. The genetic regulation of FHB resistance was found to be more complex than earlier suggested. FHB resistance QTLs were identified on chromosomes 3A, 4A, and 6B. Markers showed association with FDK resistance on chromosome 3D and at the marker *Xs12m15_4*. QTL on 2B, 4B, 5A, and 7B chromosomes were responsible for both FHB and FDK resistance and, in this case, the same QTL influenced both tracks and also possibly other tracts during pathogenesis. These QTLs were considered to be important because of their association with *Fusarium* resistance. Heading late QTL were detected on chromosomes 1A, 2D, and 7B overlapping with neither FHB nor FDK resistance QTL. The QTL identified in the Frontana/Remus population were in good agreement with results of early investigations (Szabio-Hevér et al. 2012). The mycotoxin DON produced by FHB pathogen *Fusarium graminearum* rendered barley grain unmarketable, if concentration of DON exceeded threshold values set for end-use markets. Elite hulled and hulless genotypes developed by the Virginia Tech winter barley breeding program were therefore screened in inoculated, mist-irrigated FHB nurseries over 2 years at two locations in Virginia to validate resistance levels over years and locations. The results showed that barley genotypes varied significantly for resistance to FHB incidence and DON accumulation in grain. The hulled “Nomini,” hulless “Eve,” and hulless line VA06H-48 were consistently resistant across locations to both FHB incidence and DON accumulation in grain. Screening the genotypes with molecular markers on chromosomes 2H and 6H for FHB and DON indicated quantitative loci regions (QTL) which might confer resistance in the Virginia Tech germplasm (Berger et al. 2014).

Maize (corn) inbred lines were evaluated in two agroecological regions in Nigeria to identify inbred lines resistant to *Fusarium* ear rot caused by *Fusarium verticillioides* and fumonisin accumulation in grain across two agroecological regions. *Fusarium* ear severity in 103 inbred lines ranged from 1.0 to 6.0 with a mean of 1.8 in a 1–7 scale in 2003. The mean severity of 52 selected inbred lines was 2.1 in 2004, as disease severity was higher in 2004 than in 2003. The disease severity of six inbred lines was consistently low in both years and at both locations. Total fumonisin and disease severity were positively correlated across years, when the disease was initiated from natural infection. However, a number of inbred lines with low ear rot severity had high levels of fumonisin concentration in grains, underpinning the need to select for resistance to both fumonisin in grain and ear rot severity in maize breeding programs. At both locations, no significant inbred $\times$ year interaction was observed for fumonisin concentration. Five inbred lines – 02C14585, 02C14603, 02C14606, 02C14624, and 02C14683 – consistently contained the lowest fumonisin concentration in both trials. Two of these inbred lines

02C14624 and 02C14585 had fumonisin levels of $<5.0 \mu\text{g g}^{-1}$ across trials where disease was initiated from both natural infection and artificial inoculation. These two inbred lines showed consistently low disease severity. These two inbred lines possessed potential for deployment of hybrids with good levels of resistance to *Fusarium* ear rot as sources of resistance to both disease development and fumonisin accumulation in kernels. Since the inbred lines were derived from different crosses, they might represent different resistance genes that could be useful in breeding maize for resistance to *Fusarium* ear rot disease (Afolabi et al. 2007).

The role of fumonisins in seedling colonization and induction of seedling blight symptoms by *Fusarium moniliforme* (teleomorph *Gibberella moniliformis*) was investigated using the complementary genetic analysis of host and pathogen. Only one of two fumonisins B1 (FB1)-insensitive maize backcross lines was more resistant than FB1-insensitive parent to seedling blight, indicating that the increase in FB1-insensitivity was not associated with an increase in resistance. FB1-producing and nonproducing isogenic fungal strains did not differ in their ability to cause seedling blight, but the FB1-producing strain was more effective in systemic colonization of seedlings in reciprocal strain challenge tests. The results indicated that the role of fumonisins depended on complex environmental and genetic factors in the maize-*F. moniliforme* pathosystem (Desjardins et al. 2007). New inbred lines with potential resistance to *Fusarium* ear rot and fumonisin accumulation caused by *Fusarium verticillioides* were identified from well-adapted breeding material used in the temperate conditions prevailing in South Africa. Genetically diverse maize inbred lines (24) were assessed for their levels of resistance to ear rot and fumonisin accumulation in field trials at two locations. After artificial silk channel inoculation with *F. verticillioides*, *Fusarium* ear rot development was determined at harvest and fumonisins B1, B2, and B3 were quantified. A significant effect on inbred line by location was observed for *Fusarium* ear rot severity ($P < 0.001$), although some lines proved to be consistently resistant across both locations. The individual inbred lines also differed considerably in fumonisin accumulation between two locations. None of the inbred lines was completely resistant to *F. verticillioides* or fumonisin accumulation. A significant correlation between *Fusarium* ear rot rating and fumonisin concentration in the grain was observed in the field trials. A greenhouse trial using artificial inoculation was carried out on a subset of potentially resistant and highly susceptible lines. A good correlation was observed between the results from the artificially inoculated greenhouse trial with those of field trials, suggesting that greenhouse trials could be used to accelerate progress in the selection of resistant germplasm. The inbred lines CML 390, CML 444, CML 182, VO619Y-2, and RO 549W consistently showed a low *Fusarium* ear rot ($\leq 5\%$) incidence at both locations as well as in the greenhouse assessment. Of these five inbred lines, CML 390 and CML 444 accumulated fumonisin levels of $<5 \text{ mg kg}^{-1}$. These two inbred lines could be dependable sources for breeding maize cultivars with resistance to *F. verticillioides* and the mycotoxins produced by the pathogen in maize kernels (Small et al. 2012).

Aspergillus flavus infecting corn kernels produces aflatoxin harmful to humans and animals following consumption of contaminated grains. Different techniques were used to assess the levels of resistance of corn hybrids to *A. flavus* and aflatoxin accumulation in kernels. The standard inoculation techniques (the side-needle and spray method) were compared with a dry carrier technique. Both techniques were comparable in their effectiveness in differentiating the resistant and susceptible hybrids in the first year of experimentation. However, only hybrids inoculated with the side-needle technique

showed statistically different levels of aflatoxin and *A. flavus* infection in the second year. A modified pinbar technique with inoculations near the tip or base of the ear was compared with side-needle method. When developing ears were inoculated near the base with the modified pinbar, adequate levels of aflatoxin were induced in both years to distinguish the resistant and susceptible corn hybrids. The modified pinbar technique showed potential for evaluation of corn germplasm for aflatoxin resistance (Windham and Williams 2007).

Sorghum crops are commonly infected by different fungal pathogens, some of which are carried by the seeds. Long smut of sorghum, caused by *Sporisorium ehrenbergii*, was found to be a limiting factor in increasing the yield levels in some countries. Sorghum accessions (51) including 31 commercial hybrids and 20 cultivars from the USA were evaluated for their resistance/susceptibility to long smut disease. The highest disease incidence of 44.33% and 16.47% was recorded in 2004 and 2005, respectively, on the hybrid A425, while the hybrids 3552 and A571 were entirely free of infection by *S. ehrenbergii* in two locations in Senegal, West Africa. Five hybrids showed low levels of infection. Among the cultivars and parental lines B. 9612 and R. 9645 consistently recorded low levels of disease incidence (Prom et al. 2007). Sorghum accessions were evaluated for resistance to grain mold and anthracnose diseases in Uganda. Accessions PI34117, PI34144, PI576337, PI297199, PI533833, and PI297210 and SC 748-5 were highly resistant to anthracnose during 2 years of observations. Accessions PI534117, PI297134, and PI297156 exhibited low levels of severity of grain mold disease. Grain mold fungi recorded were *Curvularia lunata*, *Fusarium thapsium*, *Alternaria* spp., and *Fusarium semitectum*. Significant negative correlation was observed between grain mold and seed-germination percentages and high temperatures during 2005–2006 (Prom et al. 2011).

Rice genotypes were evaluated for their resistance to false smut disease caused by *Ustilaginoidea virens*. A screening method was developed involving cultivation of the main culm of rice plants in the greenhouse and rapid preparation of conidial suspension for injection into the leaf sheath of 18 rice varieties/lines were tested by this method. Field trials were also conducted during 2007 and 2008 to compare with the results of greenhouse evaluation. The results indicated that the greenhouse method was more reproducible than field assessment of disease resistance. Commercial varieties were mostly resistant and all the forage varieties were highly susceptible. Rice blast resistant varieties were mostly resistant to false smut disease also. This screening procedure was useful for determining the level of resistance of rice genotypes to false smut disease and utilizing the resistance sources for breeding resistant cultivars (Ashizawa et al. 2011). As the rice sheath blight caused by *Rhizoctonia solani* is one of the major diseases in most rice-growing countries, the identification and subsequent development of resistant cultivars is a critical factor for increasing yield levels. Six moderately resistant cultivars (Teqing, Jasmine 85, Tetep, Peco, Azucdena, and Taducan), one susceptible cultivar MR219 and two advanced breeding lines (UKMRC2 and UKMRC9) were screened using micro-chamber and mist-chamber methods. The fungal isolate used for inoculation was confirmed as *R. solani* using ITS-rDNA sequencing. Tetep followed by Teqing showed a high level of resistance. A significant correlation of plant height and disease was observed with relative lesion height (RLH) indices. Significant correlations were also recorded among diseased plant affected area (DPAA), VRT (visual rating), and RLH, with VRT being the most dependable parameter. Tetep and Teqing were identified as suitable donors based on disease reactions to improve the

resistance of the susceptible cultivars UKMRC2 and UKMRC9. Mist-chamber screening procedure was found to be more reliable to evaluate the rice genotypes for sheath blight resistance under greenhouse conditions than the micro-chamber method (Hossain et al. 2014).

The resistance of rice to bacterial leaf blight (BLB) pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) increased as the plants of some rice cultivars became older, and this resistance was similar to the adult-plant resistance observed in other crops against virus diseases. Some rice cultivars exhibited resistance to four races of *Xoo*, whereas some others were resistant to one or two races only. Disease severity progressively reduced from seedling to flag leaf stage. The cultivar Wase Aikoku 3 showed adult-plant resistance to these races, but seedling resistance to another race. Adult-plant resistance to BLB in rice appeared to be race-specific (Qi and Mew 1985). Populations of *X. oryzae* pv. *oryzae* (*Xoo*) have been shown to vary greatly in most locations in the rice-growing countries. The *Xoo* isolates (78) collected from 13 different states of northwest and east India were grouped into 6 races based on disease symptoms on 12 near-isogenic lines (NIL). Race 4 and 6 were predominantly present in the northwest part of India, whereas race 1 and race 2 were in more abundance in the northeast hill and plains. Race 1 and race 2 were less virulent than other races, mixed infections by both were less virulent, and virulent races occurred in all locations. Rice cultivars with *R* genes *Xa1* and *Xa2* were susceptible to all races tested, whereas *Xa4*, *Xa11*, *Xa13*, and *Xa41* genes provided stable to moderate resistance to bacterial leaf blight (BLB) disease. *Xoo* secreted type III effectors (T3Es) directly into the rice cells to induce disease symptoms (Mondal et al. 2014).

Verticillium dahliae affects spinach seed production because the plants remain asymptomatic until bolting is initiated (conversion from vegetative to reproductive phase). The USDA spinach germplasm accessions were evaluated for their resistance to *Verticillium* wilt and to determine the range in the incidence of natural seed infection/infestation. A total of 120 *Spinacia* spp. accessions and 10 commercial *S. oleracea* hybrids were screened for resistance to *V. dahliae* in three trials during 2006–2008 under greenhouse conditions. Each accession was grown in either *V. dahliae*-infested or noninfested potting mix and rated weekly on a 7-step scale from 1 week before bolting to 4 weeks after bolting. Most of the accessions were highly susceptible. There was no evidence for qualitative resistance, but some showed greater levels of quantitative resistance than others. Plants grown in infested soil senesced faster and had significantly reduced biomass compared with plants in noninfested soil of the same accession. In the second and third year (2007 and 2008) trials, seed samples were infected to the extent of 34% and 16%. The presence of *V. dahliae*, *V. tricorpus*, and *Gibellulopsis nigrescens* (*V. nigrescens*) in the seed samples was also detected. The results may be useful for characterizing potential variability within spinach germplasm for resistance to *V. dahliae* (Villarreal-Zebello et al. 2012).

Barley covered smut caused by *Ustilago hordei* is a seedborne disease and Canadian cultivars were affected severely by this disease, resulting in appreciable yield reduction. Canadian barley cultivars, parents of six barley mapping populations and four differentials, namely Hannchen (*Ruh1*), Excelsior (*Ruh2*), Plush (*Ruh6*), and Odessa (universal susceptible), were evaluated to identify and map disease resistance genes. Six mated combinations of *U. hordei* sporidial lines harboring known alleles of virulence (*avr*) and avirulence (*Avr*) genes were used in the evaluation procedure. Most of the cvs of barley carried the *Ruh1* resistance gene, but some had *Ruh2* or *Ruh6* also. Many cultivars

showed resistance to a *U. hordei* sporidial mating of genotype (*avr1/avr1*, *avr2/avr2*, *avr6/avr6*) virulent on Hannchen, Excelsior, and Plush. The resistance indicated the presence of novel avirulence gene(s), corresponding to postulated resistance gene(s) in these cultivars. Double haploid lines (56) from mapping the population of Harrington/TR306 were inoculated with a sporidial mating of genotype SM1 (*Avr1/Avr1*, *avr2/avr2*, *avr6/avr6*), avirulent on Hannchen (*Ruh1*) but virulent on Excelsior (*Ruh2*) and Plush (*Ruh6*). Harrington (*ruh1*) was susceptible to this pathogen genotype possessing *Avr1* and TR306 (*Ruh1*) was resistant. Barley lines segregated into 27 resistant to 29 susceptible which were not significantly different from a 1:1 ratio ($\chi^2 = 0.07$, $P = 0.79$). The presence of a single major resistance gene *Ruh1* in TR306 was identified and *Ruh1* mapped to the short arm of chromosome I (7H) between markers iPgd1A and BCD129 on Harrington/TR306 map (Grewal et al. 2008).

Grain mold and head smut pathogens infecting sorghum cause considerable losses in the quantity and quality of grains. Pigments in plants were considered to protect grains against panicle diseases. Near isogenic tan or purple plant color genotypes with white grain were grown under field conditions. Harvested grain was plated into semiselective media to detect the presence of *Alternaria*, *Fusarium*, and *Curvularia* and colonization of grains. *Fusarium* and *Curvularia* spp. were present predominantly. However, there was no indication that grains of purple plants were more resistant to these pathogens. Most fungi were identified as *Alternaria alternata*, *Fusarium thapsinum*, and *F. proliferatum* using molecular techniques. In the case of *Sporisorium reilianum*, incitant of head smut disease, purple plants were more susceptible to the disease than tan plants producing white grains (Funnel-Harris et al. 2013).

The influence of plant age on the level of resistance/susceptibility to diseases caused by microbial pathogens has been studied in some pathosystems. The impact of plant age on resistance of Kabuli cultivars to Ascochyta blight of chickpea caused by *Ascochyta rabiei* was assessed by inoculating cultivars UC27 (susceptible), Sanford, and B90 (partially resistant) and desi cultivar Myles (partially resistant) with two isolates of *A. rabiei*. Seeds were sown at regular intervals to have plants at different growth stages, namely seedling, vegetative, flowering, and podding, which were inoculated at the same time under controlled and field conditions. Cultivar UC27 was highly susceptible at all stages of plant development, but plant age had a significant effect on resistant cultivars. In growth chamber experiments, blight severity was low in seedlings of the resistant cultivars (4–20% infection) and highest at podding stage (41–82%) at 2 weeks after inoculation. Under field conditions, severity at 2 weeks after inoculation showed a similar pattern, with mean severity among resistant cultivars ranging over 1–2% in seedlings and 3–28% in podding plants. The severity of blight disease at 8 weeks after inoculation on resistant cultivars inoculated as seedlings was low (6–14% and 10–23%) in 2 years of experimentation relative to those inoculated at podding stage (65–91% and 54.83%) under field conditions. The results indicated that resistance decreased with plant age in partially resistant chickpea cultivars adapted to western Canada, and it may not be desirable to depend on the varietal resistance alone as an effective approach for the management of Ascochyta blight disease of chickpea (Chongo and Gossen 2001).

Potato cultivars were tested under laboratory, greenhouse, and field conditions for their resistance to wart disease caused by *Synchytrium endobioticum* which was found to exist as two pathotypes 1 (D1) and 6 (O1). Susceptible cultivars produced large warts (>16 mm diameter) and had low levels of field resistance (1–6). Field-resistant cultivars (levels 7–9) produced small warts or no warts in all laboratory tests. In pot tests, at low

inoculum levels (1 sporangium per 25 g of soil) susceptible cultivars contracted infection and developed warts, whereas field-resistant cultivars did not develop any warts below 25 sporangia g⁻¹ of soil. All plants showed infection above 35 sporangia g⁻¹ of soil, whereas field-resistant cultivars had only minimal wart development. As the field-resistant cultivars did not support inoculum buildup in the soil, they did not create a risk of secondary infection (Baayen et al. 2005).

Several *R* genes conferring resistance to *Phytophthora infestans*, causal agent of potato late blight disease, have been identified in *Solanum demissum* (Malcolmson and Black 1966). The pathogen could produce new races capable of matching *R* genes at a faster rate than the researchers were able to produce new potato varieties with unmatched resistance genes. A shift in the objective of breeding therefore became necessary, and breeding for potato cultivars with polygenic or nonspecific resistance (field resistance) was considered as an effective alternative. Molecular techniques have been employed for characterization of different stages of interaction between host plant species and microbial pathogens. In potato, a high-resolution genetic map at the *R3* locus conferring high resistance to avirulent isolates of *P. infestans* was constructed. The *R3* locus consists of two genes *R3a* and *R3b* with distinct specificities. These genes from *S. demissum* were incorporated into potato. One accession of *S. demissum* had a natural recombination between *R3a* and *R3b* (Huang et al. 2004). A new late blight resistance (*R*) locus in *Solanum bulbocastanum* was identified. The late blight resistance genes *Rpi-blb3*, *Rpi-abpt2*, *R2*, and *R2*-like genes were identified in the same *R* gene cluster on chromosome 4 (Park et al. 2004).

An in vitro inoculation procedure was developed using potato plantlets for monitoring major *R* genes in potato and their corresponding *Avr* genes in *Phytophthora infestans*. A population of 93 clones was phenotyped for segregation of two closely linked and functionally distinct genes – *R3a* and *R3b* – in the *R3* locus. Phenotyping of the population was fully consistent with genotyping obtained from analysis of molecular markers that flank each gene. The in vitro assay was shown to be a reliable alternative to detached-leaf assay to phenotype a large population segregating in the *R3* locus. The significant correlation between the results obtained from the two assays indicated that the in vitro inoculation method may be preferred because of its rapidity (faster by 2 months), space-effectiveness, and accuracy (Huang et al. 2005).

The resistance characteristics of Phureja potatoes (*Solanum phureja*) cultivated in restricted areas of the Bolivian Andes were determined under natural conditions. Five Phureja cultivars along with Waych'a Paceña as susceptible control were inoculated by spraying sporangial suspension (8×10^5 mL⁻¹). Disease severity was assessed based on the area under disease progress curve (AUDPC). The potato varieties Chojllu Phureja, Phureja Amarilla, and Polo Phureja might possibly have larger copy numbers of the minor resistance gene against *P. infestans* than another two cultivars (Coca-Morante and Tolín-Tordoya 2013).

Investigations on the mapping and subsequent cloning of an avirulence gene from *Leptosphaeria maculans*, causal agent of blackleg disease of *Brassica* spp., were carried out. The gene *AvrLmJ1* conferred avirulence towards all three *B. juncea* cultivars tested. Analysis of RNA sequence data showed that *AvrLmJ1* was housed in a region of the pathogen genome that contained only one gene that was highly expressed in planta. The closest genes were 57 and 37 kb away. As for other avirulence genes of *L. maculans*, *AvrLmJ1* was located within the AT-rich, gene-poor region of the genome. The encoded protein with 141 amino acids had a predicted signal peptide and was cysteine-rich.

Two virulent isolates possessed a stop codon in *AvrLmJ1*. Complementation of an isolate that induced cotyledonary lesions on *B. juncea* with the wild-type allele of *AvrLmJ1* conferred avirulence towards all three *B. juncea* cultivar tests, suggesting that this gene might confer species-specific avirulence activity (Van de Wouw et al. 2014).

The infection of pepper (chilli) (*Capsicum* spp.) by anthracnose disease caused by *Colletotrichum capsici* was found to be the most common disease at pre- and postharvest stages. *Capsicum* genotypes (41) were initially screened for resistance to anthracnose under natural field conditions. The genotypes showing resistance reaction were tested by artificially inoculating them under in vitro conditions. Recording the results on disease incidence at 9 days after inoculation was the appropriate stage. Of the 41 genotypes tested, four genotypes remained symptomless and 11 genotypes were highly resistant to the pathogen. Based on data from field and in vitro assessments 9 lines, BS-35, BS-20, BS-28, Punjab Lal, Bhut Jolokia, Taiwan-2, IC-383072, Pant C-1, and Lankamura collection, were considered as resistant. The pepper fruits of 16 genotypes were inoculated with two isolates of *C. capsici* to determine their response to the pathogen isolates. The two isolates of *C. capsici* were found to be of different pathotypes. The resistant genotypes had the potential for use as sources of resistance to the anthracnose disease affecting pepper (Garg et al. 2013).

Phytophthora blight disease, which causes *Phytophthora capsici*, causes significant losses in pepper (*Capsicum annuum*). Genetic diversity and plant characteristics of 21 pepper accessions previously identified as resistant to the disease were evaluated using 22 simple sequence repeat (SSR) loci. Accessions resistant to *P. capsici* were identified. Accessions PI 201237 and PI 640532 appeared to have high levels of resistance to Phytophthora blight disease (McGregor et al. 2011). A novel inhibitor gene of resistance to *P. capsici* (*lpcr*) was identified in chilli pepper (*C. annuum*) in cultivar New Mexico Capsicum Accession 10399 (NMCA10399). The gene *lpcr* inhibited resistance to *P. capsici*, but not to other species of *Phytophthora*. In a cross between the highly resistant variety and NMCA10399, the resultant F1 populations screened for resistance were completely susceptible to *P. capsici* for root rot and foliar blight symptoms, despite the dominance inheritance of *P. capsici* resistance in chilli pepper. The F2 population displayed a 3:13 resistant-to-susceptible (R:S) ratio and a backcross population to MNCA10399 displayed complete susceptibility. The results demonstrated the presence of a single dominant inhibitor affecting *P. capsici* resistance in chilli pepper. Furthermore, when lines carrying the *lpcr* gene were challenged against six *Phytophthora* spp. the nonhost resistance was not overcome. The *lpcr* gene therefore appeared to interfere with the host-specific resistance but not the pathogen- or microbe-associated molecular pattern of nonhost responses (Reeves et al. 2013).

Phytophthora capsici infects squash and pumpkins (*Cucurbita* spp.) causing seedling mortality, crown and root rot, foliar blight, and fruit rot at different stages of plant growth. Accessions of *C. moschata* (119) from 39 geographic locations throughout the world, and a highly susceptible butternut squash cultivar, Butterbush, were inoculated with a suspension of three highly virulent *P. capsici* from Florida to identify resistance to crown rot. Mean disease rating (DR) of the *C. moschata* accessions ranged from 1.4 to 5.0 (0–5 scale). A set of 18 PIs from original screen were rescreened for crown rot resistance. The accessions PI176531, PI458740, PI442266, PI442262, and PI634693 with lowest disease severity of crown rot (<1.0) were identified. These accessions showed promise as donors of resistance to crown rot disease in cucurbits (Chavez et al. 2011). *P. capsici* was found to be a serious threat to the production of many vegetable crops. Crown rot disease resistance of *Cucurbita melo* (honeydew and muskmelon)

was investigated by inoculating seedlings under greenhouse conditions. USPIs (308) and two commercial cultivars (Athena and Diners) were grown in the greenhouse and seedlings at 2–4-leaf stage were inoculated with a 5-isolate zoospore suspension (1×10^4 zoospores/seedling) at the crown and monitored for 6 weeks for symptom development. All susceptible control plants of cultivar Athena were killed within 7 days postinoculation. The majority of the PIs (281 of 308) was highly susceptible to crown rot and succumbed to the disease rapidly; less than 20% of the inoculated plants survived. After first screening and reevaluation, more than 80% of the inoculated plants survived in only six PIs. Disease development was significantly slower on these PIs compared to the susceptible control plants. Based on higher levels of resistance, three accessions (PI420180, PI176936, and PI176940) were selected for exploiting their potential as sources of resistance to crown rot caused by *Phytophthora capsici* (Donahoo et al. 2013).

Managing black rot disease of cabbage caused by *Xanthomonas campestris* pv. *campestris* (Xcc) was found to be very difficult and uneconomical if the susceptible cabbage cultivar was grown in Tanzania. Local and introduced cabbage cultivars (31) were therefore screened for resistance to black rot disease under field conditions. Differences in resistance for both foliar and internal stem/head symptoms were observed. Open pollinated varieties were highly susceptible to black rot. Of the 31 varieties tested, 14 varieties showed partial resistance and some of these varieties possessed some undesirable characteristics (head splitting, poor taste, loose or flat heads). In another experiment, the reactions of five selected varieties were determined following artificial inoculation with four strains of the pathogen through roots or foliage. No significant difference in the cabbage varieties inoculated through roots or foliage could be observed (Massomo et al. 2004).

Bacterial fruit blotch (BFB) disease of watermelon and melon caused by *Acidovorax avenae* subsp. *citrulli* (Aac) was considered as a serious threat to the industry. None of the available commercial cultivars showed resistance to BFB disease. The levels of tolerance/resistance of the commercial varieties and breeding and wild lines to BFB disease were therefore assessed using seed-transmission assays and seedling inoculation procedures. Some of the varieties were tested in the greenhouse using mature plants. All cultivars/lines were found to be susceptible to the pathogen and most of them showed different responses to BFB disease. However, some varieties showed consistent reactions. The cultivar ADIR 339 was relatively tolerant in all tested assays and cultivar 6407 and wild lines BCB-B and EAD-B were relatively tolerant in seed-transmission assays. Evidence supporting a strong correlation between level of susceptibility of a cultivar/line and the ability of Aac to adhere to or penetrate the seed was revealed by this investigation (Bahar et al. 2009). In a later investigation, *Cucumis* spp. PIs (332) were screened for resistance to *Acidovorax avenae* subsp. *citrullii* using a newly developed seed vacuum-infusion assay. Significant difference in the reaction of the PI to bacterial fruit blotch (BFB) was observed. The majority of lines were found to be extremely susceptible to the disease. Some of the PIs had lower levels of resistance. Variability in the reaction of plants within each PI was also observed. Of the 332 PIs tested, 16 were selected for additional evaluation using a standard spraying inoculation test. The PIs 353814, PI381171, PI536573, and PI504558 (*C. ficifolius*) were found to have significantly greater levels of resistance than susceptible control cultivars or other PIs in two independent spray inoculation tests. Germplasm lines identified in this investigation might be useful as sources of resistance to BFB in *Cucumis* spp. breeding programs (Wechter et al. 2011).

Levels of resistance to common scab disease caused by *Streptomyces turgidiscabies* within potato (*Solanum tuberosum*) cultivar Russet Burbank clonal lines and other selected processing cultivars were determined. Significant variability in the resistance levels was observed. Across two-thirds, cultivars Russet Nugget and Shepody and Russet Burbank clone British Columbia were consistently grouped within the cluster of least disease incidence, whereas cultivars Russet Norkotah and Vancouver unit four grouped within the cluster showing greatest disease incidence. Russet Nugget and British Columbia were also consistently grouped within the cluster showing least disease severity, whereas Shepody and Russet Burbank clone Starks were grouped within the cluster with greatest severity scores. For some clones, consistency of rating across trials was not always apparent (Wilson 2001). Comparative resistance levels of seven potato varieties to common scab pathogen *Streptomyces turgidiscabies* were assessed in the field with a paper pot method developed in this investigation. Seedlings raised in soil in paper pots containing inocula at 1×10^3 to 10^7 CFU g⁻¹ soil were transplanted into a scab-free field and grown for 3 months. The disease severity differed across trials and years, even though their levels of resistance were approximately similar. With the paper pot method, the potato cultivar showed different resistance levels which were almost completely consistent with the results of the field trials, at more than 1×10^5 CFU g⁻¹ soil. Significant differences in disease severity between resistant and susceptible varieties could be recognized ($P=0.05$) for 2 years (Kobayashi et al. 2005).

Huanglongbing (HLB, formerly known as greening) disease of citrus caused by "*Candidatus Liberibacter asiaticus*" (CLAs) had become a serious threat to the citrus industry in Florida's Indian River region. The incidence and severity of the HLB disease was assessed in the commercial groves where HLB incidence was high. In each grove, 20 trees of each cultivar were scored for incidence and severity of HLB disease based on visual symptoms. Leaves were collected for quantitative polymerase chain reaction (qPCR) quantification of "*Ca. Liberibacter asiaticus*" (CLAs). A strong correlation between HLB rating and CLAs titer was observed as represented by Ct values. The Ct values were $r^2=0.37$ and 0.40 for whole tree and leaf sample, respectively, ($P<0.0001$) across all cultivars and groves. Although the incidence and severity of HLB differed considerably among the groves, scion-specific differences were apparent even when analyses excluded potentially confounding grove effects. The cultivar "Temple" tangor showed the least incidence and CLAs titer. In contrast, "Murcott" tangor and "Minneola" were infected heavily and CLAs titer was the highest. Levels of resistance based on the disease incidence and severity and pathogen DNA titer might be useful to identify the sources of resistance to HLB disease in *Citrus* spp. (Stover and McCollum 2011).

Some citrus accessions such as rough lemon were reported to be tolerant to huanglongbing (HLB) disease caused by "*Candidatus Liberibacter asiaticus*." In order to understand the HLB tolerance/susceptibility mechanism(s) operating in citrus, comparative anatomical analysis of tolerant rough lemon and sensitive sweet orange seedlings in response to HLB-associated bacterial pathogen were carried out. Anatomical alterations in leaf, stem, and root tissues in healthy and infected plants were determined by light and electron microscopic observations. Phloem collapse, plugged sieve elements, and accumulation of starch were observed in leaf petioles of symptomatic leaves from both HLB-disease-infected rough lemon and sweet orange, while mock-inoculated plants did not exhibit such variations. In symptomless leaves significant anatomical changes as in symptomatic leaves were only observed in diseased sweet orange, but not in rough lemon plants. Further, starch depletion, phloem cell collapse,

and the absence of phloem fibres were observed only in secondary roots of diseased sweet orange. In young green stems starch deposition occurred only in the sweet orange plants. At the whole-plant level, HLB infection induced fewer disruptive anatomical changes in rough lemon than in sweet orange; the absence of changes in rough lemon root system was considered to be critical for sustaining plant growth after infection and might contribute greatly to its HLB tolerance (Fan et al. 2013).

The resistance conferred by a gene of cultivars against viral infection is called cultivar resistance. The plant species to which the cultivar belongs is susceptible as a whole. Cultivar resistance may become ineffective when a virulent strain is formed by mutation or selection from a mixture of strains. The term tolerance is applied for an interaction between the host and virus which is not observed in the interaction between fungal or bacterial pathogens with their respective host plant species. Tolerance is resistance to symptom development rather than to the virus replication. Latent infection by a virus does not induce any recognizable symptom. When the virus becomes systemic and produces observable symptoms on other plant parts, the response is designated susceptibility (Matthews 1991). Acquired resistance is another unique phenomenon commonly associated with virus infection. When inoculated with a virus, the susceptible plant becomes resistant to subsequent infection by the same virus or its strains. Such cross-protection against related viruses or its strains has been advocated as a virus disease management strategy, especially against viruses infecting perennial crops such as citrus. Another aspect of resistance to virus diseases is the requirement of resistance to vectors which are involved in the transmission of most plant viruses from infected plants to healthy plants under natural conditions. A virus-coded function and the product of the host resistance gene may be involved in the recognition of the presence of the viral pathogen, leading to susceptibility or resistance. If the product of resistance gene is directly involved in the recognition event, it may be an inhibitor of virus infection and resistance would be constitutive in nature. Alternatively, the resistance gene product produced on meeting the virus factor may signal the induction of a host defense mechanism, leading to the production of a compound(s) inhibitory to virus replication and movement. Plants exhibit different grades of resistance to a virus which may operate at different steps of virus replication such as uncoating, synthesis of replicase, translation of virus-coded information, and production of movement protein (Narayanasamy 2011).

Resistance to viruses may be expressed by hypersensitive reaction (HR), involving necrosis of cells or tissues restricting the spread of virus in the inoculated plants. The gene for hypersensitivity in *Nicotiana glutinosa* to Tobacco mosaic virus (TMV) was incorporated into some commercial lines of tobacco (*Nicotiana tabacum*). This type of resistance is also known as field resistance. Resistance to viruses may be due to simple genetic control involving only a single locus. The resistance may be completely dominant or recessive, conditioned by a single gene or polygenes. Resistance to several viruses (69 of 87) was inherited as a monogenic dominant, incompletely dominant, or apparently recessive factor (Fraser 1990). If resistance is measured only on the basis of symptom severity, it may be considered to be dominant or recessive; if virus multiplication is the basis for assessment of resistance however, then incomplete dominance (gene-dosage dependence) of resistance may be observed. Such a situation was seen in the case of resistance due to the *Tm-1* gene to TMV in tomato (Fraser and Loughlin 1980) and resistance due to the *bc-1* gene to Bean common mosaic virus (BCMV) in *Phaseolus vulgaris* (French bean) (Day 1984). Virus multiplication as a basis of resistance

to virus may be important because symptom severity may not be always related to virus titer (concentration) in infected plants. Incomplete dominance (gene-dosage dependent) of resistance was found to be strongly associated with partial localization of the virus. The virus may be localized in the inoculated leaf itself or, if the virus is allowed to become systemic, virus multiplication was significantly reduced. Tomato cultivar with *Tm-1* gene showed systemic infection, but multiplication of TMV was inhibited (Fraser and Loughlin 1980; Watanabe et al. 1987).

A small number of recessive genes may offer complete resistance (immunity) in certain host–virus combinations. Immunity of Arlington cowpea to *Cowpea mosaic virus* was due to the constitutive expression of a single dominant gene. A protease inhibitor that specifically inhibited processing of the polyproteins of *Cowpea mosaic virus* was shown to be involved in the resistance (Bruening et al. 1987; Ponz et al. 1988b). Resistance to *Potato virus Y* in pepper plants homozygous for the recessive resistance allele y^a (Pr2') was found to be due to impaired cell-to-cell movement of the virus, rather than to interference with virus multiplication (Arroyo et al. 1996). Virus strains capable of adopting the introduced genes for resistance have rapidly restricted the usefulness of virus-resistant genotypes and made the genes ineffective in the same host–virus pathosystem. Resistance to *Cucumber mosaic virus* (CMV) was found to be “weak” and short-lived since the virus exists in the form of many strains with varying levels of virulence (Matthews 1991). The quality of the produce obtained from resistant cultivar may be poor, although the resistance gene may offer effective protection as in *Tobacco mosaic virus* (TMV-) resistant tobacco cultivar (Johnson 1983).

However, there are some virus diseases for which durable resistance was observed. The resistance genes *Tm-2²* in tomato against TMV, “Ry” in potato against *Potato virus Y*, and “N” in tobacco against TMV were found to be highly durable (Fraser 1990). Many viruses are known to be transmitted through true seeds of infected plants. Considerable difficulty was observed in eliminating the viruses from seeds. Investigations were therefore carried out to identify resistance to seed transmission of viruses with a view to reducing initial inoculum of viruses introduced through infected seeds. Resistance to seed transmission of *Barley stripe mosaic virus* (BSMV) in barley was controlled by a single recessive gene (Carroll et al. 1979). The barley cultivar Mobet had the resistance gene introduced from an Ethiopian barley cultivar Modjo Mobet barley which had high resistance to seed transmission of BSMV and desirable agronomic attributes (Carroll et al. 1983). Lines possessing resistance to seed transmission of *Lettuce mosaic virus* (LMV) in lettuce (Matthews 1991) have been identified.

Resistance to virus diseases has a unique aspect of differentiating the resistance to virus replication and resistance to symptom development. Resistance to virus ultimately results in resistance to the disease. However, necrosis as part of resistance response may prove to be lethal, as in the case of “I” gene resistance to *Bean common mosaic virus* infecting bean (*Phaseolus vulgaris*) (Collmer et al. 2000). The virus is able to become systemic in the plants of some genotypes, showing resistance to symptom expression in a manner that is distinguishable from the susceptible plants, but disease symptoms are not visible. Such plants are considered as tolerant and this trait is heritable. Tolerance to *Cucumber mosaic virus* (CMV) has been exploited as a disease management strategy in cucumber production (Hull 2002). Acquired resistance, also known as cross-protection against related strains or viruses, has been the principal management strategy adopted in the case of certain viruses infecting fruit trees such as *Citrus tristeza virus* (CTV) (Narayanasamy 2006). *Bean common mosaic virus* (BCMV) and *Bean common mosaic*

necrosis virus (BCMNV) are seed-transmitted at a high frequency, making most disease management tactics ineffective. Use of resistant cultivars therefore appeared to be the desirable approach for containing these diseases effectively. Use of molecular markers was useful to accelerate the progress in breeding programs. The *bc-1²* allele conferred resistance to BCMV strains within pathogroups I, II, and V and pathogroups III strains of BCMNV (Drijfhout 1978). The usefulness of sequence-characterized amplified regions (SCAR) markers which were tightly linked with *bc-1²* were developed (Miklas et al. 2000; Vandemark and Miklas 2002). The SCAR markers linked to *1* allele which conditioned resistance to all strains of BCMV and BCNMV was developed. For simultaneous genotyping of bean plants for the *1* and *bc-1²* alleles which condition resistance in bean to BCMV and BCNMV, a multiplex real-time PCR assay was found to be effective. The segregating bean populations could be used for assigning genotypes for both the *bc-1²* and *1* alleles. The number of F3 family progeny tests was substantially reduced. In addition, the desired genotypes could be identified at the seedling stage, resulting in acceleration of progress in germplasm enhancement and cultivar development (Melotto et al. 1996; Vandemark and Miklas 2005).

Two principal types of resistance – hypersensitive and extreme – to *Potato virus Y* (PVY) in potato have been recognized. The HR to PVY is strain-specific causing a range of necrotic reactions in both locally and systemically infected leaves (Valkonen et al. 1998). The *Ny* genes involved in *R* were identified in several potato cultivars (de Galarreta et al. 1998), but in some cultivars HR may not restrict spread of virus in plants. The *Ry* genes for extreme resistance (ER) to PVY conferred an extremely high level of protection against all PVY strains (Vidal et al. 2002). The potato chromosome contains the HR gene *Ny_{1br}* (Celebi-Toprak et al. 2002). The *Ry_{adg}* (*Solanum tuberosum* ssp. *andigena*) and *Ry_{sto}* (*S. stoloniferum*) were mapped to chromosome XI, whereas *RY_{chc}* (*S. chavense*) was located in chromosome IX (Brigneti et al. 1997; Hämäläinen et al. 1997; Hosaka et al. 2001).

Marker-assisted selection (MAS) was found to be one of the most useful applications of DNA-based markers in potato breeding programs for disease resistance. A novel locus for ER to PVY, *Ry_{fsto}*, was identified on potato chromosome XII. Bulk segregate analysis (BSA) was applied to identify an intersimple sequence repeat (ISSR) marker UC857 linked to *Ry_{fsto}*. This marker mapped to the 980 linkage group XII of a reference potato RFLP map. The RFLP markers were converted into PCR-based sequence-tagged site (STS) and cleaved amplified polymorphic sequence (CAPS) markers. The CAPS marker GP122₇₁₈ was tightly linked to the resistance gene that was reliably identified in Polish and German cultivars expressing ER to PVY. The results indicated that the CAPS marker GP122₇₁₈ has the potential for use as a diagnostic tool to monitor the distribution of specific resistance gene in a wide gene pool (Flis et al. 2005).

The incidence of *Potato virus Y* (PVY) is widespread, and as a result the yield and quality of pepper fruits are significantly reduced in the Republic of South Africa. Commercially grown pepper lines were evaluated for their resistance to PVY infection. Six commercial F1 pepper lines were challenged with PVY isolates JYW-186 by mechanical inoculation. Levels of resistance of pepper lines were assessed by visual screening for disease severity and the virus titer was determined by quantitative triple-antibody sandwich ELISA format. The pepper lines were further characterized using tetra-primer amplification refractory mutation system (ARMS-) PCR to identify and differentiate homozygous/heterozygous resistance alleles at the *pvr2* locus that conferred resistance to PVY. Two resistant lines (Double UP and Cecelia)

were identified and other lines showed susceptibility to different grades when challenged with PVY. The *pvr2* + allele was identified in all the susceptible pepper lines which confirmed the presence of this allele, dominant for PVY susceptibility (Moodley et al. 2015).

The successful deployment of the desired gene(s) into crop plant species depends more upon the identification of a positive phenotype and dissection of the phenotype, resulting in the identification of genetic markers for marker-assisted selective (MAS) breeding, and on an understanding of how the novel resistance will perform in different genetic background and under pathogen pressure in the field. The molecular markers facilitate the adoption of disease resistance strategies such as gene deployment. Pyramiding of resistance may be easily achieved using molecular markers. While breeding for resistance to one disease, the possibility of introducing susceptibility to another disease could be avoided if molecular markers are used. Pyramiding of resistance genes effective against all known pathotypes of the pathogen may be achieved using molecular markers. Manipulation of disease resistance genes through molecular approaches has opened up several opportunities to combine resistance genes of diverse origin, something which is not possible by conventional methods. However, molecular marker technology is presently very expensive and it may not be possible to use this method for routine analysis of a large number of breeding lines. Marker-assisted selection (MAS) programs may be used increasingly when the markers for a greater number of resistance genes become available at an affordable cost (Narayanasamy 2011).

12.2.1.2 Assessment of Resistance by Quantification of Pathogen Biomass

It is possible to quantify the pathogen biomass in the infected plant tissues and relate it to the level of resistance of the test plant to the pathogen concerned. A negative relationship between the pathogen biomass and resistance of plant to the pathogen has been observed in several pathosystems. Immunoassays and nucleic acid-based techniques have been demonstrated to be effective for assessing levels of host resistance to microbial pathogens causing crop diseases. The levels of resistance of wheat and triticale cultivars to leaf blotch disease caused by *Septoria tritici* and leaf and glume blotch caused by *Stagonospora nodorum* were assessed by applying a specific and quantitative biotin/avidin (BA-) enzyme-linked immunosorbent assay (ELISA) under field conditions. The antigen contents representing the target pathogens were determined in the flag leaf (F) and first leaf below the flag leaf (F-1) at a predetermined growth stage of 5 triticale and 11 wheat cultivars. *S. nodorum* was present predominantly in triticale cultivars, whereas both pathogens could be more frequently detected in wheat cultivars by BA-ELISA tests. The absorbance values correlated well with susceptibility rating in the cultivars list recommended by the German Federal Office of Plant Variety (Bundesortenamt) based on visual assessment. The diagnostic assay could be employed for detecting the pathogens before visible symptoms appeared and also when the disease pressure was low, that is, below the recognition level by visual assessment. In addition, BA-ELISA could be effectively applied to differentiate the effect of the two pathogens and provide an accurate assessment of disease severities caused by them individually. As the quantitative differences in the pathogen biomass could be ascertained by using BA-ELISA tests, the levels of resistance of cultivars and genotypes might be effectively graded (Tian et al. 2005). The BA-ELISA format has the potential for use in the assessment of levels of resistance of crop cultivars and genotypes in breeding programs.

It is well known that the responses of cultivars and genotypes differ considerably depending on the race, strain, or biotype of the pathogen. Precise identification of the isolates is therefore essential to assess the levels of susceptibility/resistance of genotypes and cultivars. The molecular identity of strains of the potato late blight pathogen *Phytophthora infestans*, present in the Toluca Valley of Mexico, was investigated. The isolates of *P. infestans* obtained from five potato varieties with differing levels of resistance were compared based on the mating type (MT), isozyme genotype for glucose-6-phosphatase isomerase (Gpi) and peptidase (Pep), and sensitivity to metal-axyl. Further, a detached-leaf assay in the laboratory and attached-leaf assay in the greenhouse was used for three potato varieties to assess the host resistance components and specificity of host-pathogen interaction. The genetically derived host factors such as efficiency of infection, number and size of lesions, latent period or time required for sporulation after infection, and sporulation capacity were considered as host resistance components. The isolate from cultivar Alpha (susceptible) did not infect cultivar Norteña (resistant) in the laboratory and greenhouse tests, indicating the existence of gene-for-gene interaction between *P. infestans* and potato cultivars. The cultivar Norteña had major or veritcal resistance genes for corresponding virulence genes of the pathogen. As the cultivar Alpha did not contain matching *R* genes, it could be infected by wild-type, virulent, and avirulent pathogen genotypes alike. The field resistance shown by cultivars Rosita and Norteña was lost when the plants of these cultivars were artificially inoculated under greenhouse conditions. However, these potato cultivars remained resistant under the natural conditions of Toluca Valley (Grünwald et al. 2002).

A real-time PCR procedure was applied for the detection and quantification of *Phytophthora capsici*, causing Phytophthora root rot disease in pepper (chilli) plants. By using the real-time PCR assay with SYBR Green Supermix (BioRad) and specific primers for *P. capsici*, the pathogen DNA could be detected as early as 8 h postinoculation. The increase in pathogen DNA was more rapid in susceptible cultivars and slower in resistant genotypes (PI1201234 and SCM331). The amount of pathogen DNA quantified in each pepper genotype was correlated with the levels of susceptibility (positive)/resistance (negative). The relationship between virulence (aggressiveness) of the pathogen and extent of plant tissue colonization was also revealed. The results demonstrated that real-time PCR with SYBR Green was sensitive and robust enough to assess both pathogen development and the levels of resistance of pepper cultivars and genotypes to *P. capsici*. The resistant genotypes selected based on the molecular technique might be used as sources of resistance to *P. capsici* in programs for development of pepper cultivars resistant to Phytophthora blight/root rot disease (Silvar et al. 2005).

Chinese wheat cultivar var. Sumai 3 and its derivatives such as Ning 7840 were used extensively as sources of resistance to Fusarium head blight (FHB) disease caused by *Fusarium graminearum*. The FHB disease resistance genes from these genotypes were characterized through molecular mapping (Bai et al. 2003a). Wangshuibai, a Chinese landrace, possessed a high level of resistance to pathogen spread within a spike and the resistance was more stable than that of Sumai 3. The recombinant inbred lines (RIL) derived from the cross between susceptible cultivars Alondra and Wangshuibai were assessed. Fifteen AFLP markers associated with three quantitative trait loci (QTL) for FHB resistance were located in two chromosomes. One QTL was mapped on 1B and two others were mapped on 3B. One QTL on 3BS had a

minor effect. The marker-assisted selection (MAS) for FHB resistance genotypes could be made using closely linked markers such as single-sequence repeat (SSR) markers. It was possible to eliminate undesirable associations between FHB resistance and other agronomic traits (Zhang et al. 2004). By making crosses between wheat and rye, common cultivars and breeding lines with FHB resistance were developed (Lu et al. 2000).

Molecular techniques have been applied for characterization of genes involved in conferring resistance to bacterial diseases affecting crop plants. Many major genes involved in resistance to rice bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) have been identified and designated in a series from *Xa1* to *Xa28*. The resistance genes (>10) were mapped on various chromosomes, especially 4 and 11 (Kinoshita 1995; Lee et al. 2003; Yang et al. 2003). The wild relative *Oryza longistaminata* is highly resistant to *Xoo*. The *Xa21* locus conferring resistance to all races of *Xoo* in India and Philippines was transgressed from *O. longistaminata* to rice cultivar IR24. The *Xa21* gene coding for a receptor kinase-like protein was inherited as a single gene in transgenic plants (Song et al. 1995; Wang et al. 1996). These plants were resistant to 29 of 32 isolates of *Xoo* from India and other SE Asian countries. The presence of a single member of multigenic family *Xa21* appeared to be sufficient to confer resistance to different isolates of *Xoo* (Wang et al. 1996; Ronald 1997). The development of *Xylella fastidiosa* (*Xf*), causing grapevine Pierce's disease (PD) in resistant and susceptible genotypes and representing a diverse selection of *Vitis* spp., was monitored by measuring pathogen movement and accumulation. The pathogen was inoculated onto the selected grapevine genotypes under greenhouse conditions. DAS-ELISA format was optimized for quantification of *Xf* populations and was employed to estimate the *Xf* population quantitatively in stem, internode, petiole, and leaf blade samples from different genotypes at 12 weeks after inoculation. Differences in *Xf* populations between stems and leaves of resistant genotypes suggested that a localized mechanism might operate within xylem, one which is not fully functional or possibly absent from xylem of petioles and leaf blades. The quantitative ELISA estimates of *Xf* in the greenhouse-grown grapevines might be an effective pointer of the level of resistance to Pierce's disease under field conditions (Krivanek and Walker 2005).

Managing the loose smut of wheat caused by *Ustilago tritici* utilizing the genetic resistance has been attempted around the world as the disease reduces the grain yield roughly in proportion to the disease incidence. DNA markers of genetic resistance have been shown to greatly reduce the time and labor resources required for screening of genotypes by employing traditional inoculation methods. In order to identify DNA markers associated with resistance to loose smut disease, double haploid progeny from the four crosses Glenlea/AC Taber, SC8021V2/AC Karma, Dimant/TD12A, and 9340-CP/AC Vista were evaluated with individual races of the pathogen and simple sequence repeat markers. Simple interval mapping was performed using MQTL software and single point analysis. With complete and broad resistance to all known races of *U. tritici* in Canada, Glenlea contributed a QTL for resistance to races T2, T10, T15, T19, and T39 at *Xwmc* 160 on chromosome 5B. Glenlea also contributed QTL for resistance to races T2, T9, and T15 at *Xgwm*302 on chromosome 7B and to races T9, T19, and T39 on chromosome 3A at marker *Xwcm* 559. The chromosome 5B QTL from Glenlea coincided with a race T39 resistance locus from AC Karma. The chromosome 5B locus in combination with either the 7A or 7B genetic loci might provide resistance to predominant races of *U. tritici* present in Canada (Knox et al. 2014).

12.2.1.3 Molecular Basis of Resistance to Fungal Diseases

Two principal kinds of defense mechanisms operate in plants to prevent/restrict the infection and invasion of microbial pathogens. Passive or pre-existing defense mechanisms involving structural barriers, such as waxy cuticle or reservoirs of antimicrobial compounds that are strategically positioned to act on the invading pathogen(s), are known to operate in some pathosystems. The role of preformed pericarp phenylpropanoids in the development of resistance in maize kernels of 11 maize genotypes was investigated. Kernel rot disease severity and fumonisin accumulation were determined after inoculation with *Fusarium verticillioides* and related to contents of pericarp phenylpropanoids in samples obtained from field trials conducted for 2 years. Grain fumonisin concentrations were highly dependent on disease severity of the genotypes ($r = 0.88$). Analysis of pericarp phenylpropanoids indicated the presence of trans-ferulic acid (tFA), cis-ferulic acid (cFA), p-coumaric acid (pCA), and five diferulates (DFAtz). The most predominant diferulates were 8.5'-diferulic acid bensofuran (8.5'-DFAtz) followed by 8.5'-DFA. Except for cFA, the most resistant genotypes exhibited high levels of phenylpropanoids which were related to low levels of disease severity and grain fumonisin accumulation. A stepwise regression analysis revealed that total diferulates contents were more related to variability of disease severity ($r^2 = 0.71$) than any other parameters considered. Grain fumonisin concentration was reflected by the contents of total diferulates, 8.5'-DFAbz and pCA ($r^2 = 0.82$). The results suggested that high levels of phenylpropanoids in the kernel pericarp is a trait associated with less disease severity and lower level of accumulation of fumonisin elaborated by *F. verticillioides* (Sampietro and Fauguel 2013).

Active defense mechanisms are induced following initiation of the infection process when structural barriers are breached by pathogens, leading to prevention of further colonization of plant tissues. Active defense responses induced by fungal, bacterial, or viral pathogens may be of three types: primary responses, secondary responses, and systemically acquired responses. Primary responses are restricted to the infected cells or to cells in close proximity to the pathogen, and the specific signal molecules produced by the pathogens have to be recognized by host cells. The outcome of this interaction may rapidly result in the phenomenon known as programmed cell death (PCD) which may be defined as a self-destructive process triggered by external or internal factors. PCD is mediated through an active genetic program which is considered to have a crucial role in the development and survival of diverse organisms (Pontier et al. 2004). The secondary responses are observed in the adjacent cells surrounding the initially infected cells in response to diffusible signal molecules (elicitors) released by pathogens. Systemically acquired response is due to the action of defense-related compounds that can be translocated throughout the plant, leading to systemic acquired resistance (SAR) (Hutcheson 1998). The gene-for-gene concept of Flor (1946) proposed for the classical flax-rust (*Melampsora lini*) could be explained based on the specific genetic interaction between the host plant resistance *R* and the pathogen avirulence (*Avr*) genes. The *R* genes of the host plant encode specific receptor site(s) for direct or indirect products of the corresponding *Avr* genes. The interaction between the gene products has been demonstrated using yeast two-hybrid system (Dodds et al. 2006).

Fusarium head blight (FHB) disease caused by *Fusarium graminearum* is responsible for both quantitative and qualitative losses. The spikes responded to infection by *F. graminearum* by activating a set of defense response genes in infected spikelets. In order to determine whether defense response genes were expressed systemically

within *F. graminearum*-inoculated spikes, the transcript accumulation of four defense response genes – peroxidase and pathogenesis-related (PR) proteins PR-1, PR-3, and PR-5 – was determined in colonized and uncolonized portions of wheat spikes of resistant and susceptible genotypes. Whether the sampled regions of point-inoculated wheat spikes were colonized or not was determined using *Fusarium* spp.-specific plate assay and a *F. graminearum* isolate expressing β -glucuronidase (GUS). The GUS-expressing *F. graminearum* isolate proved particularly useful for visualizing and following the spread of the fungus and for measuring the amount of fungus biomass in spike tissues. The results showed that transcripts of the four defense response genes accumulated in both colonized and uncolonized regions of the spikes of resistant and susceptible genotypes at 48 h after inoculation. Direct contact with the pathogen was not required for induction of defense response genes, and these genes were activated in both susceptible and resistant genotypes (Pritsch et al. 2001).

Development of Fusarium head blight (FHB) caused by *Fusarium culmorum* was investigated in seven winter wheat cultivars and one spring wheat cultivar in an outdoor pot experiment. The spring wheat cultivar Frontana alone exhibited resistance against initial infection by the pathogen. Free phenolic compounds accumulated in significantly higher amounts in the glumes, lemmas, and paleas of Frontana prior to and at all sampling times after inoculation in comparison to the winter cultivar Agent. In addition, the amount of p-coumaric acid increased significantly in the glumes, lemmas, and paleas of Frontana at 2 days after inoculation (dai) compared with that in uninoculated spike tissues. However, the amount of ferulic acid between inoculated and healthy plant tissues did not differ. Ultrastructural studies indicated more pronounced structural defense responses, such as cell-wall appositions in the infected lemma tissue of Frontana comparing with cultivar Agent (susceptible) at 3 dai. Immunogold labeling of lignin revealed no differences in the density of gold particles on the cell walls of healthy lemma tissue in the two cultivars. However, the density of particles increased in the infected lemma tissue of the resistant cultivar Frontana on cell walls adjacent to the fungal cells at 3 dai. The cultivar Agent did not show any response to the infection. The results suggested that phenolic compounds might play a role in the resistance of cultivar Frontana to *F. culmorum* (Siranidou et al. 2002).

The subcellular localization of thionins and hydroxyproline-rich glycoproteins (HRGPs) or extensins in *Fusarium culmorum*-infected wheat spikes was investigated by employing the immunogold labeling technique. A rabbit polyclonal antiserum produced against cell-wall-bound thionins from barley leaf and a mouse monoclonal antibody against HRGP from maize was used for detection of the defense-related proteins in infected tissues. The proteins were localized in the cell walls of different tissues, including the lemma, ovary, and rachis, while the cytoplasm and organelles in these tissues showed almost no labeling. However, accumulation of thionins and HRGP in infected spikes of resistant wheat cultivars differed distinctly from those of susceptible cultivars. Labeling intensities for two types of proteins in cell walls of the infected lemma, ovary, and rachis increased slightly in the susceptible cultivar Agent, compared with healthy tissues of resistant cultivar Arina in which labeling densities of thionins and HRGP increased markedly. The results indicated that accumulations of thionins and HRGP in cell walls of infected resistant wheat spikes might be involved in defense responses to infection and in spreading of *F. culmorum* (Kang and Buchenauer 2003).

The metabolic profiles of spikelets of wheat cultivars Roblin (susceptible) and Sumai 3 (resistant) were analyzed using gas chromatography/mass spectrometry (GC/MS) to

develop a procedure to discriminate resistance to *Fusarium* head blight (FHB) disease. More than 700 peaks were detected and a total of 55 compounds were tentatively identified, of which 49 were induced (upregulated following pathogen inoculation), including 23 in Roblin and 26 in Sumai 3. However, only five metabolites were significantly different both between cultivars and inoculations. Although detected in all four treatments, metahydroxy-cinnamic acid exhibited a six-fold increase in abundance in Sumai 3 following pathogen inoculation with no corresponding change in Roblin. The abundance of myo-inositol in Sumai 3 was higher than that in Roblin, and in both cultivars the abundance increased following pathogen inoculation. The compounds common to all treatments were subjected to factor analysis to identify groups of compounds based on significant factor-loadings associated with the highest scores for the first and second factors that could be used for discriminating resistance against FHB disease. The first factor was associated with the higher abundances of several fatty acids and aromatic compounds, while the second factor was associated with metabolites such as p- and m-coumaric acids, myo-inositol, and other sugars and malonic acid. The treatments involving pathogen inoculation had higher factor scores for the third factor than the water-inoculated cultivar Roblin, and might be useful in indicating the susceptible reaction. The third factor had a positive correlation with metabolites from different groups, mostly amino acids, fatty acids, and aromatics. The large number of compounds detected in susceptible and resistant wheat cultivars following infection by FHB pathogen indicated the complexity of host–pathogen interaction (Hamzehzarghani et al. 2005).

A metabolomics approach was applied for the discrimination of resistance to *Fusarium* head blight (FHB) disease in wheat cultivars. Spikelets of six wheat cultivars/lines with varying degrees of resistance were inoculated with *Fusarium graminearum* or water for control treatment. The disease severity of infected spikelets was scored and the metabolic profiles were recorded using GC/MS technique. A total of 214 metabolites were detected in spikelets and rachis, including 79 with acceptable treatment effects. Univariable analysis of variance identified 41 resistance-related (RR) metabolites, whereas multivariate analysis identified 45 resistance function-associated metabolites including 28 RR metabolites. Highly resistant cultivar Wangshuibai and line AW488 contained the maximum number of constitutive (22) and induced (14) RR metabolites. The moderately resistant line BRS 177 had 12 induced RR metabolites. The RR metabolites identified in this investigation were considered to be potential candidate biomarkers for high-throughput screening of wheat breeding lines against FHB disease of wheat (Hamzehzarghani et al. 2008).

In another investigation, the responses of wheat cultivars to trichothecene mycotoxin deoxynivalenol (DON) produced by *Fusarium* spp. during pathogenesis were determined. Infusion of wheat leaves with DON induced hydrogen peroxide (H₂O₂) production within 6 h followed by cell death within 24 h accompanied by DNA laddering, the hallmark of programmed cell death. In addition, real-time PCR analysis revealed that DON treatment rapidly induced transcription of a number of defense genes in a concentration-dependent manner. Co-treatment with DON and the antioxidant ascorbic acid reduced these responses, suggesting their induction might be at least partially mediated by reactive oxygen species (ROS) which are known to be signal molecules in plants. Wheat defense genes were more highly expressed in wheat stems inoculated with a DON-producing fungal strain than those inoculated with a DON-nonproducing mutant, but only at a late stage of infection. The results were consistent with a model in

which DON production during infection of wheat induces ROS which, on the one hand, might stimulate programmed cell death (PCD) in the host plant, assisting necrotrophic fungal growth. On the other hand, the ROS might contribute to the induction of antimicrobial host defenses (Desmond et al. 2008).

The molecular mechanisms involved in the resistance in barley to *Fusarium* head blight (FHB) pathogen *Fusarium graminearum* were studied to identify proteins that were differentially expressed in FHB susceptible and resistant barley accessions. Mature seed from six barley accessions and five pairs of barley sister lines differing in FHB resistance were subject to proteome analysis by two-dimensional gel electrophoresis (2DE). Coomassie Brilliant Blue staining was employed to visualize about 100 seed protein spots of pI 4–7 on 2DE gels. Proteins were identified by liquid chromatography and tandem mass spectrometry (LC-MS/MS) sequencing after in-gel digestion by trypsin. The 2DE seed protein profiles of two FHB susceptible cultivars AC Lacombe and Stander were compared with four partially FHB-resistant barley accessions CI4196, Chevron, Penco/Chevron, and I92130. A total of 171 protein spots differed in abundance between resistant and susceptible FHB barley genotypes, and 97 protein spots differed in abundance between resistant and susceptible sister lines. Overall, 11 proteins associated with disease or stress resistance were identified. Proteins identified at a higher abundance in barley lines resistant to FHB included α -amylase inhibitor (BDAI-1), aldehyde dehydrogenase (BIS1), aconitate hydratase, and NADP-dependent malic enzyme (Zantinge et al. 2010).

The metabolomics approach was applied in another investigation to identify the metabolites in barley that were related to resistance against FHB caused by *Fusarium graminearum* (teleomorph *Gibberella zeae*). Barley genotypes Chevron and Stander were inoculated with mock or pathogen during anthesis stage. The disease severity was determined as the proportion of spikelets diseased. The genotype Chevron (0.33) showed higher level of quantitative resistance compared with Stander (0.88). Spikelet samples were harvested at 4 h postinoculation and metabolites were extracted and analyzed using an LC-ESI-LTQ-Orbitrap (Thermo Fisher, MA, USA). Of the 1826 peaks retained, a t-test identified 496 metabolites with significant treatment effects. Among these, 194 were resistance-related (RR) constitutive metabolites whose abundance was higher in resistant mock-inoculated than in susceptible mock-inoculated genotypes. The RR metabolites mainly belonged to phenylpropanoid, flavonoid, fatty acid, and terpenoid metabolic pathways. Selected RR metabolites were assayed in vitro for anti-fungal activity on the basis of fungal biomass production. Some of the RR metabolites showed potential for use as biomarkers for screening for resistance of barley genotypes against FHB pathosystem (Bollina et al. 2010).

The host responses of susceptible and resistant maize plants to *Fusarium verticillioides*, causative agent of ear rot disease of corn, were investigated. The susceptible and resistant maize kernels were sampled in the area neighboring the point of inoculation in the early stages of infection. The pathogen growth assayed by quantitative (q) RT-PCR quantification of the *tub2* gene was eight times lower on an average in resistant kernels than that detected in susceptible line over the time course of 96 h. During the very early stages of infection (12–24 h after inoculation) a small portion of the host transcripts was induced. The number of differentially regulated genes reached 147 at 48 h after infection and decreased to 140 and 98 at 72 h and 96 h postinoculation, respectively. About 1% of the genes assayed were differentially expressed and 7.1% of them were assigned to the category cell rescue, defense, and virulence. Pathogenesis-related protein (PR)-5,

endo-1,3-1,4-beta-D-glucanase PRm6, chitinase, PRm3, and MYB-like DNA binding protein had a higher level of expression in the resistant line compared to the susceptible line. The results indicated that the resistant line might activate a battery of defense genes before the invasion by *F. verticillioides* in nondamaged tissues or in adjacent tissues. In the resistant kernels, a basal defense system might be activated and the expression of defense genes might be controlled by both the host genotype and pathogen virulence (Lanubile et al. 2012).

Fusarium graminearum, incitant of wheat Fusarium head blight (FHB) disease, produces a potent mycotoxin deoxynivalenol (DON) which is a virulence factor involved in pathogenesis. The expression of the core DON biosynthetic gene *Tri5* during wheat head infection of susceptible and resistant cultivars was investigated. DON was quantified to correlate expression with toxin accumulation. The highest *Tri5* expression relative to housekeeping genes occurred at the infection front. As infection progressed the earliest infected kernels showed diminished relative *Tri5* expression, but *Tri5* expression was observed throughout the period of experimentation (21 days). The *Tri5* transcripts remained detectable but reduced in the infected tissue of the susceptible cultivar Wheaten, after the plant tissue had completely senesced. In the resistant cultivar Alsen, initial fungal invasion was arrested and the pathogen appeared to retreat by the later stages of sampling. The resistant cultivar showed minimal spread of the fungus, with no fungus detected at 21 days after inoculation. In Wheaten, DON levels were negligible at 8 days postinoculation (dpi) with detectable DON at later-sampling time points. *Tri5* expression was detected in fully senesced kernels at 21 dpi. The presence of *Tri5* transcripts in the susceptible cultivar could be detected over a much longer period than previously reported (Hallen-Adams et al. 2011).

The ability of two mutants of *Fusarium graminearum* (*tr6Δ* and *noxABA*) to prime immune responses in wheat cultivar Roblin was determined against challenge with pathogenic *F. graminearum*. Priming treatments generated FHB-resistant wheat phenotypes and reduced accumulation of mycotoxins produced by the pathogen in infected tissues. Microarray analysis detected 260 transcripts that were differentially expressed during the priming period. Expression changes were observed in genes associated with immune surveillance systems, signaling cascades, antimicrobial compound production, oxidative burst, secondary metabolism, detoxification, and transport. Genes related to jasmonate, gibberellins, and ethylene biosynthesis exhibited differential expression during priming. In addition, induction of the phenylpropanoid pathways that lead to flavonoid, coumarin, and hydroxycinnamic acid amide accumulation was also observed. The results indicated the usefulness of employing nonpathogenic mutants to elicit and delineate stages of defense responses in wheat (Ravensdale et al. 2014).

Stagonospora nodorum blotch (SNB), caused by *Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*), is responsible for significant yield losses in wheat. Development of wheat cultivars with resistance to SNB disease was considered as an effective option for the management of the disease, in addition to adoption of cultural practices to eliminate infected plant debris. Wheat cultivars (29) with varying levels of resistance to SNB representing 10 eastern US breeding programs were infiltrated with culture filtrates from *S. nodorum* isolates in randomized complete block design. *S. nodorum* produced several necrotic effectors (NEs, also known as host-selective toxins) and the host sensitivity (Snn) corresponding to the NEs was determined. Three single-NE *Pichia pastoris* controls, two *S. nodorum* isolate controls, and six Snn-differentiated wheat controls were used. Cultivar-isolate interactions were visually

determined for sensitivity at 7 days after infiltration. Of the 39 isolates, 17 produced NEs different from those previously characterized in the Upper Great Plains region. Only 3, 0, and 23% of the 39 isolates produced SnToxA, SnTox1, and SnTox3, respectively, by the culture filtrate test. A Southern dot-blot test showed that 15, 74, and 39% of the isolates carried the genes for those NEs, respectively. Only two wheat cultivars appeared to contain known *Snn* genes, although half of the cultivars displayed sensitivity to culture filtrates containing unknown NEs. Effector sensitivity was more frequent in SNB-susceptible cultivars than in moderately resistant (MR) cultivars ($P = 0.008$), although some susceptible cultivars did not exhibit sensitivity to NEs of multiple isolates. The results suggested that sensitivities to NEs could influence, but might not be the only determinant of, wheat cultivar resistance to *S. nodorum* (Crook et al. 2012). In order to understand the mechanisms regulating the expression of maize resistance genes against *Aspergillus flavus*, the potent roles of six WRKY transcription factors and the expression of these pathway indicator genes in response to inoculation of *A. flavus* in B73 (susceptible) and TZAR101 (resistant) genotypes were investigated. The genes *ZmWRKY19*, *ZmWRKY53*, and *ZmWRKY67* showed elevated expression in resistant TZAR101 plants. *ZmNPR1* expression was induced by inoculation in TZAR101 without concurrent induction of *ZmERF1*. The results indicated that WRKY transcription factors were involved in resistance and salicylic acid (SA) ethylene signaling might coordinate defense responses (Fountain et al. 2015).

Sorghum grain molds reduce the yield quantitatively as well as qualitatively. Protein that could effectively reduce the growth of the fungi-causing grain molds in vitro were identified in the sorghum seeds. The ability of antifungal proteins (AFPs) present during grain development to restrict the development of fungal pathogens was assessed. Sureño (resistant) RT x 430 (susceptible) and their F1 hybrids were planted at two moisture levels. Caryopsis were collected from each genotype at 7 days interval after anthesis (daa) during development and maturity of the grain. Levels of four AFPs, namely sormatin, chitinases, β -1,3-glucanases, and ribosomal-inhibiting proteins (RIPs), were determined using immunoblotting technique. During grain development (7–35 daa), Sureño and the F1 hybrid showed higher levels of sormatin and chitinase than RT x 430. The levels of RIPs in Sureño and in the F1 hybrid were higher than those of RT x 430 after the grain reached physiological maturity. The results indicated that AFPs might be associated with grain mold resistance, since Sureño and the F1 hybrid induced and/or retained higher AFPs levels under grain mold infection pressure than RT x 430 (Rodriguez-Herrera et al. 2006).

The interaction between an avirulent isolate of the rice blast pathogen *Magnaporthe grisea* and the resistant rice plant was investigated. Several AVR genes were identified in *M. grisea* through classical genetic experiments. The AVR genes from *M. grisea* designated Avirulence Conferring Enzyme 1 (ACE1) was identified in a cross between Guy11, hermaphrodite isolate from South America, and ML25, a male isolate from Africa (Silué et al. 1992; Notteghem et al. 1994). The rice resistance gene *Pi33* was used to recognize the *M. grisea* isolates carrying *ACE1* gene (Berruyer et al. 2003). *ACE1* encoded a putative polyketide synthase (PKS) and a nonribosomal peptide synthase (NRPS). The expression of *ACE1* during infection was examined by fusing the green fluorescent protein (GFP) reporter gene to *ACE1* promoter and terminator sequences. GFP fluorescence was first observed in mature appressoria at 15 h after inoculation (hai) of barley or rice leaves and strongest fluorescence was detected during penetration of the leaf surface at c. 24 hai. GFP fluorescence disappeared rapidly when the secondary infection hyphae began to

spread within the leaf tissues. *ACE1* was strongly expressed during penetration of leaves, the stage at which plant defense reactions were triggered. The fungal transformants with the vector pACE1:GFP, expressing strong GFP fluorescence in appressoria, were avirulent towards rice cultivars Bala and C101Lac carrying *Pi33* but were fully virulent towards susceptible cultivar Martelli. The results indicated that the ACE-GFP fusion protein was functional, since it confirmed avirulence when introduced in a virulent strain of *M. grisea*. The fungal signal recognized by the resistant rice plant seemed to be the secondary metabolite whose synthesis is governed by *Ace1* (Böhnert et al. 2004).

Expression of defense-related genes in plants susceptible and resistant to rice blast pathogen *Magnaporthe grisea* during pathogenesis was investigated. The defense-related genes were induced within 1 h after inoculation with *M. grisea* in rice plants on which the conidia had just germinated on the leaves. Treatment of rice cells with water soluble and heat-sensitive molecule(s) released from the conidia induced rapid defense gene expression but not cytoplasmic pH decrease, which was reported to be induced by elicitors originating from the pathogen mycelium. The results suggested that, prior to penetration into plant cells, a rapid defensive response to the presence of blast pathogen was triggered by conidia-originating elicitor(s) in both resistant and susceptible rice genotypes (Kuroda et al. 2006).

The defense responses of susceptible and resistant rice cultivars against two fungal pathogens of agricultural importance, *Magnaporthe grisea* and *Cochliobolus miyabeanus* (anamorph *Helminthosporium oryzae*), incitant of rice brown spot disease, were compared. Early infection processes of these pathogens share common characteristics such as conidial attachment to host surface, appressorium formation, and direct penetration of host cuticle, using the infection hyphae differentiated from the appressorium. In cultivar Tetep, resistant to both pathogens, inoculation with them rapidly triggered the hypersensitive reaction (HR), resulting in macroscopic cell death. In rice cultivar Nakaong, susceptible to both pathogens, *M. grisea* did not induce HR whereas *C. miyabeanus* caused rapid cell death similar to that associated with HR and the reaction observed in cultivar Tetep. However, the necrotic response failed to block fungal ramification in cultivar Nakdong. Treatment with conidial germination fluid (CGF) from *C. miyabeanus* induced rapid cell death in both cultivars, suggesting the presence of phytotoxins in CGF. Pretreatment of cultivar Nakdong with CGF significantly increased resistance to *M. grisea*, while the same treatment did not enhance resistance to *C. miyabeanus*. Spray application of CGF from *C. miyabeanus* strain HIH-1 caused HR-like lesions on leaves of both cultivars Nakdong and Tetep within 12 h of application only at a concentration of CGF of ≥ 100 ppm. CGF treatment significantly increased the level of PR-1 transcripts in cultivars Tetep and Nakdong within 1 day of treatment. No expanding invasive mycelial growth of *M. grisea* (strain KJ401) was observed in the CGF-treated leaves. CGF treatment modified the expression patterns of four marker genes in cultivar Nakdong infected with KJ401 strain as in plants treated with benzothiadiazole (BTH), a known inducer of resistance against several pathogens. The modified expression patterns resembled those in cultivar Tetep infected with KJ401. Transcripts from all four genes in CGF-treated cultivar Nakdong peaked at 1 day postinoculation, whereas the transcripts of these genes in untreated Nakdong noticeably increased only at 2–3 dpi. The results indicated that distinctly different mechanisms of resistance in rice against *M. grisea* and *C. miyabeanus* might operate. The HR-associated host cell death was effective against *M. grisea*, but not against *C. miyabeanus* which produced phytotoxin during pathogenesis as a major virulence factor (Ahn et al. 2005).

Mechanisms underlying resistance in rice to *Ustilaginoidea virens*, incitant of false smut disease, were investigated. The RNA sequence analysis was performed to study the transcriptional modulation varieties resistant and susceptible rice to *U. virens*. Differentially expressed genes (DEGs) in resistant IR28 and susceptible HXZ cultivars were determined to be 1405 and 1066, respectively. Gene ontology (GO) enrichment analysis revealed a set of GO to be differentially enriched in the two rice varieties. Functional analysis of DEGs showed that a large number of genes encoding secondary metabolites flavin-containing monooxygenases and peroxidases were differentially regulated. Classification analysis of DEGs showed that brassinosteroids may have more important roles than salicylic acid (SA) and ethylene in response to *U. virens* infection. Recognition of patterns, cellular metabolic changes, and hormone signaling constitute a comprehensive network to manipulate the responses of host rice plant to *U. virens* infection (Yang et al. 2014).

Active penetration of a plant cell by producing appressoria and penetration pegs is the characteristic feature observed during the infection process of *Colletotrichum* spp. Deposition of a melanin layer in the appressoria is essential for the development of high internal turgor pressure within the appressorium. Three nonpathogenic strains of *C. lindemuthianum*, causal agent of bean anthracnose disease, with defects in each of the appressorial development stages, were generated. The strain H1 was blocked at the differentiation stage (with no appressorium production), H191 was blocked at appressorium maturation stage (appressoria defective in pigmentation), and H290 was impaired in the appressorium function (failure to penetrate the cell wall). Inoculation of bean with wild-type strain UPS9 and mutant strain H290 elicited strong accumulation of PAL3, CHS, and PvPR2 transcripts. The early induction of these genes might be linked to the perception by the plant of fungal conidia upon inoculation and/or conidial adhesion and growth of germ tubes on leaves. The mutant strains and the wild-type strain could be discriminated based on defense responses induced by them in bean plants. Appressorium maturation (but not its function) attained in the wild-type UPS69 and H290 strains was both necessary and sufficient for triggering most defense responses such as early plant defense genes and accumulation of major PR-proteins. However, appressorium function (entry into the first host cell) was required for avirulence-mediated recognition of the pathogen by the bean plant (Veneault-Fourrey et al. 2005).

Transcription of specific genes is activated early in the plant defense response against pathogens. The upregulation of genes in the early resistance reaction of bean (*Phaseolus vulgaris*) against *Colletotrichum lindemuthianum* was investigated. The effect of the light-dark transition, ultraviolet (A-Po) light, and temperature stress on these genes was also assessed. A calcium-binding protein and previously identified SUMO genes were overexpressed in the plant reaction to pathogen under environmental stress. These genes appeared to act as integrative signals and convergence points in early plant responses to pathogens and several forms of environmental stress. The calcium-binding protein gene was present in both plant and fungal genomes (Alvarado-Gutiérrez et al. 2008). The race-specific and ulvan-induced defense responses to *C. lindemuthianum* race T3 in resistant and susceptible bean cultivars were monitored and compared. When sprayed under greenhouse conditions, ulvan (a water-soluble algal polysaccharide) reduced anthracnose disease severity locally and systemically in susceptible plants by 60% and 40%, respectively. Neither race-specific resistance nor ulvan treatment affected conidial germination or appressorial formation of *C. lindemuthianum* on leaves. Race 73-specific resistance was associated with a rapid recognition of the

pathogen, expressed by a more frequent hypersensitive response (HR) in bean epidermal cells. Ulvan increased peroxidase activity in resistant but not in susceptible plants, and increased glucanase activity in both resistant and susceptible plants inoculated with the pathogen. The results indicated that ulvan application might protect bean plants against anthracnose without affecting HR (De Freitas and Stadnik 2012).

Verticillium wilt disease of potato is caused by *Verticillium dahliae* which infects a large number of crops worldwide. A differential potato-*V. dahliae* pathosystem consisting of two potato cultivars, susceptible (S) and moderately resistant (MR) *V. dahliae* isolates (weakly (WA) and highly aggressive (HA)), was used to evaluate the expression of five defense-related genes PAL-1, PAL-2, PR-1, PR-2, and PR-5 which are generally associated with the salicylic acid (SA) defense-signaling pathway. Expression levels of these genes were assessed in potato roots and leaves at 0, 4, and 21 h postinoculation (hpi) and 3, 7, and 14 days postinoculation (dpi). In the roots, the expression of PAL-1, PR-1, and PR-2 in the MR cultivar was higher than in S cultivar in response to inoculation with either one of the tested *V. dahliae* isolates. PAL2 gene expression increased gradually starting at 21 hpi in the MR cultivar, as opposed to the susceptible cultivar. Higher expression of PR-1 was detected at 7 dpi in roots. In the leaves, both *PAL1* and *PAL2* genes showed higher expression in the MR cultivar relative to the S cultivar. Combined data analyses revealed the differential transcriptional levels in response to infection by *V. dahliae*. The results indicated that SA pathway was also involved in potato defense against *V. dahliae* and added to the data gathered to elucidate the signaling mechanisms in potato-*V. dahliae* pathosystem (Derksen et al. 2013).

Fusarium oxysporum f.sp. *cubense* tropical race 4 (TR4) of Panama (*Fusarium*) wilt pathogen has a major negative impact on banana production in all countries. In order to understand the molecular interactions underlying banana defense responses, a proteomic approach was adopted. The responses of a susceptible cultivar Williams and a resistant cultivar GCTCV-119 to inoculation with race TR4 were investigated using molecular techniques. Sixteen protein spots were subjected to MALI-TOF-MS procedure. All of the proteins identified were related to metabolism, energy, immunity and defense, and unknown functions. In the resistant variety, eight spots were in higher concentrations and six spots were less concentrated compared to control. Of these, a total of five protein spots showed significant changes at at least two time points. In the susceptible variety, five spots were more abundant and three spots were at a lower concentration compared to the control. Of these, six spots changed significantly after challenge inoculation with TR4 strain. Chitinase and β -1,3-glucanase and superoxide dismutase were estimated quantitatively by employing qRT-PCR assay to determine whether the observed proteome changes were associated with changes in the mRNA levels or were due to posttranscriptional regulation. β -1,3-glucanase and chitinase were present only in pathogen-challenged plants of the resistant variety, compared with control plants at 6, 12, 24, 48, and 72h postinoculation, but β -1,3-glucanase and chitinase were expressed both in resistant and susceptible varieties in the roots of TR4-infected banana plants (Lu et al. 2013).

The defense responses of pepper (*Capsicum annuum*) infected with incompatible and compatible strains of *Phytophthora capsici*, causative agent of Phytophthora blight disease, were studied. The activities of β -1,3-glucanase and peroxidase (involved in defense mechanisms) increased substantially in the incompatible interaction. The expression patterns of four defense-related genes, *CABPR1*, *CABGLU*, *CAP01*, and *CaRGA1* in the leaves and roots of pepper inoculated with different strains of *P. capsici*

were determined. All gene expression levels were higher in the leaves than in the roots. Markedly different expression patterns were observed between incompatible and compatible host–pathogen interactions. In the incompatible interactions, the expression levels of three genes showed an increase by a factor of 13.2–20.5, but *CaRGA1* gene expression level was to a lesser degree (by a factor of 6). In contrast, the expression levels of the four defense-related genes in compatible interactions increased by the lower factor of 2.0–11.2 compared to the controls. The expression levels of the four genes were much lower in the roots than in the leaves. The highest levels of mRNA were those of *CABPR1* gene, which increased by a factor of 5.1 at 24 h in the incompatible and by 3.2 at 48 h in the compatible interactions. The results revealed the involvement of defense-related genes in the defense responses of pepper to *P. capsici* infection (Wang et al. 2013).

The role of key biochemical defense mechanisms operating in seedlings of winter oilseed rape cultivars differing in tolerance to infection with *Leptosphaeria maculans*, incitant of blackleg disease, was investigated. In the resistant cultivar, a significant increase in the activity of chitinase and β -1,3-glucanase was observed and this could be considered an indication of a systemic defense reaction. Furthermore, the resistant cultivar had a more efficient antioxidant system, as revealed by the higher activity of specific SOD isoforms and the higher level of low molecular antioxidants. Only the resistant cultivar had three Fe-SOD sub-isoforms. A correlation between the content of cell-wall-bound phenolics and hydrogen peroxide in the resistant cultivar might indicate that an important method of H_2O_2 neutralization was its participation in the process of phenolics incorporation into the cell wall. Higher concentrations of cell-wall-bound phenolics significantly enhanced the cell-wall integrity which became a more effective firewall against *L. maculans*. In addition, high utilization of soluble sugars during synthesis of the phenolic compounds in the pathogenesis was demonstrated in the resistant cultivar. The key factors contributing to a greater tolerance to *L. maculans* were shown to be an effective antioxidant system and higher activity of PR proteins such as chitinase and β -1,3-glucanase (Hura et al. 2014).

To understand disease resistance mechanisms in plants, the identification of defense response genes in the plants with different levels of resistance/susceptibility is considered to be one of the most essential steps. A suppression subtractive hybridization (SSH) library was constructed to identify the genes involved in response to infection by *Fusarium oxysporum* f.sp. *cubense*. The cDNAs from tolerant genotype *Musa acuminata* spp. *burmannicoides* “Calcutta-4” infected by wilt pathogen were used as a tester and cDNAs from uninfected “Calcutta-4” as a driver population. After hybridization and cloning, the EST library of 83 nonredundant clones was assigned to different functional categories. The expression pattern of selected eight defense-related genes, namely peroxidase, glutaredoxin, polyphenol oxidase, glutamate synthase, S-adenosyl methionine synthetase, 14-3-3, heat shock protein, and mannose-binding lectin, were analyzed through real-time PCR in contrasting genotypes. The expression of these genes during initial progression of disease was higher in tolerant “Calcutta-4” genotype, compared with the susceptible “Kadali” genotype (Swarupa et al. 2013).

12.2.1.4 Molecular Basis of Resistance to Bacterial Diseases

During pathogenesis, the plant bacterial pathogens have to successfully breach the protection offered by exterior surfaces of plants and preformed antimicrobial compounds, followed by cell walls forming an effective second barrier to invaders that are able to

gain entry into interior spaces. In addition, the pathogens have to overcome the formidable plant immunity responses. Plant immunity comprises two components operating at different time scales. The base defense system operates early in the interaction between the host and pathogen, whereas the resistant *R* gene-mediated defense operates on the time scale of hours. The pathogen-associated molecular patterns (PAMPs) that mediate early basal responses include lipopolysaccharide, peptidoglycans, bacterial flagellin, and yeast mannans (Nurenberger et al. 2004; Pemberton et al. 2005). The innate response may also be induced by the bacterial elongation factor EFTu (Kunze et al. 2004). The receptors located in the plasma membrane recognize PAMPs, activating a phosphorylation cascade upon binding, leading to the induction of early basal resistance. This basal resistance may be responsible for preventing colonization of nonpathogenic bacteria (Gómez-Gómez and Boller 2002).

The well-characterized example of plant host sensing and responding to PAMP at the cell surface is exemplified by the rice *Xa21* receptor kinase. Recognition of bacterial strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) expressing AvrXa21 activity was due to *Xa21* receptor kinase. The rice plants lacking *Xa21* gene were susceptible to *Xoo* (Gómez-Gómez and Boller 2002). Rice has been considered as a model cereal crop to understand the functioning of the defense machinery. A large-scale analysis of rice gene expression in response to bacterial leaf blight (BLB) disease incited by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and rice blast caused by *Magnaporthe grisea* was performed during the early stages of compatible and incompatible interactions. Using a gene chip containing 10,254 rice cDNAs representing 9240 unique genes, 794 and 612 genes differentially expressed in the incompatible and compatible interactions, respectively, were detected with 274 genes coregulated during both interactions. In the rice-*Xoo* pathosystem, 454 and 498 differentially expressed genes were identified in the incompatible and compatible interactions, respectively, including 237 coregulated genes in both interactions. By clustering differentially regulated genes from all these interactions, 29 coregulated genes from all these interactions were identified in all four interactions and 86 and 74 coregulated genes in the two incompatible and two compatible interactions, respectively. These differentially expressed genes could be classified into three categories, including *M. grisea*- and *Xoo*-regulated, *M. grisea*-specific, and *Xoo*-specific genes. The expression patterns of representative defense-related genes were further confirmed by RT-PCR assay. The results of microarray analysis indicated the existence of distinctive as well as shared defense pathways between the rice-*M. grisea* and rice-*Xoo* interactions (Li et al. 2006).

Lipopolysaccharides (LPS) covering the cell surface of Gram-negative bacterial species represent typical pathogen-associated molecular patterns (PAMP) capable of inducing defense-related responses including suppression of HR, the expression of defense genes, and systemic resistance in plants. Various LPS molecules from *Pseudomonas syringae* pv. *syringae* (*Pss*), *Erwinia chrysanthemi*, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), and *Ralstonia solanacearum* and nonpathogens were shown to induce defense responses such as ROS generation and defense gene expression in rice cells. Furthermore, global analysis of gene expression induced by LPS and chitin oligosaccharide (PAMPs) also showed a correlation between the gene responses induced by them. It is possible that there could be a convergence of signaling cascades downstream of their corresponding receptors. The defense responses induced by LPS in the rice cells were demonstrated to be associated with programmed cell death (PCD) (Desaki et al. 2006).

Infection before the pathogen becomes established may be restricted by the PAMP-triggered immunity, as indicated by the evidence obtained from investigations on PAMP flagellin. The connection between inhibition of pathogen development and the recognition of the PAMP flagellin by the receptor FLS2 was demonstrated by Zipfel et al. (2004). Flagellin is the major protein of flagella and also a well-characterized PAMP which is recognized by the Leu-rich repeat receptor kinase FLS2 in the model plant *Arabidopsis*. FLS2 is located in the plasma membrane and is considered to be involved in the early bacteria–plant interaction by recognizing and binding flagellin (Dangl and McDowell 2006). Treatment of plants with flg22, a peptide representing the elicitor-active epitope of flagellin, induced the expression of several defense-related genes and triggered resistance to phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) in wild-type plants, but not in plants carrying mutations in the flagellin receptor gene *FLS2*. This induced resistance seemed to be independent of salicylic acid (SA) and jasmonic acid (JA). Wild-type and *fls2* mutant expressed enhanced resistance when treated with crude bacterial extracts even devoid of elicitor-active flagellin, indicating the existence of functional perception systems of PAMPs other than flagellin. The results indicated that flagellin perception could restrict bacterial invasion, possibly at an early step, thus contributing to disease resistance in plants (Zipfel et al. 2004).

Phospholipid signaling has been suggested to have an important role in plants, including responses to biotic and abiotic stresses. In rice plants challenged by an avirulent strain of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the incitant of bacterial leaf blight (BLB) disease, expression of phospholipase C (PLC) and phospholipase D (PLD) genes were induced. PLD was localized in the plasma membrane, especially at the part adjacent to *Xoo* in incompatible interaction (Young et al. 1996). The effects of N-acetyl chito-oligosaccharide elicitor in rice cells were assessed. Biphasic generation of reactive oxygen species (ROS) induced by the elicitor in rice cells was associated with activation of PLC and PLD. The synthetic diacetylglycerol (DG) and phosphatidic acid (PA) also were able to induce the expression of elicitor-responsive genes in the absence of elicitor. They themselves induced the biosynthesis of phytoalexins. However, they significantly increased the elicitor-induced phytoalexin accumulation. The results revealed the active involvement of phospholipid signaling in eliciting defense responses in treated plants (Yamaguchi et al. 2005).

The AvrXa21 activity of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was governed by four genes, namely *raxA*, *raxB*, *raxC*, and *raxST*, which encoded components of type I secretion system and sulfation system (da Silva et al. 2004). Two additional genes encoding a two-component regulatory system that were also required for AvrXa21 activity (*raxR* and *raxH*) were also identified (Burdman et al. 2004). Sequence analysis of three naturally occurring *Xoo* strains no longer recognized by *Xa21* showed alterations in the *raxST* and *rax* genes. The results indicated that bacterial type I secretion was essential for *Xa21*-mediated recognition and immunity. Further, type I secretion and modification of pathogen-associated molecules might play a significant role in triggering the immunity response in rice. Characterization of the *rax* genes indicated that type I secretion (*raxA*, *raxB*, and *raxC*) and sulfation (*raxST*, *raxP*, and *raxQ*) were found to be essential for *Xa21*-mediated recognition of *Xoo* (Shen et al. 2002; da Silva et al. 2004). The rice *NPR1* homologous genes were isolated and transgenic rice over-expressing these genes were generated. The rice homologous gene *NH1* was shown to be the rice *NPR1* ortholog (also termed as *OsNPR1*). Overexpression of *OsNPR1* conferred high levels of resistance to rice bacterial leaf blight (BLB) caused by *Xoo*.

In contrast, *OsNPR1* knockout/knockdown plants were highly susceptible. The wild-type *OsNPR1* protein (in GFP fusion) was located in the cytoplasm and was able to move into the nucleus after redox change. Different subcellular localization of *OsNPR1* antagonistically regulated salicylic acid (SA-) and jasmonic acid (JA-) responsive genes, but not SA and JA levels. The results suggested that SAR-like disease resistance might be activated against rice BLB disease through SA signaling which might be less dependent on the elevation of SA levels in rice cells. This investigation indicated the possibility of engineering rice with broad spectrum resistance to *Xoo* using site-mutated *OsNPR1* gene (Yuan et al. 2007).

Expression of pathogenesis-related (PR-) proteins in the interactions between rice plants with bacterial blight resistance gene *Xa21* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was studied. Three PR genes – *OsPR1a*, *OsPR1b*, and *OsPR10a* – were cloned and fusion proteins were expressed in *Escherichia coli* to generate polyclonal antibodies (PABs). The proteins isolated at 0, 12, 24, 36, 48, 60, 72, and 144 h postinoculation (hpi) from rice leaves inoculated with incompatible *Xoo* strains were analyzed using Western blotting. All three PR proteins were significantly induced in the late stages after inoculation. Expression of PR proteins among four different modalities, namely incompatible interactions (R), compatible interaction in the absence of R gene (SR-), compatible interactions in the absence of avirulence factor in *Xoo* (SAvr-), and mock-inoculated control treatments (M), were compared. Results showed that three PR proteins were enhanced in the R, SR- and Avr- interactions more than in the control treatments. Induction of PR proteins reached the peak in the R interaction, suggesting the involvement of PR proteins in the defense mechanism in the interactions between rice plants and BLB pathogen (Wu et al. 2011).

The rice near-isogenic line (NIL) CBB23, derived from a cross between a wild rice *Oryza rufipogon* accession (RBB 16) and a susceptible *indica* rice variety (Jingang 30) was highly resistant to all field strains of *Xoo* tested. Although the bacterial leaf blight (BLB) resistance of CBB23 has been widely used in rice breeding programs, the mechanisms of its extremely broad spectrum resistance remained unclear. Molecular cloning of an avirulence gene *avrXa23* from *Xoo* strains PX099A was performed to understand the resistance mechanism against *Xoo* in rice. *AvrXa23*, a novel transcription activator-like effector, specifically triggered the broad-spectrum BLB resistance in CBB23. The presence of *avrXa23* in all *Xoo* strains tested may indicate the basis for the broad-spectrum resistance of CBB23 to *Xoo* strains. The results may provide leads, facilitating the molecular cloning of the corresponding resistance (*R*) gene in the host, leading to a better understanding of the molecular mechanism for broad-spectrum disease resistance in plants (Wang et al. 2014).

Plant bacterial pathogens produce several kinds of type III effectors which are designated avirulence (Avr) factors involved in elicitation of resistance to the pathogen species/strains concerned. In the presence of disease resistance *R* genes in specific plant genotypes, Avr factors trigger potent gene-for-gene resistance and HR-localized programmed cell death (PCD) (Dangl and Jones 2001). The functions of Avr effectors may be related to either triggering resistance or enhancing pathogen virulence (Shan et al. 2000). The *avrPto* and *avrPtoB* were the only avirulence genes in *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (causal agent of tomato bacterial speck disease) that elicited Pto-mediated defense responses in tomato. The *avrPtoB* deletion mutant of *Pst* DC3000 retained PtoB-specific avirulence on tomato. The double mutant DC3000 Delta *avrPtoDelta avrPtoB* caused less severe disease symptoms than single mutants and grew

slower than single mutants on susceptible leaves (Lin and Martin 2005). *P. syringae* pv. *tomato* (*Pst*) has unrelated effector proteins AvrPto and AvrPtoB that can trigger disease resistance in tomato cultivars containing the *Pto* gene. The Pto, a protein kinase, interacts directly with each effector through overlapping surface areas (Pedley and Martin 2002; Wu et al. 2004). In *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), the AvrPtoB type III effector elicited immunity-associated programmed cell death (PCD) when expressed in tomato plants carrying the Pto resistance protein. In contrast, in the absence of Pto the same AvrPtoB functioned as suppressor of PCD and immunity in tomato. The PCD eliciting and cell death suppressor (CDS) activities of AvrPtoB were mapped to distinct regions of the protein. The deletion of as few as 44 amino acids from the C-terminus resulted in the loss of CDS activity (Abramovitch and Martin 2005).

In response to pathogenic infection or abiotic elicitors, a group of low-molecular-weight compounds known as phytoalexins accumulate in treated plants (Kuč 1995). In cotton, cadinene-type sesquiterpenes including cadalene derivatives (7-hydroxylated cadinanes) are produced mostly as phytoalexins in response to stress (Bell 1986). Cotton leaves infected by incompatible races of *Xanthomonas campestris* pv. *malvacearum*, causal agent of bacterial blight, induced the cadalene derivatives as the major phytoalexins as part of HR which formed an important component of resistance to bacterial blight disease (Pierce et al. 1996). The enzyme (+)- δ -cadinene synthase (CDNS) catalyzes the first step in the biosynthesis of cadinene-type sesquiterpenes, such as gossypol, which provide constitutive and inducible protection against diseases. Infection by *X. campestris* pv. *malvacearum* induced CDNS expression in cotton. Constructs for the constitutive or seed-specific antisense suppression of *cdn-1-C4* were introduced into cotton by *Agrobacterium*-mediated transformation. The induction of CDNS mRNA and protein in response to bacterial blight infection of cotyledons was completely blocked in the constitutive antisense plants. The results suggested that *cdn1-C4* expression might be specifically induced in response to bacterial blight infection of cotton plants (Townsend et al. 2000). The gene-for-gene interaction was indicated between cotton (*Gossypium hirsutum*) cultivars and races of *Xanthomonas campestris* pv. *malvacearum* (*Xcm*). The interaction between cotton cultivar Reba B50 and race 18 of *Xcm* resulted in hypersensitive response (HR). However, this cultivar was susceptible to race 20, leading to the ultimate death of infected plants. The HR was correlated with the activity of 9S-lipoxygenase (LOX) responsible for lipid peroxidation. A LOX gene (*GhLOX1*) whose activity was involved in cell death during HR was characterized. The role of LOX in the development of resistance to bacterial blight disease was investigated using different cotton cultivars and *Xcm* race combinations. Various combinations of *R-avr* genes were selected to determine whether *GhLOX1* gene transcription and LOX activity could be correlated with the resistance phenotype. Cotton genotypes with the *B2B3*, *B2b6*, or *B12* genes or cotton near-isogenic lines containing single *B* genes (*B2*, *B6*) were infected by races 1, 18, or 10 of *Xcm*. Cotton phenotypes were analyzed for all interactions tested. For incompatible interactions, the LOX response appeared to be concomitantly triggered during cotton resistance, and this reaction might be a key event of hypersensitive cell death (Sayegh-Alhamdia et al. 2008).

Different species of *Erwinia*, namely *E. carotovora* subsp. *atroseptica* (*Eca*), *E. c.* subsp. *carotovora* (*Ecc*), and *E. chrysanthemi* (*Ech*), are involved in potato tuber soft rot disease. Potato-tuber slices and small tubers of 12 potato cultivars were inoculated with isolates of *Eca*, *Ecc*, and *Ech*. Cultivar resistance was expressed as the mean diameter of the rotted tissue area. Grouping of cultivars into classes of relatively high, intermediate, or low

resistance was different after incubation in air in comparison to incubation under anaerobic conditions, and depended on the bacteria (subspecies) used for inoculation. Grouping of the cultivars was not significantly affected by the isolates of the same subspecies of the pathogen. The results of experiments with either field-grown seed tubers or glasshouse-grown small tubers from 2 years were reproducible, except when tuber tissue was inoculated with *Ech* and incubated aerobically. In the latter case, the cultivar only showed relatively small differences for resistance. Correlation coefficients between slice and small tuber screenings were 0.71 and 0.64 for *Eca* and *Ecc*, respectively. The results showed that soft rot resistance of clones to different soft rot pathogens could be determined effectively, if standardized oxygen concentration during incubation was used (Allefs et al. 1995). *Erwinia carotovora* subsp. *atroseptica* (*Eca*) (renamed *Pectobacterium atrosepticum*) causes soft rots on tubers and blackleg on stems of potato, reducing the yield levels significantly. Partial resistance in some cultivars was recognized. The mechanistic basis of this type of resistance was investigated using a proteomic approach to identify pathways specifically activated during the interaction between potato tubers and *Eca*. Protein profiles on silver-stained gels in the 5–8 pH range were obtained from healthy and infected tubers from two cultivars differing for resistance level, analyzed by a 2DE and nano-LC-MS/MS procedure. Thirteen proteins were differentially upregulated in the partially resistant cultivar Keerpondy. In contrast, no significant difference in protein profiles of inoculated and control tuber could be observed in the susceptible cultivar Bintje. Mass spectrometry and database search showed that these proteins were involved in energetic metabolism (glyceraldehydes-3-phosphate dehydrogenase, 2-phosphoglycerate dehydratase or enolase, fructose biphosphate, aldolase, and ATPase α -subunit), cytoskeleton structure (actin), protein catabolism (cysteine protease inhibitor), and patatins or patatin precursors. A proteomic approach appeared to be efficient, providing an insight into the mechanisms and pathways leading to resistance in potatoes against *E. c. atroseptica* (Barzic and Com 2012).

12.2.1.5 Molecular Basis of Resistance to Virus Diseases

Resistance to virus diseases may be expressed at different stages of virus replication and movement to other plant organs, restricting the spread of the virus followed by development of visible symptoms, when infection becomes systemic. Cellular level resistance may be characterized as a state where virus replication is not likely to occur or occurs at an undetectable level in inoculated cells. This type of resistance was observed in cowpea cultivar Arlington challenged with *Cowpea mosaic virus* (CpMV). The involvement of a protease inhibitor in preventing CpMV polyprotein processing was suggested for the inhibition of virus replication (Ponz et al. 1988a). Another type of mechanism occurring at cellular level involves an active resistance to virus infection that limits virus replication rapidly before cell-to-cell movement is initiated. No symptoms or extremely limited necrosis may be observable in such plants. Resistance offered by *Tm-1* gene against *Tobacco mosaic virus* (TMV) in tomato (Watanabe et al. 1987), by *Nx* and *Rx* genes against *Potato virus X* (PVX), and *Ry* gene against *Potato virus Y* (PVY) in potato (Solomon-Blackburn and Barker 2001) and *Rsv1* gene against *Soybean mosaic virus* in soybean (Hajimorad and Hill 2001), has been reported. The host response of this type of extreme resistance (ER) in potato was revealed by the absence of any visible symptoms when challenged with PVY. By applying RNA hybridization and ELISA, accumulation of PVY could not be detected in the inoculated leaves of potato carrying *Ry* gene. In addition, the protoplasts isolated from these plants did not support replication of PVY.

As no HR could be seen, it was hypothesized that inhibitors of virus accumulation might be encoded by the resistance genes (Solomon-Blackburn and Barker 2001).

Pepper mild mottle virus (PMMoV) was unable to replicate in tomato plants because *tm-1* protein inhibited RNA replication. Resistance of tomato to PMMoV was durable both in the field and in vitro conditions. Several PMMoV mutants were constructed and their abilities to replicate in tomato protoplasts and plants were determined. The mutants PMMoV-899R, F976Y, PMMoV-899R, F976Y, and D1098N were able to replicate in tomato protoplasts, but only the latter mutant could multiply in tomato plants. The D1098N mutation of the replication proteins weakened the inhibitory effect of the *tm-1* protein and enhanced the replication efficiency of PMMoV-988R, F9764, and D1098N. The infectivity of the viruses decreased in the order wild-type PMMoV > PMMoV-899R, F976Y > PMMoV-899R, F976Y, and D1098N in original host plants pepper and tobacco plants. In contrary, the single mutation in D1098N abolished PMMoV replication in tobacco protoplasts. The results suggested that the deleterious side-effects of mutations in replication proteins might prevent the emergence of PMMoV mutants capable of overcoming *tm-1*-mediated resistance (Mizumoto et al. 2014).

Resistance to the movement of viruses within and between cells may occur, resulting in the failure of development of disease symptoms. In some pathosystems, the virus may be able to establish infection in or on a few cells, but the virus fails to move to other cells because of a lack of interactions between plant and viral factors required for cell-to-cell movement or due to active host defense responses that may rapidly restrict virus spread. In the case of DNA viruses, crossing the nuclear membrane is a formidable barrier for virus movement into and out of the cell nucleus (Whittaker and Helenius 1998). The viral protein required for import of virus into the nucleus and export of progeny genomes back to the cytoplasm for translation and virion assembly have been identified for some viruses. Systemic infection represents the susceptibility response of the host plant. The rate of systemic movement of virus may markedly reduce in some pathosystems as an expression of resistance. Resistance to *Potato leafroll virus* (PLRV) was associated with an exclusion of virus from external phloem bundles (Derrick and Barker 1997), whereas disrupted movement of *Cucumber mosaic virus* (CMV) in phloem tissue and confinement of CMV in the lower portions of stems of resistant pepper plants were considered to contribute to the resistance against the virus (Dufour et al. 1989). In the case of *Potato virus A*, the vascular movement of the virus was completely blocked in potato with a homozygous recessive *ra* allele (Hämäläinen et al. 2000). The genes involved in the protection of plants against viruses are yet to be identified in most pathosystems. Plasmodesmata are elaborate and highly regulated structures with which viruses can interact for both cell-to-cell and long distance movement, establishing symplastic connectivity between the epidermal/mesophyll cells and cells with the vascular elements, including sieve elements (SE) (Santa Cruz 1999). For the virus entry into the companion cell the virus may have direct access to the sieve tube, the conducting element of the phloem through which nutrients and viruses have to move. Only a few host factors that may affect the long-distance movement of viruses have been identified. Long-distance movement of *Tobacco mosaic virus* (TMV) was impaired due to down-regulation of pectin methylesterase (PME), resulting in the blocking of virion exit from phloem (Chen and Citovsky 2003).

Avirulence genes have been recognized by their requirement for disease resistance in plants possessing corresponding *R* genes. Large numbers of genomes of a single virus may be present in a single host plant cell across many cells per host. Avirulence

determinants are conventionally identified by producing chimeric clones derived from viral genotypes with different virulence levels followed by assessment of their infectivity on suitable host plant species. Avirulence factors with respect to *R* genes have been shown to be virtually any fragment of the viral genome, conferring resistance against the target virus. The viral RNA polymerase subunits, movement of protein, and CP of different viruses may function as virulence determinants. The HC-Pro and P3 proteins of *Soybean mosaic virus* function as virulence determinants for *Rsv1* in soybean (Eggenburger and Hill 1997). Evaluation of virus resistance using molecular markers was shown to be successful in several crops. Biochemical markers associated with disease resistance may be useful for accelerating the selection of resistant plants or sources of resistance to viruses affecting perennial horticultural crops. Apricot cultivars showing resistance to *Plum pox virus* (PPV) had enhanced levels of activities of catalase, ascorbate peroxidase (APX), and dehydroascorbate reductases (DHAR) compared to susceptible cultivars, suggesting the possibility of using the activities of these enzymes as biochemical markers for selecting genotypes resistant to PPV (Hernández et al. 2006).

Investigations to determine defense responses of potato cultivar Désirée to infection by *Potato virus X* were carried out. Salicylic acid (SA) biosynthesis and expression of several defense genes, including PR-1 and glutathione-S-transferase (GST) involved in ET- and ROS-dependent signaling, were highly upregulated in upper inoculated leaves of PVX-infected potato plants compared to mock-inoculated control plants. Further, the β -phenylethylamine alkaloids such as tyramine, octopamine, and norepinephrine were found to be highly induced upon PVX infection. These alkaloids may be involved in the elicitation of plant defense responses by forming hydroxycinnamic acid amides (HCAA), considered to enhance cell-wall stability by extracellular peroxidative polymerization. Infection by PVX substantially enhanced the expression of tyramine-hydroxycinnamoyl transferase (THT) and apoplastic peroxidase (POD) in systemically infected leaves. The metabolic and transcriptional changes observed in PVX-infected potato plants might represent the systemically acquired resistance (SAR) response against subsequent pathogen infection (Niehl et al. 2006).

RNA silencing is an important mechanism involved in gene regulation in several organisms, including plants. Posttranscriptional gene silencing is an RNA silencing-based approach employed to reduce the level of expression of a (viral) gene of interest. RNA silencing may be of two types: RNA-mediated transcriptional gene silencing (TGS); and posttranscriptional gene silencing (PTGS). The PTGS mechanism may be mediated through a host-encoded RNA-dependent RNA polymerase (RdRp) and RNA helices (Dalmay et al. 2001). The PTGS in plants inactivates some aberrant or highly expressed RNAs in a sequence-specific manner in the host cell cytoplasm and it is an innate and viral defense strategy of plants (Soosaar et al. 2005). Many RNA and DNA viruses stimulate PTGS shortly after infection, because of the formation of double-stranded structures (transient replication intermediates). Plants transformed with constructs that produce RNAs capable of duplex formation induce virus immunity with almost 100% efficiency when targeted against viruses (Smith et al. 2000; Wang et al. 2000; Kalantidis et al. 2002). Regardless of the origin, the occurrence of ds-RNAs in the cytoplasm induced PTGS. The RdRp was also shown to be involved in PTGS (Dalmay et al. 2000). PTGS could generate mobile silencing signals with sequence-specific information that spread from cell to cell through plasmodesmata and systemically through vascular system to different plant organs (Mlotshwa et al. 2002; Himber et al. 2003).

The potential of ds-RNA-mediated interference in plant virus infections has been assessed to exploit this phenomenon for the management of crop virus diseases. Three viruses, *Pepper mild mosaic virus* (PMMoV), *Tobacco etch virus* (TEV), and *Alfalfa mosaic virus* (AMV), belonging to the genera *Tobamovirus*, *Potyvirus*, and *Alfalfamovirus*, respectively, were investigated. The primary principle of RNAi technology is the ability to deliver ds-RNA as potent activator of RNA silencing into an organism or cell with the aim of triggering sequence-specific degradation of homologous target RNAs. Triggering RNA-interference (RNAi) could be successfully demonstrated by mechanically inoculating the host plants with in vitro transcribed ds-RNA to leaf cells together with the target virus (Tenllado and Díaz-Ruiz 2002). Homologous ds-RNA was able to interfere with virus infection in a systemic host infection. The ability of ds-RNA to specifically interfere with virus infection appeared to require a minimum length of ds-RNA. When a mixture containing infectious viral genomic RNA transcripts was used to inoculate test plants, none of the inoculated plants developed symptoms of infection. Further, AMV-RNAs 1, 2, and 3 could not be detected in the test plants (Tenllado et al. 2004). The genome of *Grapevine virus A* (GVA) was screened for the presence of a gene potentially capable of suppressing RNA silencing. Four of the five proteins encoded by GVA genome were screened using green fluorescent protein (GFP-) based transient expression assay. The expression product of ORF5 (protein p10) had the property of suppressing silencing induced by a transiently expressed ss-sense RNA. GVA P10 was found to be a suppressor belonging to a new family. This protein reduced the levels of siRNAs markedly and the recombinant protein bound ss- and ds-forms of siRNAs and microRNAs, suggesting the existence of a potential mechanism of suppression based on RNA sequestering (Zhou et al. 2006).

Satellites associated with some viruses and viroids have been shown to have small, noncoding, and highly structured RNA genomes. As in the case of viruses, the ability of viroids and viral satellites to induce RNA silencing has been observed. Tomato plants inoculated with *Potato spindle tuber viroid* (PSTVd) were analyzed for the presence of small RNAs with sequence specificity to PSTVd. Both mild and RG1 strain of PSTVd induced the production of small RNAs in infected tomato plants, but mock-inoculated tomato plants did not have the small RNAs. The siRNAs showed sequence specificity to PSTVd, indicating the operation of PSTVd-induced RNA silencing (VdIRS). However, the variations in symptoms caused by the mild and severe strains (RG1) of PSTVd did not seem to be related to development of RNA silencing (Itaya et al. 2001). The siRNAs induced following infection of tomato by PSTVd were 22- and 23-nt, representing different domains of the viroid genome. Production of the siRNAs in PSTVd infected plants indicated that nuclear replication of PSTVd RNA was able to induce PTGS. The siRNAs were slightly more abundant at 30 days postinoculation (dpi) than at late stages. No apparent relationship was noticed between the concentrations of PSTVd-specific siRNAs and the titers of viroid strain attained in infected plants (Papaefthimiou et al. 2001). Tomato plants expressing harpin (protein coded by *hrp* genes) RNA derived from *Potato spindle tuber viroid* (PSTVd) developed symptoms similar to those of PSTVd infection. Further, if RNA silencing in tobacco was prevented using a silencing suppressor, symptoms induced by *Cucumber mosaic virus* (CMV) satellite Y were markedly reduced. The results suggested that viroids and satellites appeared to cause disease symptoms by directing RNA silencing against physiologically important host genes. Viroid and satellite RNAs exhibit remarkable resistance to RNA silencing-mediated degradation that is effective against viruses. Most viroids, including PSTVd, replicate in

the nucleus and this subcellular localization possibly protect them from RNA-induced silencing complex (RISC) believed to be functioning in the cytoplasm. Nevertheless, viroids need to traverse the cytoplasm during cell-to-cell movement, during which time they are likely to be exposed to the RISC-degradation. Probably the extensive intramolecular base pairing may protect them from the degradation by the RISC complex (Wang et al. 2004).

Early evidence that PTGS occurred during viroid replication was reported by Wassenegger et al. (1994), whereas overexpression of viroid RNAs resulted in methylation of *Potato spindle tuber viroid* (PSTVd) genes incorporated into the tobacco genome. Accumulation of short RNA fragments of 21-24-nt representing different regions of PSTVd genome occurred both in pospiviroid- and avsunviroid-infected plants (Dierman et al. 2010; Owen et al. 2012). These fragments are characteristic of PTGS or RNA silencing which provides a multilayer defense system and protects plants from invasion by RNA replicons, including viruses and viroids. RNA-dependent RNA polymerase 6 (RDR6), which catalyzes the amplification producing double-stranded precursors of secondary-silencing RNAs (siRNAs), plays a role in the restriction of PSTVd spread to floral and vegetative meristems (Di Serio et al. 2010). Evidence suggests that most of these short fragments may originate from restricted regions of the viroid molecule, including the pathogenicity and the potential binding sites to host genes (Machida et al. 2007; Wang et al. 2011).

An alternative mechanism suggested is that viroid genomic RNAs or the replication process could be the effectors of pathogenesis and may impair the normal function of the RNA silencing machinery in the host. The presence of small viroid-specific RNAs in the cytoplasm of viroid-infected plants indicates that viroids can trigger RNA silencing in a host and may also be substrates for dicer-like cleavage to produce short interfering RNAs (siRNAs) (Itaya et al. 2001). However, mature monomeric, partially double-stranded, highly structured viroid molecules can overcome this RNA interference (RNAi-) mediated machinery (Gómez and Pallás et al. 2007; Schwind et al. 2009). There may be many reasons for the failure of the RNA-induced silencing complex (RISC) to target viroid RNAs (Kovalskaya and Hammond 2014).

12.3 Improvement of Disease Resistance Through Biotechnological Approaches

The use of genetically resistant cultivars for management of crop diseases is known to be the most economical option for profitable cultivation of various crops. However, the lack of durability of resistance for longer periods has been found to be the major obstacle because of selection pressure exerted on the microbial pathogens, which may sooner or later, to adapt themselves to the newly introduced resistance genes by producing new strains. In addition, the unavailability of dependable sources of resistance to several economically important diseases has necessitated the development of alternative methods of containing the diseases caused by the microbial pathogens. Rapid developments in molecular biology and genetic engineering during the past few decades have indicated the possibilities of obtaining crop cultivars resistant to diseases that have evaded the crop protection umbrella in the near future. Plants possess natural protective systems provided by a variety of constitutive barriers already present in the plant before

infection by a microbial pathogen and this type of resistance, known as constitutive resistance, is usually observed in wild relatives of the cultivated plant species. On the other hand, protective defense system(s) may be activated in response to treatment/infection by a pathogen or abiotic agent; such resistance is designated induced or acquired resistance. Investigations of the molecular biology of disease resistance have clearly elucidated various aspects of plant–microbe interactions, resulting in the expression of compatible or incompatible interactions between crop cultivars and pathogen strains or races with varying levels of resistance/virulence. Genetic engineering procedures have been shown to be effective for isolation of disease resistance gene(s) and expressing them in the desired crops. Resistance genes from diverse sources have been incorporated into cultivars to provide effective protection against pathogens that could not be contained by other techniques. Various biotechnological approaches that can be employed to improve resistance levels and restrict the incidence and spread of diseases transmitted via seeds and propagules are discussed.

12.3.1 Tissue and Cell Culture Techniques

Tissue and cell culture procedures have been employed primarily for producing disease-free seeds and mother plants which form the nuclear stock. This aspect was discussed in Chapter 10. Another important aspect is the identification of clones from the same culture which show greater levels of tolerance/resistance to target pathogen(s).

12.3.1.1 Somaclonal Variation

Variations in several characteristics including disease resistance have been observed in plants regenerated from organ cultures, calli, and protoplasts. The variability expressed by regenerated calli was named somaclonal variation by Larkin and Scowcroft (1981). Regenerants from callus cultures resistant to *Sugarcane Fuji virus* were selected (Krishnamurthi and Thaskal 1974). Later, somaclones regenerated from potato cultivar Russet Burbank protoplasts were found to be resistant to the late blight pathogen *Phytophthora infestans* and also to the early blight disease caused by *Alternaria solani* (Shepherd 1981; Doke and Furnichi 1982). Somaclones generated from individual cells or organs may exhibit resistance to pathogens or their metabolites. Sugarcane somaclonal variants displaying resistance to smut disease caused by *Ustilago scitaminea* were investigated to identify genes that are activated and associated with resistance in sugarcane, following inoculation with *U. scitaminea*. Differentially expressed transcription-derived fragments (TDFs) that were induced following challenge inoculation of resistant and susceptible somaclones were isolated using a cDNA-AFLP technique. Of these 62 differentially regulated genes, 10 were downregulated while 52 were induced; these differentially induced TDFs were isolated and sequenced. Of these TDFs, 11 were isolated from the smut-resistant somaclones. The differential expression of 19 TDFs was confirmed by Northern blots, and the mRNA levels of genes that were homologous to 7 of those TDFs were highly induced in resistant somaclones inoculated with *U. scitaminea*; only low or no expression could be detected in the susceptible parental lines, however. Only one of the 52 differentially expressed TDFs was highly induced in *U. scitaminea* and also *Bipolaris sacchari* (another important sugarcane pathogen causing eye spot disease) interactions with sugarcane somaclones. The TDF was derived from a gene encoding a putative serine-threonine kinase and might contribute to disease resistance in sugarcane (Borrás-Hidalgo et al. 2005).

Fusarium (Panama) wilt pathogen, *Fusarium oxysporum* f.sp. *cubense* (*Foc*), exists in the form of four races (1, 2, 3, and 4). The race 4 attacked Cavendish cultivar Taiwan and had a host range different from the other three races. The Cavendish plantlets from tissue culture were screened for their resistance to *Fusarium* wilt disease by planting them in a pathogen-infested field. To increase the inoculum level, diseased tissues were ploughed into the soil at 300–1000 propagules per gram of soil. Plantlets (20,000) were grown for 2 months. Suckers from each of the 18 surviving clones were selected after 1 year. Rhizomes were shaved with a knife to remove any infected tissues, revealed by the presence of reddish or brown streaks. Symptomless suckers were planted in the disease nursery again. All of the first-generation plants from suckers of six surviving clones remained healthy after 1 year. The unaffected plants in the second and third generations were tested in a similar manner. These suckers were highly resistant to *Foc* race 4, in comparison to the parent plantlets. Large numbers of plants from six clones were tested in banana farms where they were highly resistant to the wilt disease. The resistant plants were also tested with high inoculum levels in the greenhouse. Among the variants, only one (GCTCV-2/8) possessed better horticultural parameters in addition to resistance to *Fusarium* wilt disease, and the fruits were well received by consumers in Taiwan and Japan (Hwang and Ko 2004).

Powdery scab disease of potato caused by *Spongospora subterranea* is an economically important disease occurring in several countries. Variations in the resistance of somaclones to *S. subterranea* were determined by inoculating the somaclonal variants. Most of the variants consistently showed increased resistance to powdery scab disease, both under glasshouse and field conditions, when compared to parental cultivar Russet Burbank. The best variant showed reduced powdery scab incidence by 51% and disease severity (based on tuber surface coverage) by 64%. However, no improvement was observed in the severity of root infection and root galling caused by *S. subterranea*. The results suggested that mechanisms of defense responses to the root and tuber infections might be different. Correlation analyses of disease indices among the selected variants did not show any association either between root infection and gall scores, or between root infection and tuber disease severity. However, a weak positive association was indicated between root gall score and tuber disease and a strong correlation between tuber disease incidence and severity scores. The results indicated positive correlations between the levels of resistance to powdery scab and common scab (caused by *Streptomyces scabies*) expressed and incidence and severity of diseases within variants, suggesting a common defense mechanism against both diseases (Tegg et al. 2013).

12.3.1.2 In Vitro Selection for Disease Resistance

Microbial plant pathogens produce different kinds of toxic metabolites and/or resistance-inducing compounds that may play a role in host–pathogen interactions, leading to disease development or induction of resistance depending on the nature of the compounds involved. It was considered that plant cell/organ cultures showing resistance to pathogen-derived toxic compounds might give rise to plants with resistance to infection by the same pathogen-producing toxic compounds. The defense responses of calli and seeds of two rubber cultivars showing resistance (BPM-24) and susceptible reaction (PRIM600) were determined by treating them with zoospores and elicitor purified from *Phytophthora palmivora*. Both zoospores and elicitor induced the biosynthesis of phytoalexin scopoletin (Scp) in *Hevea* calli at concentrations ranging from 5×10^5 to 4.5×10^6 zoospores mL⁻¹ and 0.5–2 µg elicitor per gram fresh weight of calli.

The rate of Scp production was fastest at the highest concentration of zoospores and elicitor tested. Decline in the Scp level at the highest zoospore/elicitor concentration was correlated to the amount of cell death. The Scp and peroxide (POD) accumulations were approximately 2–3 times higher in resistant cultivar compared to susceptible cultivar. The responses of the calli to elicitor were faster than those to zoospores. The patterns of Scp and POD activities monitored in elicitor-treated *Hevea* seeds was similar to that of *Hevea* calli after treatment with zoospores or elicitor. The results showed that callus culture could be used as a tool to determine the defense mechanisms operating in rubber plants against *P. palmivora* (Chirapongsatunkul et al. 2008).

The pathogenic strains of *Streptomyces scabiei*, the primary pathogen of potato common scab disease, produce the phytotoxin thaxtomin A, whereas nonpathogenic strains do not produce the phytotoxin. This phytotoxin could induce common scab symptoms when applied on developing tubers in the absence of the pathogen. It was suggested that resistance to thaxtomin A in potato seedlings might be correlated with decreased scab disease lesions on tubers (Hiltunen et al. 2006). The effectiveness of using thaxtomin A as a positive selection agent for somatic cell selection of potato with enhanced resistance to common scab disease was assessed. A somatic cell selection procedure was developed to use thaxtomin A as a positive selection agent and 113 potato cell clones of cultivar Iowa were recovered. From these cell colonies, 39 plants were regenerated within a single subculture period. Regenerated variants (13) were selected after artificial inoculation, based on mean tuber coverage with lesions and the proportion of disease-free tubers. The best variant had an average reduction of 85–86% in disease score over that of disease severity in particular cultivar. Two (65A and 65B) of the 13 variants produced more tubers than parent cultivars in two of three trials conducted. Although not immune to infection, these two variants did not exceed an estimated 1.3% lesion tuber surface coverage and showed less tuber surface score (85–86%) than the unselected parent cultivar (Fig. 12.3). The disease-resistant variants differed in their response to

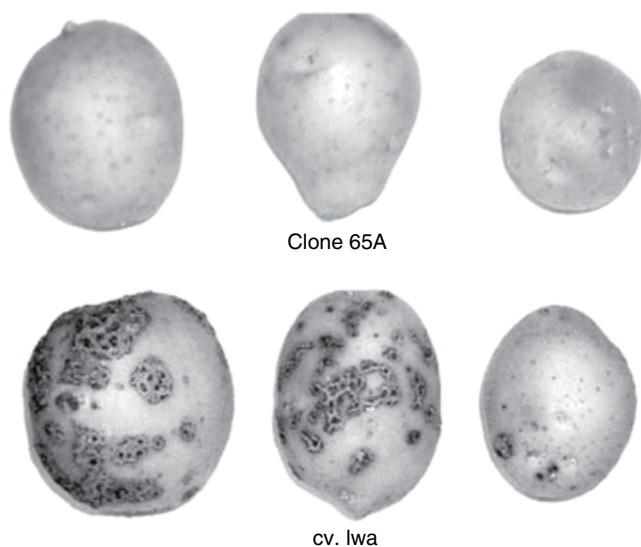


Figure 12.3 Disease severity induced by *Streptomyces scabiei* in resistant clone 65A and susceptible parent Iwa. Source: Wilson et al. (2009). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

thaxtomin A in detached leaflet and tuber slice bioassays. The results suggested that the cell selection procedure and thaxtomin A screening had limited success in selecting for toxin tolerance, and that thaxtomin A tolerance was not necessarily the critical factor for induction of disease resistance observed. Variations in the relative necrosis scores between the leaflet and tuber assays for individual variants possibly suggested differential expression of thaxtomin A tolerance in different tissues. Yield estimates from the glasshouse-grown potatoes showed that the majority of disease-resistant variants produced yields equivalent to the unselected parent cultivar, indicating the commercial viability of growing the resistant variants of potato (Wilson et al. 2009).

12.3.1.3 Induction of Mutation Using Chemicals

With the development of in vitro culturing of plant cells/organs, in vitro mutagenesis has been shown to be useful for genetic improvement in many characteristics including disease resistance/tolerance. Banana cultivars with wilt (*Fusarium*) resistance have been derived from in-vitro-induced mutants (somaclonal variants) such as cultivars Tai Chiao No.1 and Formosana (Hwang and Ko 2004). Ethyl methanesulfonate (EMS) was used for mutagenesis of banana plant and apical tissues to produce mutants (Yang and Lee 1981; Omar et al. 1989). Micro-cross-section derived from banana leaf bases were used for callus and shoot bud induction with subsequent regeneration of healthy plants (Okole and Schulz 1996). This procedure of regenerating banana plants was employed for inducing mutation in vitro using EMS and for screening for *Fusarium* wilt-resistant lines of Brazil banana (*Musa* spp.). The results indicated that the optimum EMS concentration and duration of treatment of micro-cross-sections obtained from the pseudostem of tissue-cultured plantlet were 300 mM and 60 min, respectively. One hundred generated plantlets were used for screening for *Fusarium* wilt-resistant lines by inoculating *F. oxysporum* f.sp. *cubense* race 4 (5×10^6 spores mL⁻¹). The initial disease symptom of yellowing in lower leaves of susceptible plants appeared at 2 weeks after inoculation. After 2 months, only six plants survived the preliminary field test. The early screening technique eliminated 94% of the plantlets regenerated from micro-cross-sections with mutation induction by EMS. The selected plants showed marked reduction in wilt disease incidence. The results showed that EMS-induced mutation of banana through the micro-cross-section cultural system might be useful for improvement of resistance of banana to *Fusarium* wilt disease (Chen et al. 2013).

12.3.2 Transgenic Resistance to Crop Diseases

Genetic engineering has provided numerous options for selecting the desired gene(s) from a wide range of sources, including the pathogens themselves, which could not be accessed by other methods. The disease resistance genes available in various sources, especially wild relatives of crop plant species, have been located at specific sites by the action of restriction endonucleases. The DNA fragment(s) carrying the desired gene(s) are identified and used as molecular markers. Random amplified polymorphic DNA (RAPD) technique in conjunction with PCR assay has been employed to identify resistance genes rapidly. The PCR assay has been demonstrated to be very useful to amplify the desired sequences in the DNA. Transgenic plants constitutively expressing defense-related genes from desired sources may be generated. The exogenous DNA fragment introduced artificially via genetic engineering techniques has to pass through the germ line of the plant to be transformed, following incorporation of the foreign DNA into the plant's nuclear material. Crop varieties with resistance to diseases caused by microbial

pathogens were developed within a short period by employing biotechnological methods; classical breeding procedures require several years, in addition to being hampered by obstacles posed by interspecific sterility and linkage with undesirable genes. Incorporation of genes from bacteria, fungi, viruses, or various species of plants has been accomplished by employing the Ti (tumor-inducing) plasmid containing TDNA of phytopathogenic bacterial species *Agrobacterium tumefaciens* (At). Ti DNA can be disarmed, rendering the plasmid nononcogenic but still retaining its ability to integrate with host plant genome. Depending on the nature of pathogens and their pathogenic potential (virulence), various techniques have been used for the generation of cultivars with higher levels of resistance to target pathogen(s).

12.3.2.1 Transgenic Resistance to Virus Diseases

12.3.2.1.1 Pathogen-Derived Resistance

Transgenic expression of pathogen sequences has been utilized to provide protection against respective pathogens themselves by interfering with pathogen development or multiplication. Pathogen-derived resistance (PDR) is the principal approach by which transgenic protection is offered against many viruses infecting various crop plants. Incorporation of coat protein (CP) gene of *Tobacco mosaic virus* (TMV) in tobacco was demonstrated to result in enhanced protection against TMV for the first time by Abel et al. (1986). The transgenic tobacco plants only showed resistance against intact TMV but not against viral RNA, since inoculation of transgenic plants with viral RNA resulted in the development of symptoms of TMV infection in as many plants as in nontransformed control plants (Nelson et al. 1997). As the functions encoded by different viral genes have been determined, it has been possible to transform plants with different viral genes encoding structural and nonstructural proteins with varying degrees of success.

Transgenic plants expressing the CP gene of *Potato leafroll virus* (PLRV) exhibited resistance to PLRV when inoculated by viruliferous aphids (Kawachuk et al. 1990). The CP gene of some of the plant viruses was reported to have the ability to function as an elicitor of resistance. The L^2 gene in *Capsicum* is known to confer resistance to all known tobamoviruses except *Pepper mild mottle virus* (PMMoV). A hybrid virus consisting of the CP gene of *Paprika mild mottle virus* (PsMMV), which could induce a hypersensitive reaction (HR), and the PMMoV-S sequences was able to elicit HR in *Capsicum frutescens* (L^2L^2) plants, suggesting that the CP gene had the sequences that might affect induction of HR-mediated L^2 resistance in pepper. The CP but not the RNA appeared to be the elicitor of HR in pepper (de la Cruz et al. 1997). The CPs of U2 strain of *Tobacco mosaic virus* (TMV) and *Odontoglossum ringspot virus* (ORSV) also were able to elicit HR whereas the CP gene of *Cucumber mosaic virus* (CMV) could not elicit HR in eggplant, indicating that elicitation of HR may be associated with CPs of specific tobamoviruses (Dardick and Culver 1997). The potato cultivar Igor was seriously affected by NTN isolates of *Potato virus Y* (PVY^{NTN}), causing potato necrotic ringspot disease (PNRD). This cultivar was transformed with the CP gene of PVY^{NTN} and several transgenic lines were highly resistant, showing no visible symptoms when they were graft-inoculated. The virus could not be detected by either ELISA or infectivity tests. Among the 34 transgenic lines, two showed resistance to PVY^{NTN} and PVY^O and one line was resistant to PVY^O (Racman et al. 2001). Transgenic potato plants expressing CP gene of *Potato virus X* (PVX) were found to be resistant to both intact virion and viral RNA. It was hypothesized that PVX CP might either interact with the

origin of assembly and restrict replication or interfere with translation of the replicase gene (Hemmenway et al. 1988; Spillane et al. 1997).

The T-36 strain of *Citrus tristeza virus* (CTV) expressing green fluorescent protein gene (CTV-GFP) was used to detect replication of CTV in wild-type (WT) “Hamlin” sweet orange plants, and transformed with the use of *Agrobacterium tumefaciens* (At) strain harboring binary vector with a coat protein gene from either T-36 or T-30 isolate of CTV. Soil-adapted WT and transgenic plants were challenged with CTV by grafting shoots from CTV-GFP infected plants. None of the transgenic plants appeared to be able to inhibit CTV replication, as CTV-associated GFP fluorescence was detected in all of them by fluorescent microscopy. CTV multiplication in transgenic plants was also examined by ELISA tests. GFP-labeled CTV represented a useful aid for estimating the susceptibility of citrus cultivars or transgenic lines to CTV infection. The method using CTV-GFP was found to be simpler, more economical, and less time-consuming than ELISA tests (Orbović et al. 2011).

The possible role of PTGS in the development of coat-protein-mediated resistance (CP-MR) against PVX infection was investigated. Transgenic tobacco plants expressing PVX CP were challenged with PVX under conditions in which PTGS was suppressed by low temperatures or by using PVY that encoded HC-Pro, a known suppressor of PTGS. The transgenic plants expressing PVX CP were maintained at 15 and 24°C for 10 days. The transgenic plants were resistant, producing very few small sites of infection as against production of a high number of sites of infection in nontransformed control plants. The results showed that PVX CP-MR was not temperature-dependent, indicating that resistance was not dependent on PTGS (Bazzini et al. 2006).

Plum pox virus (PPV), causing sharka disease of huge economic importance, infects all commercial stone fruit crops (*Prunus* spp.). *Agrobacterium tumefaciens* (At-) mediated transformation procedure was applied to obtain transgenic clones of *Prunus domestica* from plum hypocotyles with PPV CP gene (Scorza et al. 1994). One of the clones, C5, showed high level of resistance to PPV under glasshouse conditions (Ravelonandro et al. 1997). In another investigation, six transgenic clones (C2, C3, C4, C5, C6, and PT-6) expressing CP gene of PPV and PT-23 transformed with marker genes only and a nontransformed line were evaluated for their resistance to PPV in Poland and Spain under field conditions where disease pressure was high for 7 years. A high level of resistance to PPV was noted only in one clone C5 and none of the C5 trees showed infection by PPV under high disease pressure conditions. In contrast, all other plum trees (100%) exhibited disease symptoms and were positive for PPV by ELISA and RT-PCR assays. The C5 trees, showing distinct posttranscriptional gene silencing (PTGS), were found to be uninfected by PPV during the entire period of experimentation (7 years), representing a significant part of the productive life of plum orchards (Malinowski et al. 2006).

The posttranscriptional gene silencing (PTGS) process may be negatively impacted by some biotic and abiotic conditions, which represents potential concern for production of stable engineered virus resistance traits. The *Plum pox virus* (PPV-) *Nicotiana benthamiana* pathosystem was used to evaluate the stability of intron-harpin (ihp-) mediated virus resistance under conditions known to adversely affect PTGS. The ihp plants grown at low or high temperatures were fully resistant to multiple PPV challenges, different PPV inoculum concentrations, and even to a PPV isolate differing from the ihp construct by more than 28% at the nucleotide level. In addition, infections of ihp plants with viruses belonging to *Cucumovirus*, *Potyvirus*, or *Tombusvirus*, all known to

affect PTGS at different steps, were unable to defeat PPV resistance in transformed plants. Low temperatures did not affect the accumulation of transgenic small interfering RNAs (siRNAs), whereas a clear increase in the amount of siRNAs was observed during infections sustained by *Cucumber mosaic virus* (CMV) and *Potato virus Y* (PVY). The results indicated that stress factors tested did not appear to be important factors influencing transgenic resistance in plant species with robust virus resistance traits (Di Nicola et al. 2014).

Global climate changes may have potential influence on the distribution and profile of crop pathogens. The mechanisms of virus suppression by small RNAs (sRNAs) have been investigated. High-throughput silencing (HTS) can generate large sets of sRNA sequences which can be analyzed to recognize the abundant sRNAs that result from transcript degradation and to identify the regulatory microRNAs (miRNAs) involved in gene regulation and small inhibitory RNAs (siRNAs) that confer resistance to plant viruses. Transgenic potato plants (*Solanum tuberosum*) that were glasshouse or field grown, and that expressed the *Potato virus Y* (PVY) coat protein (CP) inverted hairpin RNA (ihRNA) from the 409S promoter with GBS S5 intron as a spacer, exhibited resistance to PVY. Southern blots of genomic DNA indicated high levels of siRNA production from the transgenic hairpin constructs. Up to 47% of the top 200 most frequent sRNA reads were attributable to the PVY CP transgenic construct in Ranger Russet line #5. This combination of transgenic and transcription strength is functional for constructing efficient resistance cassettes for crop production. Up to 95 of the top 200 sRNA sequences were 21-nt in length with as many as 19 sequences corresponding to known miRNA transcripts. Additional endogenous sRNAs with homology to resistance gene analogs may be indicative if additional or complementary resistance mechanisms are produced in concert with PVY siRNAs arising from PVY-CP RNA (McCue et al. 2012).

Grapevine fan leaf virus (GFLV) belonging to the genus *Nepovirus* enclosing the nematode-transmitted viruses contains three types of particles, namely T (top), M (middle), and B (bottom) which remain at the sequential positions in a purified virus suspension. The top (T) component contains empty virus capsids (coat protein), whereas the M and B components contain viral RNA2 and RNA1 + RNA2, respectively. RNA2 encodes viral CP and also determines vector specific transmission by the nematode *Xiphinema index*. Grapevine (*Vitis vinifera*) cultivar Russalka plants were transformed by transferring the CP gene including nontranslatable and truncated forms via At-mediated transformation. The transgenic sequences were detected by PCR in all lines. Southern blot analyses also revealed the number of inserted T-DNA copies varying from 1 to 6. Accumulation of GFLV-CP could not be detected by ELISA tests in any one of the 36 transgenic lines analyzed. Application of RT-PCR analysis revealed the presence of GFLV CP mRNA, expressed at variable levels. The levels of resistance to GFLV in grapevine transgenic lines were not assessed using the nematode vector to determine the potential value of this approach for development of GFLV-resistant grapevine cultivar (Maghuly et al. 2006).

Potyvirus form a major group of plant viruses with genome in the form of a single large ORF encoding a large primary polyprotein. The virus-encoded proteins are produced by self-proteolysis due to the action of products of three viral cistrons. The nuclear inclusion body is composed of two virus-encoded proteins, namely NIa and NIB. Transgenic peas, containing a copy of the *Pea seedborne mosaic virus* (PSbMV) isolate DPD1 NIB sequence, developed a “recovery” phenotype when challenged with either the homologous DPD1 or a heterologous (NY) PSbMV isolate. However, the

specificity of the subsequent resistance differed with respect to the initiation by and targeting of different virus isolates. Analysis of tissue in which recovery had been induced by either of the isolates revealed the presence of low molecular RNA molecules (siRNA) derived from degradation of the NIB transgene mRNA. When nontransgenic scions were grafted onto transgenic stocks in which recovery had been induced all the scions were infected, indicating that the virus might be exported from the recovered tissue. In the reciprocal grafting experiments, recovered scions were grafted onto nontransgenic stocks. The recovery phenotype could be maintained in the apparent absence of a source of virus. However, many side shoots developing on the nontransgenic stocks became infected. The results indicated that the recovered pea tissues contained extremely low levels of infectious virus with potential directly or indirectly to confer the resistance specificity. Indirectly the viral genome could act as a source of specific siRNA molecules which are present in infected tissues but were below the level of detection in recovered tissues. This could act in conjunction with siRNAs derived from the transgene mRNA to maintain a level of posttranslational gene silencing (PTGS) activity which is effective in preventing further accumulation of heterologous or related viruses (Van den Boogaardt et al. 2004).

The concept of using an RNA interference construct to silence the *Cowpea severe mosaic virus* (CPSMV) proteinase cofactor gene and the *Cowpea aphid-borne mosaic virus* (CABMV) coat protein gene was test-verified to generate resistant transgenic cowpea plants. Ten cowpea transgenic lines were produced, presenting a normal phenotype and transferring the transgene to the next generation. Plants were tested for resistance to both CABMV and CPSMV by mechanical coinoculation. Seven lines exhibited milder symptoms when compared to the control, and three lines showed enhanced resistance to both viruses. Northern analyses were performed to detect the transgene-derived small interfering RNA (siRNA) in leaves and revealed no correlation between siRNA levels and virus resistance. In addition, in the symptomless resistant lines the resistance was homozygous-dependent. Homozygous plants only remained uninfected, while hemizygous plants showed milder symptoms (Cruz and Aragão 2014).

12.3.2.1.2 Protection by Expression of Antiviral Proteins

Crop plants may be protected by transformation with genes encoding antiviral compounds. The ribozymes construct, based on the nucleotide sequence of a viral gene, may cleave the target sequence efficiently in vitro. By integrating the ribozymes sequence into the host plant genome, the invading virus may be inactivated. The construct targeting *Plum pox virus* (PPV) that contained a hammerhead ribozymes provided significantly higher level of protection compared with the antisense RNA construct (Liu et al. 2000). The presence of antiviral proteins has been shown in several nonhost plant species. A ribosome-inhibiting protein (RIP) present in the cell wall of pokeweed (*Phytolacca* spp.) was reported to have antiviral properties. A cDNA clone for the pokeweed antiviral protein (PAP) was isolated and introduced into tobacco or potato plants by transformation with *Agrobacterium tumefaciens* (At). Transgenic potato or tobacco plants that expressed PAP or double mutant derivative of PAP exhibited resistance to infection by a broad spectrum of plant viruses such as *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Cucumber mosaic virus* (CMV). Resistance to the viruses was effective when transgenic plants were either inoculated by sap or aphid inoculation. PAP protected the plants by inhibition of early events in the infection process (Lodge et al. 1993). Plants expressing a low level of a C-terminal

deletion mutant of PAP showed resistance to PVX (Tumer et al. 1997). Another RIP from pokeweed PAP-II was found to be less toxic than PAP. The plants expressing PAP-II were resistant to *Tobacco mosaic virus* (TMV) and PVX (Wang et al. 1988). Another antiviral protein PAP-2 from *Phytolacca insularis* exhibited antiviral activity against TMV (Song et al. 2000).

In a later investigation, the effectiveness of pokeweed antiviral protein (PAP) in protecting *Petunia hybrida*, an important ornamental plant species, against CMV was assessed. Mutant PAP efficiently inhibited viral gene expression at both translational and transcriptional levels without causing host cell toxicity. The noncytotoxic mutant PAP gene was transferred into petunia cell via *At* transformation technique. Forty-two putative transgenic regenerated lines were obtained from selected explants. Forty-six plants immune to CMV infection were recovered from nine transgenic lines. Another 51 plants tolerant to CMV infection were obtained from 10 transgenic regenerated lines. Successful integration of the mutant PAP gene into the genome of transgenic petunia was confirmed by PCR and Southern blot analysis. Expression of the PAP gene was further confirmed by RT-PCR and Western blot analysis. These results were consistent with the assay of resistance to CMV (Li et al. 2013).

12.3.2.1.3 Protection by Engineered Mild Strains of Viruses

Cross-protection of crop plants using the naturally occurring mild strains has been successfully applied as a virus disease management strategy, as in the case of *Citrus tristeza virus* (CTV). *Papaya ringspot virus* (PRSV-) W strains can infect cucurbitaceous plants, but not papaya (infected by P strain only). Chimeric mild strains were constructed from HA5-1, a mild strain of PRSV (artificially produced from a severe Hawaii PRSV type P strain using nitrous acid) to carry a heterologous CP coding region and/or a 3' UTR (untranslated region of HA5-1 Wep 3U) of a type W strain that originated from Taiwan (PRSV W-CI). The chimeric mild virus HA5-1 Wep 3U provided a much higher level of cross-protection against the type W virus (W-CI strain) than the mutant HA5-1, while still providing a high degree of cross-protection against the type P virus P-HA in cucurbits. The results indicated that broad spectrum protection against two strains (that are differentiated by the ability or inability to infect papaya) could be accomplished by employing a mild potyvirus strain carrying a heterologous 3' genomic region, since the 3' UTR of the potyvirus was involved in the replication of positive-strand viral RNA. Sequence-homology-dependent PTGS is considered an antiviral defense mechanism in virus-infected plants, playing an important role in cross-protection against the same virus or related strains (Voinnet 2001).

12.3.2.2 Transgenic Resistance to Fungal Diseases

Fungal pathogens have more complexity in structure than bacterial and viral pathogens. Fungal pathogens possess the capacity for adopting different strategies to weaken the host responses that may restrict pathogen development. Resistance to fungal pathogens may be enhanced in different ways: (i) transformation of host plants for biosynthesis of a novel protein with adverse effect on pathogen growth; (ii) activation of expression of endogenous gene(s) normally induced following infection; (iii) transfer of gene(s) from other sources to direct the synthesis of a novel low-molecular-weight antimicrobial compound(s); and (iv) introduction of gene(s) to detoxify or deactivate pathogen factors required for pathogenesis. The development of plants resistant to fungal pathogens depending on the effective interference in the process of assembling pathogen cell wall

and membrane, and/or degrading them after assembly by introducing the gene(s) encoding the synthesis of antifungal compounds, formed the most frequently applied approach. Introducing pathogenesis-related (PR) proteins with chitinase or β -1,3-glucanase activities has been found to be more effective in arresting development of fungal pathogens. Biosynthesis of chitinase is governed by single genes; it may therefore be comparatively easier to transfer these genes than compared to the multiple genes that control other defense-related compounds such as phytoalexins and lignins. Chitin constitutes one of the major components of the cell walls of many fungal pathogens such as *Rhizoctonia*, and is hydrolyzed by chitinase. *Brassica napus* var. *oleifera* plants transformed with the endochitinase gene from tomato showed a greater level of resistance to *Phoma lingam*, *Sclerotinia sclerotiorum*, and *Cylindrosporium concentricum* compared with nontransformed control plants under field conditions (Grison et al. 1996).

Trichoderma harzianum has been employed as a biocontrol agent (BCA) against several fungal pathogens. Various strains of *T. harzianum* produce endochitinases, CHIT31, CHIT33, CHIT36, CHIT37, CHIT42, and CHIT52 which have been reported to be effective against fungal pathogens (Viterbo et al. 2001; Markovich and Kononova 2003). *T. harzianum* TM strain produced CHIT36 activity when expressed in planta. A plasmid containing *chit36* gene under the control of CaMV 35S promoter and the *nptII* selection gene was introduced into polyethylene glycol (PEG-) treated carrot (*Dacus carota*) protoplasts. The transgenic plants expressing CHIT36 were used in resistance assays to determine their levels of susceptibility/resistance to fungal pathogens *Alternaria radicina*, *A. dauci*, and *Botrytis cinerea*. Laboratory-based assays on detached leaves and petioles showed that transgenic carrots had less severe disease symptoms induced by these pathogens. All transgenic clones showed elevated chitinolytic activity. The highest activity observed for one clone (No. 178) was almost double the activity of the other transgenic lines and ten-fold higher than that of the control (nontransgenic parent lines). Comparison of infected leaflet area showed that transgenic clones differed in their reactions to fungal pathogens. Two clones (No. 96 and 178) had significantly enhanced resistance (35% and 19% disease) compared to the parent line. The clone No. 178 was tolerant to three pathogens tested, whereas clone No. 180 showed tolerance to *A. radicina* and *B. cinerea* but not to *A. dauci*. In general, disease severity of transgenic clones expressing CHIT 36 was reduced by approximately 50% relative to the control plants. For plants produced from seeds (Koral), a strong correlation was observed between chitinolytic activity measured in leaf extracts and the susceptibility to *A. radicina* ($r=0.94$; $P=0.008$) and to *B. cinerea* ($r=0.93$; $P=0.11$) (Fig. 12.4). The results indicated that expression of CHIT36 in planta may be an effective approach for improving the resistance of carrot to fungal pathogen (Baranski et al. 2008).

The genes encoding antifungal proteins (AFPs) from antagonistic biocontrol agents (BCAs) have been used to transform plants to improve the resistance to diseases caused by microbial pathogens. The AFPs encoded by the antifungal activity against several economically important fungal pathogens, including *Magnaporthe grisea*, have been identified. The transgenic rice plants constitutively expression *afp* gene were generated via *Agrobacterium tumefaciens* (At-) mediated transformation procedure. The presence of AFP protein in the leaves of transgenic rice plants was detected by Western blot analysis using polyclonal antibodies (PABs) raised against the purified AFP protein. Constitutive expression of the *afp* gene in rice resulted in a higher level of resistance to *M. grisea* in detached-leaf assay. Among the transgenic lines, the line 10* harboring the

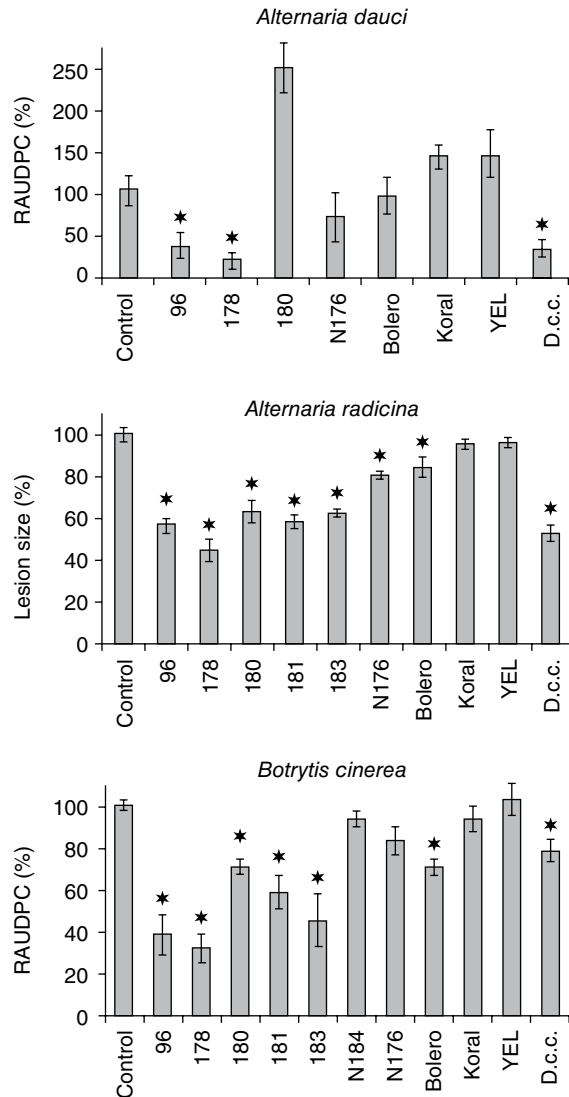


Figure 12.4 Reactions of transgenic carrot plants expressing chitinase *CHIT36* gene to the fungal pathogens *Alternaria dauci*, *A. radicina*, and *Botrytis cinerea* following inoculation of detached leaflets and petioles. Values represent the percentage of nontransgenic “Koral” control for transgenic line 96-183. N176 and N184: the nontransgenic clones not expressing *CHIT36*; D.c.c.: *Dacus carota* ssp. *Communitatus*; YEL: yellow leaf mutant; bar: standard error; star: clones with significantly lower values than the control based on Dunnett’s test at $P=0.05$ for *A. dauci* and $P=0.01$ for *A. radicina* and *B. cinerea*. Source: Baranski et al. (2008) and with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

synthetic *afp* gene was more resistant to rice blast pathogen. The level of *afp* expression appeared to be correlated to the level of disease resistance. The resistance of transgenic rice plants was considered to be due to the inhibition of germination of conidia of *M. grisea* at infection site (Coca et al. 2004).

In another investigation, the activity of the promoters from three maize PR genes *ZmPR-4*, *nipi*, and *PRms* in transgenic rice expressing *gfp* gene from *A. giganteus* was evaluated. The *ZmP4* promoter was strongly induced in response to infection by *M. grisea* and also treatment with fungal elicitors and mechanical wounding, as revealed by histochemical assays of GUS activity in transgenic rice plants. The *nipi* promoter was responsive to infection and wounding. On the other hand, *PRms* was not responsive to any of the treatments tested. The transformants expressing the *afp* gene under the control of *ZmPR4* promoter exhibited resistance to *M. grisea* to different degrees (Moreno et al. 2004). Proteomics analysis of rice phylloplane-secreted proteins was performed to identify proteins secreted during infection by the rice blast pathogen *Magnaporthe grisea*. Apoplastic proteins were analyzed by two-dimensional electrophoresis and MALDI-TOF mass spectrometry. The biological functions of proteins induced by *M. grisea* infections were related to metabolism (47%), stress/defense (36%), and proteolysis (13%). Most secreted proteins were predicted to retain a signal peptide and were highly expressed in incompatible interactions. A domain of unknown function 26 (*OsDUF26*) apoplastic protein was most abundantly secreted in response to rice blast infection. Transgenic rice and *Arabidopsis* plants expressing *OsDUF26-promoter::GFP* were generated to assess the role of *OsDUF26* in response to biotic stress. The *OsDUF26* promoter was activated in response to *Xanthomonas oryzae* pv. *oryzae*, *M. grisea*, and fungal elicitor. The results indicated that plant apoplastic proteins might have key roles in plant responses to biotic stress (Wu et al. 2014).

Fungal pathogens produce endopolygalacturonases (endo-PG) which have an important role during early stages of pathogenesis, spreading into the host tissues in advance of the invading mycelium. Polygalacturonase-inhibiting proteins (PGIPs) are basic proteins present in the cell wall of most dicotyledonous plants. PGIP is structurally similar to several resistance gene products, as it belongs to the superfamily of leucine-rich repeat (LRR) proteins (Matthei et al. 2001). The apple PGIP inhibited PGs secreted by *Verticillium dahliae* grown on cell walls and pectin. The usefulness of apple PGIP to confer resistance against wilt disease caused by *Verticillium dahliae* affecting potato was therefore assessed. Transgenic potato cultivar BP1 plants containing apple *pgip1* gene under the control of the constitutively enhanced CaMV 35S promoter were generated. The PCR and Southern blot analyses confirmed the stable integration of the apple *pgip1* transgene into the potato genome. The extracts prepared from all transgene lines except one effectively inhibited PGs of *V. dahliae*. Active PGIP1 was expressed both in the leaves and roots of transgenic potato plants. Apoplastic localization of PGIP 1 activity in the *pgip1* transgenic potato plants was observed. Six transgenic potato lines showed reduced intensity of disease symptoms compared with controls and other lines grown in sick soil. However, the reduction in disease intensity could not be attributed to inhibition of *V. dahliae* PGs, although a high level of expression of PGIP1 occurred in transgenic potato plants (Gazendam et al. 2004).

Puroindolines (PINs) form the principal components of wheat grain hardness locus (Ha) and have in vitro antimicrobial activity against bacteria and fungi. The effect of variation in PINA and/or PINB content on the inhibition of *Penicillium* spp., causing wheat seed rot, was assessed. Seed-germination assays were performed after incubating seeds in soil contaminated with *Penicillium* sp. As the first step, wheat genotypes consisting of two sets of transgenic isolines created in the varieties Bobwhite and Hi-Line having overexpression of PINA and/or PINB were tested. Another set of genotypes consisting of near-isogenic lines (NILs) varying for mutations in PINA or PINB created

in varieties Explorer and Hank was also tested. Transgenic wheat seeds overexpressing PINA in both Hi-Line and Bobwhite and both PINs in Hi-Line, when incubated in *Penicillium* sp.-contaminated soil, exhibited significantly reduced fungal infection and increased seed germination. No significant differences in pathogen infection or germination rates could be seen in seed of NILs. The results indicated that puroindolines might increase seed viability and that, when overexpressed as transgenes, the puroindolines might function as effective antifungal proteins, resulting in reduction of disease incidence (Kim et al. 2012).

Fusarium graminearum, incitant of Fusarium head blight (FHB) disease of wheat, produces the mycotoxin deoxynivalenol (DON) which is a potent inhibitor of eukaryotic protein synthesis and is believed to have a role in the pathogenesis of diseases affecting cereal crops. The 60S ribosomal protein L3 (RPL3) was suggested as the putative site of actions of the toxin. The rice cDNA encoding the ribosomal protein RPL3 was modified so that the amino acid 258 was changed from tryptophan to cysteine, a change considered to confer resistance to similar mycotoxins in yeast. Both types of the rice *Rpl3* genes were introduced into two species of tobacco by *Agrobacterium tumefaciens* (At) cocultivation and expressed under the control of a constitutive promoter. When cells, tissues, and protoplasts of these transgenic tobacco plants were compared for growth in the presence of DON, a significant difference in growth rate and the ability to undergo differentiation was observed among those plants expressing the modified version of the *Rpl3* gene (Rpl3:c258) compared to those expressing the wild-type *Rpl3* gene. The results indicated a possible mechanism of enhancing host plant resistance to *F. graminearum* among the susceptible cereal crop plants based on the expression of modified *Rpl3* genes (Harris and Gleddie 2001).

The rice cultivar Pusa Basmati 1 was transformed with the gene for Acc-AMP 1 from onion coding for an effective antimicrobial protein CAMP homologous to plant non-specific lipid transfer proteins (Ns-LTPs). The Acc-AMP1 protein was cloned under an inducible rice phenylalanine-ammonia lyase (PAL) or Ub1 (a constitutive maize ubiquitin) promoter. Accumulation of Acc-AMP1 predominantly in the extracellular space in transgenic rice plants was demonstrated by immunolocalization using antibodies against Acc-AMP1. This protein caused morphological deformation of the growing pathogen hyphae of the blast pathogen *Magnaporthe grisea* by membrane permeabilization. The transgenic rice lines exhibited enhanced levels of resistance, as reflected in a reduction of 86% in disease severity induced by *M. grisea*. In addition, the transgenic rice lines expressing Ace-AMP1 also showed resistance to other economically important diseases; the disease severities of sheath rot disease caused by *Rhizoctonia solani* and the bacterial leaf blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) were reduced by 67% and 82%, respectively. The transgenic rice lines also possessed the desirable attribute of retaining all agronomic parameters of nontransformed parent Pusa Basmati without any recognizable adverse effect on the rice plant phenotype (Patkar and Chattoo 2006).

Rice blast disease management using cultivars with *R* gene-mediated resistance has been shown to be an environmentally sustainable approach. A unique *R* gene of rice was identified on the chromosome 11 of *Oryza sativa* ssp. *indica* cultivar Tetep. Using complementation assay, the functional validation of the *Pi-K^h* (*Pi54*) gene was obtained. The blast resistance candidate gene *PiK^h* (*Pi54*) was cloned into a plant transformation vector and the construct was used to transform a japonica cultivar of Taipei 309 susceptible to rice blast pathogen, *Magnaporthe grisea*. Transgenic lines containing *Pi54* gene

showed a high degree of resistance to diverse isolates of *M. oryzae*. The deposition of callose in transgenic and nontransgenic rice lines was compared. In transgenic rice lines showing incompatible interaction, callose deposition was found to be widespread at infection sites. Successful complementation of *Pi54* gene revealed the role of this gene in the development of resistance to rice blast pathogen. The gene *Pi54* was pathogen-inducible, but expressed constitutively. Callose deposition in the resistant plants containing *Pi54* gene indicated the involvement of callose in the initiation of integrated defense responses to infection by *M. grisea* (Rai et al. 2011).

Plants produce several compounds such as phytoalexins involved in the enhancement of resistance to diseases. Stilbenes, a class of phytoalexins known to be present in grapevines and peanut (groundnut), are produced by the enzyme stilbene synthase. The gene for stilbene synthase was transferred from grapevine into the protoplasts of the japonica cultivar Nipponbare, using the polyethylene glycol (PEG-) mediated direct gene transfer technique. The stilbene synthase gene was stably integrated into the genome of the transgenic rice plants and was also inherited in the offspring. The transgenic rice plants exhibited a greater level of resistance to the rice blast disease (Stark-Lorenzen et al. 1997). The *Rop* proteins of plants, small GTPases (guanosine triphosphates), are important molecular switches in plant signal transduction and the *Rop* gene is also referred to as *Rac* gene. Overexpression of the constitutively active form of *OsRac1* in rice led to a hypersensitive reaction (HR) at points of infection by *Magnaporthe grisea* race 007 and consequently to disease resistance via reactive oxygen species (ROS) production. In contrast, overexpression of dominant negative forms of *OsRac1* consistently resulted in increased susceptibility to *M. grisea* (Kawasaki et al. 1999; Ono et al. 2001). The gene *OsRacB* cDNA was isolated to determine its function through gene expression and transgenic approach. In the rice genome, *OsRacB* present as a single gene shared 98% identity with *HvRacB* at the amino acid level. Strong expression of mRNA of *OsRacB* was recorded in the rice leaf sheath and panicles, while its expression in young and mature leaves was weak. Upon inoculation with *M. grisea*, the transgenic rice plants showed a dramatic regulation in the *OsRacB* transcripts. Overexpression of *OsRacB* resulted in enhancement of symptom severity. In addition, fluorescence microscopy of green fluorescent protein (GFP):*OsRacB*-transformed onion cells and *Arabidopsis* protoplasts revealed the association of *OsRacB* with plasma membrane. The results suggested that functioning of *OsRacB* may require its localization in plasma membrane and its function as a potential regulator for a basal disease resistance pathway in rice (Jung et al. 2006).

The usefulness of fungal viruses to improve the resistance of crops to diseases caused by fungal pathogens causing wheat smut diseases was demonstrated. The presence of “killer proteins” (KPs) in *Ustilago maydis* viruses was detected and the possibility of employing KPs to enhance resistance of wheat to smut fungi was indicated. Three non-homologous genes from three different viruses encoding *KP1*, *KP4*, and *KP6* with antifungal properties were identified. *KP4* was used to transform two Swiss spring wheat varieties Golin and Greina susceptible to stinking smut disease caused by *Tilletia caries*. Specific antifungal activity of the *KP4* gene product in the transformed lines was demonstrated in vitro and in planta in the glasshouse (Clausen et al. 2000). In a later study, the transgenic lines expressing *KP4* protein did not segregate for the transgenes and they were considered to be homozygous for *KP4* gene. The antifungal specificity of the KP system was observed in both transgenic plants. *KP4* in transgenic wheat plants inhibited the growth of *T. caries* and *Ustilago tritici*, incitant of loose smut disease.

The transgene functioned as a quantitative resistance gene and was highly specific for fungal pathogens belonging to Ustilaginales. The antifungal effect of *KP4* was indicated by an enhanced level of resistance of transgenic lines under field conditions (Schlaich et al. 2006).

The improvement of resistance to rice sheath blight disease caused by *Rhizoctonia solani* of transgenic rice lines was attempted by overexpressing the rice *oxalate oxidase 4* (*Osoxo4*) gene in a green tissues-specific manner which breaks down oxalic acid (OA), the pathogenesis factor secreted by *R. solani*. Transgenic plants showed higher enzyme activity of oxalate oxidase (OxO) than nontransgenic control plants which could be visualized by histochemical assays and SDS-PAGE technique. Transgenic rice leaves were more tolerant than control rice leaves to exogenous OA. Transgenic plants showed a higher level of expression of other defense-related genes in response to pathogen infection. A higher level of durable resistance to *R. solani* in transgenic rice leaves was their most desirable attribute. Overexpression of *Osoxo4* in rice did not affect any phenotypic or agronomic parameters. The results indicated that rice OxO could be utilized effectively in plant genetic manipulation for sheath blight resistance and possibly to other diseases caused by necrotrophic pathogens secreting OA (Molla et al. 2013).

In another investigation, the rice basic chitinase gene (RCH10) and the alfalfa β -1,3-glucanase gene (AGLU1) were tandemly inserted into transformation vector pB1101 under the control of 35S promoter with its enhancer sequences to generate a double-defense gene expression cassette pZ100. The pZ100 cassette was used to transform rice cultivar Taipei 309 by *Agrobacterium tumefaciens* (At-) mediated transformation. More than 160 independent transformants were obtained and confirmed by PCR. Northern analysis of inheritable progenies revealed similar levels of both RCH10 and AGLU1 transcripts in the same individuals. Disease resistance to both sheath blight and blast was challenged in open field inoculation. Immunogold detection revealed that RCH10 and AGLU1 proteins were initially located mainly in the chloroplasts and they were delivered to the vacuole and cell wall upon infection, suggesting that these subcellular compartments act as the gathering and execution site for these antifungal proteins. In addition, transgenic seeds showed reduction in seed germination and seedling vigor, indicating that enhancement of resistance might occur at the expense of plant development (Mao et al. 2014).

12.3.2.3 Transgenic Resistance to Bacterial Diseases

Alternate strategies involving biotechnological methods have been found to be useful for developing crop cultivars resistant to bacterial diseases for which traditional breeding procedures were either ineffective or difficult. The *Xa21* locus conferring resistance to all known races of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) occurring in the Philippines and India was transferred from *Oryza longistaminata*, the wild relative of rice to the rice cultivar IR24. The *Xa21* gene coding for a receptor kinase-like protein was inherited as a single gene in transgenic progenies (Song et al. 1995; Wang et al. 1995). Transgenic plants expressing *Xa21* were resistant to 29 of 32 isolates of *Xoo* from SE Asian countries, including India and Nepal, and Colombia. In a further study to improve the level of resistance to different strains of *Xoo*, an elite *indica* rice cultivar IR72 was transformed with a cloned *Xa21* gene. The transformed rice lines showed higher levels of resistance to rice bacterial leaf blight (BLB) disease due to pyramiding of *Xa21* with *Xa4* which had already been transgressed into the rice cultivar IR72 (Tu et al. 1998). In a later investigation, rice genotype IRBB13 was transformed with a

DNA fragment encompassing the *Xa13* candidate, including the promoter region isolated from another rice cultivar IR 64 and 29 independent transformants were produced. In order to characterize the function of *Xa13*, the expression of *Xa13* or *xa13* was suppressed in rice lines using an RNA interference (RNAi) strategy. The level of resistance to BLB in the cultivar Zhonghua 11 to PX099 strain was significantly increased in 9 of the 12 transgenic lines containing *Xa13*, as indicated by the reduced accumulation of *Xa13* transcripts. With significant reduction in the expression of *Xa13* or *xa13*, the transgenic plants had reduced fertility which was associated with male sterility. The *Xa13* recessive allele conferring BLB resistance plays a key role in both disease resistance and pollen development (Chu et al. 2006).

Attacins constitute a class of antimicrobial peptides detected in the insect haemolymph in response to bacterial infection (Engstrom et al. 1984). Attacins act on the outer membrane of Gram-negative bacteria by altering permeability and protein synthase activity (Carlsson et al. 1998). Attacin genes have been identified in the haemolymph of the giant silk moth *Hyalophora cecropia* (*attacin E*), *Drosophila melanogaster* (*attacin A*), and *Trichoplusia ni* (*attacin A*). Transgenic *Citrus sinensis* cultivar Hamlain plants expressing attacin A gene (*attA*) were generated via *Agrobacterium tumefaciens* (*At*-) mediated transformation for improving the resistance to citrus canker disease caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*). The cDNA clone was used to construct a binary plasmid vector (pCattA2300) and the construct included the native signal peptide (SP) responsible for directing the introduced protein to the extracellular space, where the bacterial pathogen is likely to accumulate in vivo. The presence of the transgene in citrus was confirmed by PCR and Southern blot hybridization analyses in regenerated plants. The transcription of *attA* gene could be established by Northern blot analysis in all transgenic plants. When inoculated with *Xac* (10^6 CFU mL⁻¹), 7 out of 8 transgenic lines exhibited significant reduction in the susceptibility to citrus canker disease. However, no correlation could be seen between disease resistance and level of transcription of *attA* (Boscariol et al. 2006).

The rice *Xa21* gene has been used to transform rice plants to enhance resistance to bacterial leaf blight (BLB) disease. Sequences of citrus genome (52) were shown to have high similarities to the rice *Xa21* gene, with amino acid sequence identity ranging over 54.9–59.7% (Deng and Gmitter 2003). Transgenic lines of four sweet orange cultivars (Hamlin, Natal, Pera, and Valencia) expressing the rice *Xa21* gene were evaluated for their resistance to canker disease. The *Agrobacterium tumefaciens* (*At*-) mediated transformation was performed and the transgene integration was confirmed by Southern blot analysis. The transgenic lines were propagated and challenged with citrus canker pathogen *Xanthomonas axonopodis* pv. *citri* and a nontransformed plant of each cultivar was used as control. All seven transgenic lines of cultivar Hamlin showed significant reduction in susceptibility to canker pathogen. The transformed plants had undergone 1–3 independent transgene integration events. The genetic transformation efficiency was 18.6% for Valencia sweet orange. Transgene transcription was confirmed by Northern blot analysis in most transgenic lines analyzed. Transgenic lines with a higher level of resistance to citrus canker were identified for cultivars Hamlin, Natal, and Pera. The lowest diseased leaf area values were detected for transgenic lines of Natal or Pera which were considered moderately resistant to citrus canker in the fields, indicating the influence of cultivar genetic background on the effectiveness of the *Xa21* gene restricting the incidence and spread of rice BLB disease (Fig. 12.5). The results indicated that the resistance conferred by the transgene was added to the original

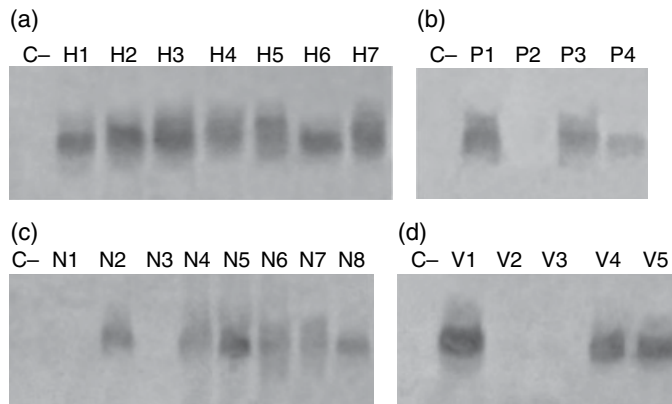


Figure 12.5 Northern blot analysis revealing transcription of *xa21* gene of rice in *Citrus sinensis* cultivars (a) Hamlin; (b) Pera; (c) Natal; and (d) Valencia leaves. Lanes 1–8: transgenic lines expressing *xa21* mRNA; lane C: nontransgenic plant (control). Source: Mendes et al. (2010) and with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

resistance level of the cultivar. Integration and expression of the rice *Xa21* gene in citrus could be achieved and the possibility of employing *R* genes for the management of citrus canker disease in a heterologous genetic background was indicated in this investigation (Mendes et al. 2010).

The possibility of producing grapevine cultivars resistant to crown gall disease caused by *Agrobacterium vitis* was explored. Grapevine cultivar Chardonnay was transformed with a construct containing two antimicrobial peptides (AMPs): Magainin-2 and PGL. Five lines expressing Magainin-2 showed enhanced level of resistance to two strains of *A. vitis*: TM4 and CG450. The effects of nine AMPs on the in vitro growth of four grapevine pathogens, *A. tumefaciens* and *A. vitis* (bacterial pathogens) and *Botrytis cinerea* and *Erysiphe necator* (fungal pathogens), were investigated. Cecropin B, MSI-99, and Shiva 1 were most effective against the bacterial pathogens, while ESF-12 had no effect. The growth of *A. vitis* strain TM4 was arrested by the AMPs to a greater extent than that of strain CG450, a pattern parallel to the levels of resistance to crown gall disease among transformants expressing Magainin-2. *B. cinerea* required more than M a-Purothionin, PGL, or ESF 39 for effective growth inhibition. None of the AMPs showed a consistent inhibitory effect on the conidial germination or lesion formation of *E. necator*. MSI-99 and Cecropin B were found to be more potent to provide increased resistance to crown gall disease in grapevine (Rosenfield et al. 2010).

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13

Crop Disease Management**Biological Management Strategies**

Crop plants at different stages of development from seed germination to harvest of the crop produce are exposed to a large number of microorganisms that may be pathogenic or beneficial to plant growth. Some of the microorganisms present along with the microbial plant pathogens in the same environment have been found to be our allies in the fight against pathogens. These microorganisms have the potential to restrict pathogen development to different degrees. The biological management strategies applicable to crop disease management seek to be a component of a synergistic orchestrated symphony, the benefit of which has to reach both the producer and consumer. The biological management may be defined as “the utilization of biotic and abiotic agents that act through one or more mechanisms to reduce the potential of the microbial pathogens directly or indirectly by acting on the host defense systems to reduce disease incidence and/intensity.” Several investigations have demonstrated that the combination of biotic and abiotic agents may result in synergism and consequent improvement in the effectiveness of disease suppression and enhancement of crop yield (Narayanasamy 2013).

Biological control agents effective against diseases caused by microbial pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses, and viroids – may be divided into two major groups as (i) biotic agents and (ii) abiotic agents. Biotic biological control agents are nonpathogenic living organisms that are antagonistic to potential microbial pathogens causing economically important crop diseases in various agroecosystems. They may suppress the pathogen development through one or more mechanisms. Abiotic agents are derived from inorganic or organic sources. The abiotic agents may also act on the microbial pathogens directly by inhibiting their growth and/reproduction or indirectly act on the host plants by activating their defense systems. The abiotic agents may be classified based on their chemical constitution or on the mode of action on the target microbial pathogen(s). Some of the abiotic agents have been synthesized for application against the plant pathogens and are available as commercial products. The microbial pathogens causing seed- and propagule-borne diseases are known to exist in the form of several races and strains, differing in their pathogenic potential (virulence). Likewise, biotic biological control agents (BCAs) also vary in their efficacy against the target pathogens. Strains or subspecies of the same morphologic species of the biological control agents differ in their biocontrol potential due to their differing sensitivity to environmental conditions, adaptation to new locations, rhizosphere competence, and the nature and quantity of antibiotics, enzymes, or toxic metabolites

produced by them. The precise identity of the effective BCAs therefore has to be established accurately by applying efficient techniques based on their biological, biochemical, and genomic characteristics. The biocontrol efficacy of the strains existing in a location has to be assessed by employing appropriate screening methods performed under in vitro, greenhouse, and field conditions to select the most efficient species/strains for large-scale application.

13.1 Evaluation of Biotic Agents for Biological Control Potential

13.1.1 Fungal Biological Control Agents

Dual culture procedure was applied to evaluate the biocontrol potential of eight isolates of *Trichoderma harzianum* and two isolates each of *T. atroviride* and *T. koningii* to suppress the development of *Pyrenophora tritici-repentis*, causing tan spot disease of wheat. The efficiency of inhibition of mycelial growth by *Trichoderma* spp. varied over 50–74%. The growth inhibition by the fungal biocontrol agents (BCAs) might be due to plasmolysis of conidia and hyphal cells of *P. tritici-repentis*, as observed under the microscope (Perelló et al. 2003). Two yeast strains *Cryptococcus* sp. OH71.4 and *C. nodaensis* OH 182.9 and two bacterial strains *Bacillus subtilis* AS43.3 and *B. subtilis* AS43.4 were evaluated for their efficacy in suppressing the development of Fusarium head blight (FHB) disease of wheat caused by *Fusarium graminearum* (teleomorph *Gibberella zeae*), using two cultivars of durum wheat under greenhouse and field conditions. All antagonists reduced FHB severity on cultivar Renville and three of the four strains reduced disease severity on cultivar Ben. *B. subtilis* strain AS43.3 decreased FHB severity to the maximum extent (90%). All antagonists/production medium combinations (except one) were effective in reducing disease on both durum cultivars. Of the six antagonists tested at field sites, *Cryptococcus* sp. OH71.4 and *C. nodaensis* OH182.9 reduced disease severity by as much as 57% at one site (Peoria, IL), while *Cryptococcus* sp. OH18.1 reduced disease severity by 59% at another location (Langdon, ND). The bacterial antagonist strains *B. subtilis* AS43.3 and AS43.4 were generally more effective in reducing disease in the greenhouse environment than the yeast antagonists, regardless of the durum cultivars. Conversely, the yeast antagonists *Cryptococcus* sp. OH71.4 and *C. nodaensis* OH182.9 were more effective under field conditions. The relative performance indices for four antagonists in the greenhouse and field sites showed that the location, but not the cultivar, influenced the relative performance of the antagonists. The yeast biocontrol agents seemed to have greater potential for reducing the FHB disease on durum wheat in the field (Schisler et al. 2002).

The biocontrol potential of isolates of *Clonostachys rosea* was evaluated against seed-borne pathogens *Alternaria dauci* and *A. radicina* in carrot seeds. The suspension of the spores of *C. rosea* was used to coat the seeds of carrot. Biopriming with addition of *C. rosea* reduced the incidence of *A. radicina* and *A. dauci* to <2.3% and 4.8%, respectively, in seeds with initial infection levels of 29% and 11%, respectively. With low initial infection level (4.7%) in seeds, biopriming of seeds reduced the incidence to less than 0.5%. In addition, biopriming enhanced seedling stand, compared with hydroprimed or nonprimed seeds. The biopriming of carrot seeds with *C. rosea* IK726 transformed with a green fluorescent protein (GFP) reporter gene revealed the presence of the BCA covering the seeds with a fine web of sporulating mycelium of *C. rosea* when observed

under the microscope. Biopriming with *C. rosea* was able to provide protection to healthy and infected seeds without any risk of adverse effect on seedling establishment (Jensen et al. 2004). In a later investigation, the comparative efficacy of fungal and bacterial antagonists, physical methods (hot water, aerated steam, electron treatment), and natural agents (resistance inducers, plant products) was assessed for suppression of the development of *A. dauci* and *A. radicina*, infecting carrot. Based on the average of five field trials, *Clonostachys rosea* strain IK726 and *Pseudomonas* sp. strain MF 416 were the most effective against both fungal pathogens. Hot water treatment, when combined with *C. rosea* IK726 or *Pseudomonas* sp. strain MF416 treatment, was more effective in suppressing the pathogen development (Koch et al. 2010).

Rhizoctonia solani, a soilborne fungal pathogen, causes stem canker and black scurf disease in potato plants and tubers, accounting for significant quantitative and qualitative losses. Six fungal endophytes isolated from Norway maples were screened for their ability to suppress the development of *R. solani*. The endophytes were identified based on the morphological characteristics and sequences of internal transcribed spacer (ITS) regions of rDNA as *Alternaria longipes*, *Epicoccum nigrum*, *Phomopsis* sp., and *Trichoderma atroviride*. Both *T. atroviride* and *E. nigrum* inhibited the mycelial growth of *R. solani* to a greater extent than other endophytic fungi tested, with greatest inhibition zone observed for *E. nigrum* in dual cultures. However, the culture filtrates of *T. atroviride* induced greatest inhibition. Under greenhouse conditions, the disease index differed significantly among different treatments, ranging from 0.1 for *T. atroviride*, 1.13 for *E. nigrum* E18, 1.86 for *Phomopsis* sp., to 2.86 for *A. longipes* with 1.00 for control (uninoculated). The in situ interaction between the pathogen *R. solani* and the endophytic BCAs was visualized using the confocal microscope. *T. atroviride* was able to establish close contact with the hyphae of *R. solani* by coiling. The coils were very dense and appeared to encircle the pathogen hyphae tightly. After 7 days of contact with the pathogen, the BCA penetrated into the pathogen hyphal cells which lost their turgor. The results indicated that *T. atroviride* functioned as a mycoparasite and a competitor for nutrients. On the other hand, *E. nigrum* and *A. longipes* acted on the pathogen by producing secondary metabolites toxic to the pathogen, whereas *Phomopsis* sp. competed for nutrients and space. *T. atroviride* and *E. nigrum* improved the potato yield significantly and decreased the stem disease severity index in sensitive potato cultivar, as indicated by greenhouse assessment (Lahlali and Hijri 2010).

A fungal isolate identified as *Aspergillus niger* var. *niger* was able to transform the phytotoxin, thaxtomin A produced by the potato scab pathogen *Streptomyces scabies*, into much less toxic compounds. The rate and extent of transformation of thaxtomin A was assessed under a variety of conditions including different growth media, quantum of biomass, incubation periods, and shaker speeds. Under optimum conditions, the fungal isolate converted thaxtomin A into two major and five minor metabolites which were fully characterized by a combination of mass spectral and nuclear magnetic resonance techniques. When assayed on aseptically produced minitubers, the major metabolites proved to be much less phytotoxic than thaxtomin A (Lazarovits et al. 2004). The potential of ten strains of wild yeasts isolated from vineyards in Washington State, USA, was assessed for protecting potatoes against *Phytophthora infestans*, causal agent of potato late blight disease. Potato leaf assays and dual culture assay in plates were performed for determining the efficacies of *Metschnikowia pulcherrima*, *Curibasidium pallidicorallinum*, and *Candida saitoana* in restricting lesion development on potato leaves and suppression of pathogen growth in culture plates. *M. pulcherrima* was the most effective in suppressing the development of disease symptoms in a manner suggestive of

induced resistance when applied prior to inoculation with *P. infestans*. Other yeast species also suppressed development of disease symptoms (Hadwiger et al. 2015).

Isolates of *Trichoderma* (23) were screened to select the isolates for effective suppression of four genetically distinct isolates of *Phytophthora capsici*, infecting bell pepper (*Capsicum annuum*) under greenhouse conditions. Three isolates GL12, GL13, and Th23 suppressed the pathogen development in at least two assays. These isolates were compared with *Trichoderma virens* isolates GL3 and GL21 for suppression of the pathogen in the presence and absence of the harpin-based natural product, Messenger. Isolates GL3 and Th23 provided significant suppression of disease development in 3 of 4 assays, whereas GL12, GL13, and GL21 were effective only in 2 of 4 assays. Messenger application was not found to be beneficial in any of the assays performed. The *Trichoderma* isolates were differentiated using phylogenetic analyses (Roberts et al. 2010). The biocontrol potential of *Clonostachys rosea*, a saprophytic strain of *Fusarium oxysporum*, and strains of *Pseudomonas putida* against *Colletotrichum lindemuthianum* causing anthracnose in bean, and *Ascochyta* spp. causing Ascochyta blight of pea, was assessed. *Bacillus subtilis*-based formulations provided the most effective protection against anthracnose, while *P. putida* and saprophytic *F. oxysporum* were also effective against bean anthracnose pathogen. None of the treatments significantly suppressed the development of Ascochyta blight pathogen. A strain of *C. rosea* showed some antagonistic activity against *Ascochyta* sp. (Tinivella et al. 2009).

Banana Fusarium (Panama) wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (FOC) is an economically important disease, negatively impacting banana production in all countries. Isolates of *Fusarium oxysporum* including the biocontrol agent (BCA) Fo47, nonpathogenic isolates from banana rhizosphere, and two field strains of *Trichoderma* (T2 and T5) were evaluated for their biocontrol potential against FOC. The pathogen was multiplied on millet seeds and the inoculum was used to infest sterilized soil in which banana plants treated with test BCAs had been planted. Disease development was determined by cutting the rhizome open using a scalpel, and recorded based on the disease severity rating as per Inihab's Technical Guidelines. The nonpathogenic isolates and Fo47 effectively suppressed the development of banana Fusarium wilt disease (Nel et al. 2006). In a later investigation, the biocontrol potential of nonpathogenic *Fusarium oxysporum* endophytes isolated from healthy banana roots were evaluated for their efficacy in suppressing *F. oxysporum* f.sp. *cubense*. The nonpathogenic *F. oxysporum* isolates were identified and grouped into 14 haplotypes by employing PCR-RFLP analysis. Representative isolates were evaluated for the control of the banana wilt disease. Under greenhouse conditions, 10 nonpathogenic *F. oxysporum* isolates reduced the wilt disease significantly. The isolate that protected banana plantlets most effectively in the greenhouse tests and *Pseudomonas fluorescens* WCS417 were tested under field conditions. Neither the nonpathogenic *F. oxysporum* isolate, *P. fluorescens*, nor their combinations could reduce the wilt disease under field conditions. The lack of control of the disease to the desired levels was attributed to the soil microbial populations and chemical composition and reduced survival of biocontrol organisms in banana roots (Belgrove et al. 2011).

13.1.2 Bacterial Biological Control Agents

The isolates of bacterial biocontrol agents (BCAs) obtained from seeds/planting materials, soil, or other substrates are evaluated for their biocontrol potential against the target

pathogen(s) under laboratory, greenhouse, and field conditions. Bacterial strains (52) and six *Trichoderma* spp. isolated from the wheat rhizosphere were evaluated for their biocontrol potential against *Fusarium graminearum*, causal agent of Fusarium head blight (FHB) and seedling blight of wheat, under in vitro and greenhouse conditions. Isolates differed in their capacity to inhibit the mycelial growth of *F. graminearum* in agar plate bioassays by 0–79%, but this characteristic was not related to the biocontrol efficacy in vivo. All isolates were initially tested in the greenhouse on wheat cultivars Klein Centauro (moderately resistant) and ProINTA (susceptible) planted in sterilized soil artificially infested with *F. graminearum*. Among the 25 bacterial strains and 6 fungal isolates that exhibited suppressive effect, the most efficient 10 isolates were further assayed on 8 cultivars potted in cultivated-inoculated soil. Among the antagonists, only *Streptophomonas maltophilia* was able to improve the plant stand, height, and dry weight in eight cultivars, compared to other antagonists. *S. maltophilia* reduced disease incidence to nonsignificant level only. Three *Bacillus cereus* and one isolate of *Trichoderma harzianum* decreased the percentage of infection in some wheat cultivars (Dal Bello et al. 2002).

Fusarium graminearum caused several epidemics in Argentina and has been a devastating disease, causing extensive yield and quality losses, due to the contamination of grains with mycotoxins (mainly deoxynivalenol, DON). Potential biocontrol agents (BCAs) isolated from wheat anthers were evaluated in Argentina. A total of 354 bacterial strains were screened in two selection steps using index of dominance (ID) assay, considering environmental parameters such as temperature and water activity in the interaction between pathogen and antagonist. Among the antagonists, 22 strains reduced the production of DON on irradiated grains by 60–100%. The ability of these strains to reduce FHB and DON production was evaluated under greenhouse conditions. Nine strains (40.9%) significantly reduced the disease severity by 49–71% ($P=0.05$). The DON content in spikes produced by plants in the greenhouse trial was significantly reduced by 32–100% compared with control. Five strains belonging to *Brevibacillus* sp. BRC263 and *Streptomyces* sp. BCR87B decreased mycotoxin content in wheat grains to undetectable levels, indicating their effectiveness and potential for use against *F. graminearum* (Palazzini et al. 2007).

The efficacy of 18 bacterial isolates was evaluated for suppressing the development of *Fusarium graminearum* under in vitro and greenhouse conditions. One isolate each of *Bacillus subtilis* MAA03 and *Pseudomonas fluorescens* MAA10 were identified using International Identification Keys and Biolog System. These two isolates were selected based on their effectiveness against *F. graminearum*. The 16S rDNA sequences of these isolates had more than 99.5 identity to the strains already described. When used individually or in combination as a mixture, *B. subtilis* and *P. fluorescens* significantly increased final seed germination percent, the mean daily germination, and germination index of wheat cultivar, while the mean germination time was significantly reduced relative to the infested control. The final infection percent, the mean daily infection, and infection index were decreased, while the mean infection time was significantly increased compared to the infested control. *P. fluorescens* was more efficient than *B. subtilis* or in combination. The best application method of the bacterial BCAs was seed coating. Application of *B. subtilis* and *P. fluorescens* separately or in combination, as seed coating or seed soaking treatments, increased the root length, while soil drench treatment nonsignificantly reduced root length. Likewise, root and shoot fresh and dry weights were also increased by seed coating or seed soaking treatment with these two bacterial antagonists (Moussa et al. 2013).

Rhizoctonia solani infects several crops including potato and lettuce, causing appreciable yield losses. Three potato-associated ecto- and endophytically growing strains of *Pseudomonas fluorescens* B1 and B2 and *Serratia plymuthica* B4 were evaluated against *R. solani* in potato and lettuce. Disease suppressive ability of the bacterial biocontrol agents (BCAs) was assessed in growth chamber as well as in the field. All three BCAs entirely or significantly limited the dry mass (DM) losses on lettuce and disease severity (DS) caused by *R. solani* on potato sprouts in growth chamber assays. Strain B1 was the most efficient strain in suppressing pathogen development (52%) in potato. Under field conditions, the disease severity on both potato and lettuce which were bacterized decreased significantly and the biomass losses on lettuce decreased significantly. The strain B1 was more effective (37%) in suppressing the development of *R. solani* under field conditions than B2 strain (32%) and B4 strain (31%). The marketable potato tuber yield was increased up to 12% by B1 strain, compared to 6% by B2 strain and 17% by B4 strain under high disease pressure conditions. The strain B4 was the most effective in all experiments, indicating its suitability for development as a commercial product against *Rhizoctonia* diseases (Grosch et al. 2005).

Strains of fluorescent pseudomonads (303) isolated from roots and rhizospheric soil of tomato plants were tested in vitro against *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), causative agent of bacterial canker disease of tomato. Strains showing high antagonistic capacity were evaluated for their ability to colonize tomato roots. The most effective fluorescent pseudomonads were present in the rhizoplane soil and endorhizosphere. Among the 42 spontaneous antibiotic-resistant mutants obtained by treatment of the wild-type isolates with antibiotics (rifampicine, nalidixic acid, ampicillin, and chloramphenicol), 28 isolates could colonize the roots of all tomato seedlings efficiently. All wild-type isolates (42) were used for in vitro screening using cotyledon test. Eight of 42 isolates reduced the disease incidence and severity significantly ($P < 0.001$) when applied as seed and root treatments (Amkraz et al. 2010). Citrus canker disease caused by *Xanthomonas citri* pv. *citri* (Xcc) was suppressed by *Pseudomonas* sp. strain LV. The antibiotic activity of the BCA was demonstrated using scanning electron microscope (SEM) and transmission electron microscope (TEM). The antibiotic activity of dichloromethane phase (DP) was greater than all other fractions from vacuum liquid chromatography (VLC), as reflected by inhibition of the pathogen development. The toxic metabolites produced by the BCA prevented formation of biofilms by *Xcc* and destroyed exopolysaccharides (EPS) present around *Xcc* cells. The cells of *Xcc* were found to be shrunken. Finally the cell wall and cytoplasmic membrane of *Xcc* became indistinct, followed by apparent lysis of pathogen cells. Lack of EPS production and formation of biofilm resulted in reduction in lesion formation in plants treated with metabolic fractions of *Pseudomonas* sp. LV (de Oliveira et al. 2011).

Detached-leaf assay was performed to determine the antagonistic activity of isolates of *Bacillus subtilis* against *Phytophthora* blight of red pepper (*Capsicum annuum*) caused by *Phytophthora capsici*. The suspensions of bacterial isolates (10^8 cell mL⁻¹) were sprayed individually on the detached leaves and they were placed in the petriplates lined with moistened filter paper. Spore suspension (10^6 spores mL⁻¹) of the pathogen was sprayed on the leaves already treated with different bacterial isolates. The leaves were incubated in a growth chamber at 20°C with 12h of light and dark periods for 10 days. Disease severity in different treatments was determined using 0–4 disease rating scale as follows: 0 = no visible symptom; symptom severity: 1 = 1–12%; 2 = 13–25%; 3 = 26–50%; and 4 = 51–100% of leaf area covered by the disease. All the antagonistic

isolates showed varying levels of antagonism against *P. capsici*. Two isolates R33 and R13 were more effective in suppressing the disease severity by 86.8% and 71%, respectively, under in vivo. Based on the 16S rDNA sequencing, these two isolates were identified as *Bacillus subtilis*. In addition, the root and shoot length of plants were increased by the isolates of *B. subtilis* (Lee et al. 2008).

Strains of *Paenibacillus polymyxa* (25) were screened for their antimicrobial activity against *Phytophthora capsici*. Based on the inhibitory activity 15 strains were categorized as strongly antimicrobial, among which the strain GBR-462 was the most active, and 5 strains each as weakly or no antimicrobial activity. The antimicrobial activity was influenced by the initial inoculum density. The strains of *P. polymyxa* with a strong antimicrobial activity (including the strain GBR-462) showed antimicrobial activity against *P. capsici* and could only form biofilm when they were applied at the higher initial population levels of 10^8 CFU mL⁻¹ of *P. polymyxa* GBR-462. However, formation of sporangia and release of zoospores were significantly reduced at the lower population levels. Further, the sporangia showed aberrant structures with no or few healthy nuclei, indicating that sporangium and zoospore formation was inhibited at the lower levels of BCA density, as revealed by the light microscopy examination. Application of *P. polymyxa* GBR-462 into potted soil suppressed the disease development and also the disease severity by 30%, compared with untreated control treatment (Kim et al. 2009).

Paenibacillus polymyxa E681 strain produces fusaricidin, a cyclic depsipeptide with antifungal property. *P. polymyxa* E681 effectively suppressed the development of Phytophthora blight disease of pepper caused by *Phytophthora capsici*. The effects of fusaricidin on the pathogen development and disease suppression were investigated. The minimal inhibitory concentration (MIC) of fusaricidin was 16 ppm against *P. capsici*. Application of fusaricidin at 0.1 ppm concentration reduced disease severity significantly (40%) compared with water-treated control (81.7%) on postinoculation, whereas the disease severities on pretreatment were 45% and 83.3% in fusaricidin treatment and control, respectively. Fusaricidin was effective in suppressing the disease severity when applied as soil treatment or foliar spray at 1.0 ppm concentration. The results indicated that fusaricidin might protect plants functioning as an antibiotic directly or *P. polymyxa* might secrete elicitors of disease resistance against plant pathogens (Lee et al. 2013).

The effectiveness of *Paenibacillus ehimensis* KWN38 in suppressing the development of Phytophthora blight disease of pepper caused by *Phytophthora capsici* was assessed. The BCA grown in a grass medium showed high antifungal and lytic enzyme activities. Under greenhouse conditions, 1 month old pepper plants were treated with the strain KWN38 or fungicide. The shoot dry weight was increased and zoospore infection was reduced in plants treated with the bacterial BCA, compared to control treatment. The plants survived better due to treatment with BCA strain or fungicide. Soil cellulase activity was promptly increased shortly after zoospore infection and reached the maximum in BCA-treated plants. Likewise, the β -1,3-glucanase activity of BCA-treated plants increased to the maximum level after zoospore infection. The results indicated that inoculation of the strain KWN 38 in the root zone of pepper plants in the pots resulted in enhancement of plant growth and alleviation of the root death caused by infection by *P. capsici* zoospores (Naing et al. 2014).

Isolates of fungi, actinomycetes, and bacteria (342), isolated from soil and plants from a potato field in which wild oat (*Avena strigosa*) had been precultivated, were screened to select the microorganism(s) effective against *Streptomyces turgidiscabies*, causative agent of potato common scab disease. Based on the initial pot trials, 26 isolates were

selected as antagonistic candidates with disease suppressive potential. After a second pot trial, five actinomycetes isolated either from the rhizosphere soil of wild oat or the soil adhering to potato stolons and tubers, were selected as antagonists because of their consistent suppressive activity. Based on the partial sequence analysis of the 16S rRNA genes from the five isolates, they were assigned to the genus *Streptomyces*. Of these five isolates, WoRs-501 was found to be the most effective in inhibiting the growth of *S. turgidiscabies* and was also the most effective in suppressing potato common scab disease development in the field trial. In the pot trial, a 10% (v/v) mix of WoRs-501 (6.2×10^8 CFU g⁻¹ dry mass) decreased the disease severity by 78–94% compared to the untreated control at the pathogen inoculum of 5×10^4 to 5×10^6 CFU g⁻¹ dry soil. The isolate WoRs-501 showed desirable level of biocontrol potential for the control of potato common scab pathogen *S. turgidiscabies* (Kobayashi et al. 2012).

Root-associated/endophytic (RAE) bacterial isolates (72) obtained from main peanut (groundnut) -producing provinces of China were evaluated for their biocontrol potential against *Aspergillus parasiticus* causing peanut aflaroot disease, which produces the mycotoxin aflatoxin in peanut kernels harmful to humans and animals. The 16S rRNA sequence and phylogenetic analyses placed the RAE bacterial isolates in the class Bacilli (49 isolates) and Gammaproteobacteria (23 isolates). The majority of bacteria belonging to Bacilli belonged to two genera *Bacillus* (48) and *Lysinibacillus* (1), while those isolates in Gammaproteobacteria belonged to the genera *Enterobacter* (7), *Serratia* (11), *Stenotrophomonas* (3), and *Pseudomonas* (2). *Lysinibacillus xylanilyticus* was found for the first time to be an effective antagonist against *A. parasiticus*. All the selected RAE bacteria exhibited antagonistic activities against the growth of *A. parasiticus* and/or aflatoxins (AFs) production in visual agar plate assay and tip culture method. Majority of RAE bacterial strains (96%) reduced the mycelial growth significantly or AFs production levels by >50%. Further, 22 strains (50%) could degrade colloidal chitin on the chitin medium plate. Ten selected chitinolytic RAE bacteria significantly reduced the mycelial growth of *A. parasiticus* and AFs production by >90%. The results indicated the presence and wide distribution of bacteria capable of suppressing the development of aflatoxin production by *A. parasiticus* (Wang et al. 2013).

Detached-leaf assay was performed to assess the biocontrol potential of bacterial strains to suppress the development of potato late blight pathogen *Phytophthora infestans*. The bacterial antagonist was applied at a concentration of 10^8 CFU mL⁻¹ into the Russet Burbank potato leaflets by dipping and incubating for 5 h at room temperature. The leaflets were challenged by applying the pathogen (20 μ L droplets of 10^4 zoospores mL⁻¹ suspension) into the lower surface of the leaflet. The inoculated and control leaflets were then placed on moist sterile filter paper kept in a covered sterile petridish and then transferred to a moist chamber to provide high humidity (95%) for the development of the pathogen. The bacterial-broth supernatant was prepared by inoculating the nutrient broth in flasks and keeping them on a shaker for 3 days continuously at room temperature. After centrifuging at 4500 rpm for 10 min, the supernatant was decanted and frozen. The effect of supernatant bacterial nutrient broth was assessed, as in the case of live bacterial cells. The inoculated leaves treated with live bacteria or water were scanned using the image analysis software Asses® to determine the area of leaflets infected by *P. infestans*. The percentage of inhibition of disease development was calculated using the formula:

$$\text{Percent inhibition} = 100 - \left[\left(\frac{\text{BP}}{\text{WP}} \right) \times 100 \right]$$

where BP is infected area in leaflets treated with bacteria/supernatant and WP is infected area in leaves treated with water (control). The disease intensity is expressed using a rating scale of 1–5 as follows: 1 = <10%; 2 = 10–20%; 3 = 21–40%; 4 = 41–75%; and 5 = >75%. Some isolates of *Pseudomonas fluorescens* DF37 and *Bacillus pumilus* W1 were effective inhibitors of *P. infestans* in vitro, but they did not provide any protection in detached potato leaves when applied as suspension, and had no effect in treated leaves when challenged with *P. infestans*. In contrast, their culture filtrates provided protection against *P. infestans*. *B. pumilus* W1 induced 47% systemic protection, even though it did not provide any local protection against *P. infestans* on either detached leaves or whole plants. The detached-leaf technique might be useful to study the mechanism of action of the biocontrol agents isolated from different substrates (Daayf et al. 2003).

Native bacterial strains were evaluated for their efficacy in suppressing the development of *Colletotrichum falcatum*, causing the devastating red rot disease in sugarcane under greenhouse and field conditions. The antagonistic bacterial strains of *Bacillus subtilis* NH-100, NH-160, *Bacillus* sp. NH-217, and NH-69 effectively suppressed the development of red rot disease under greenhouse conditions. The performance of these four strains was assessed under field conditions, using two sugarcane cultivars SPF-234 and CO 1148, for 3 consecutive years. Two different trials were carried out. The red rot pathogen was inoculated by different methods to determine the extent of suppression as well as induction of systemic resistance by the bacterial antagonists. Three strains reduced the disease incidence by 45–49% in sugarcane plants challenged by pathogen inoculation in the stem and by 48–56% in plants inoculated in soil near the roots. The results indicated the usefulness of the bacterial strains existing in the location concerned (Hassan et al. 2012).

Infection of wheat seeds by *Fusarium graminearum* and related species of *Fusarium* cause wheat Fusarium head blight and scab and maize ear rot and also the produce of mycotoxins such as deoxynivalenol and zearalenone, which induce ailments in humans and animals. The biocontrol potential of a patented endophytic bacterial strain *Bacillus mojavensis* RR101 was assessed in growth rooms. All strains of *B. mojavensis* were antagonistic to *F. graminearum*, *F. pseudograminearum*, and *F. verticillioides*. Seed germination percentage of the highly susceptible wheat cultivar 2552 was increased from 77 to 97% due to treatment of seeds with the BCA, followed by planting in *Fusarium* spp.-infested soil. The seedling emergence was also substantially increased from 20 to 82% following treatment of seeds with the BCA strains. Seed treatment with bacterial antagonists might provide effective protection against the mycotoxin producing fungal pathogens (Bacon and Hinton 2007).

Phytoplasmas are wall-less pathogenic bacteria belonging to the class Mollicutes and are capable of causing several diseases in economically important fruit trees, vegetables, ornamentals, and cereal crops. They are phloem-limited and transmitted by leafhoppers and psyllids (Weintraub and Beanland 2006; Bertaccini 2007). Biological management of phytoplasma diseases introduces the fourth component, the biological control agents. The effectiveness of using bacterial biocontrol agents for the suppression of chrysanthemum yellows phytoplasma (CYP) was assessed. The biocontrol potential of *Pseudomonas putida* SPIf1Rif strain was determined by measuring plant biomass, root architecture, symptom severity, phytoplasma titer, and viability in CYP-inoculated and control plants. The pathogen reduced plant growth and root development. The CYP titer in young apical leaves was not reduced by treatment with *P. putida*, which however

improved the growth of CYP-infected plants. The phytoplasmal viability was reduced over time, regardless of the presence of the BCA in treated and untreated control plants. The phytoplasma cells in fully developed leaves of CYP-infected plants often appeared to be degenerated following treatment with the BCA. The results indicated that the BCA might alleviate adverse effects of the disease, although it could not affect the viable phytoplasmas in young leaves of infected plants (Gamalero et al. 2010).

Antagonistic interactions among strains of “*Candidatus* Phytoplasma mali” infecting apple were investigated by graft-inoculating *Catharanthus roseus* and *Nicotiana occidentalis* (bioindicators) with mild strains 1/93Vin and 1/93Tab as suppressors and three aggressive strains as challengers. Inoculation of suppressors was carried out either in the cross-protection mode prior to grafting of the challengers or by inoculating suppressors and challengers. Multiplex real-time PCR assay was employed to monitor the presence/movement of the suppressors/challengers. In long-term cross-protection experiments with *C. roseus*, the presence of suppressor 1/93Vin was detected in all root and randomly collected stem samples over the entire observation period. In contrast, the challengers were never detected in such stem samples and rarely in the roots. Following simultaneous inoculation, the suppressor successively colonized all stem and root regions whereas detection of challenger AT steadily decreased. However, this strain remained detectable in up to 13 and 27% of stem and root samples, respectively. In the cross-protection experiments with *N. occidentalis* similar results were obtained. Comparison of the symptomatology of infected apple trees with the presence of putatively suppressive strains indicated that the suppression of severe strains also occurred in apple. Phylogenetic analysis using a variable fragment of AAA + ATPase gene AP460 of “*Ca. P. mali*” revealed that suppressors 1/93Vin and 1/93Tab, together with several other mild strains maintained in apple, cluster distantly from obviously nonsuppressive strains that were predominantly highly virulent (Schneider et al. 2014).

The efficacy of plant growth-promoting rhizobacteria (PGPR) has been examined for suppressing the development of virus diseases affecting various crops and providing different degrees of protection to treated plants. Single strains of PGPR were applied as seed treatments to cucumber and tomato for protection against *Cucumber mosaic virus* (CMV), which has a wide host range. There was a delay of 7 days in the onset of symptoms in some PGPR-treated plants compared with nonbacterized CMV-inoculated control plants. The PGPR-treated plants that did not develop symptoms at 7 days post-inoculation (dpi) remained symptomless for 4 weeks (end of experimentation) and did not contain a detectable concentration of CMV in noninoculated top leaves. In tomato plants grown from bacterized seeds, the area under disease progress curve (AUDPC) was reduced relative to the nonbacterized CMV-inoculated plants, indicating that the protection of tomato plants against CMV was due to induction of resistance by PGPR strain. Protection was observed when tomato plants were treated with PGPR strain followed by inoculation with CMV in the greenhouse and later transplanted in the field. The induced resistance was sustained under field conditions (Zehnder et al. 2000).

Due to the variability in efficacy of single PGPR strains, combinations of two strains of PGPR formulations with the carrier chitosan (biopreparations) were evaluated for their potential to promote plant growth and to enhance resistance to infection of tomato plants by CMV. Each PGPR combination included GB03 strain of *Bacillus subtilis* and one of the following PGPR strains of *B. pumilus* SE34: *B. amyloliquefaciens* IN937R; *B. subtilis* IN937b; *B. pumilus* INR7; or *B. pumilus* (T4). Tomato plants treated with each of the biopreparations appeared phenotypically and developmentally similar to

nonbacterized control plants that were 10 days older (older control). The biopreparation significantly enhanced the growth parameters of treated plants. CMV disease severity ratings were lower for biopreparation-treated tomato plants at 14 and 28 dpi. Accumulation of CMV in young noninoculated leaves was significantly less for all biopreparation-treated plants. The results indicated that the biopreparations promoted the growth of treated plants and also protected them from CMV infections in a manner that mimics natural (adult) plant resistance that is exhibited by most plant species as they become older (Murphy et al. 2003).

13.1.3 Viral Biological Control Agents

Virus infections in all organisms from prokaryotic bacteria to human beings are known. The possibility of using viruses as the biological control agents of fungal and bacterial plant pathogens has been explored in some pathosystems. Viruses and virus-like particles have been detected in plant pathogenic fungi and bacteria and have been evaluated for their biocontrol potential for application against phytopathogens.

Among *Fusarium* spp., causing various kinds of diseases of crops, the presence of ds-RNA mycoviruses (viruses infecting fungi) was detected in *Fusarium graminearum*, causal agent of Fusarium head blight (FHB) disease of cereals. Colonies of *F. graminearum* with unusual morphology, slow growth, amoeboid mycelia, and increased pigmentation were observed (Chu et al. 2002). Derivatives of strain DK21 free of the ds-RNA required only 3 days to cover the plates as against 9 days needed for virus-infected isolates of *F. graminearum*. Conidial production by the hypovirulent (with reduced virulence) was significantly reduced and elimination of ds-RNA through conidial passage resulted in faster mycelial growth and less pigmentation. Isolates of *F. graminearum* carrying ds-RNA produced much less mycotoxins (DON) compared to the ds-RNA-free isolates. Development of FHB disease in plants inoculated with isolates carrying ds-RNA was much slower. As there are several vegetative compatibility groups (VCGs) of *F. graminearum*, this condition may serve as a major obstacle for transmission of ds-RNA to virus-free isolates (Chu et al. 2002).

Cycloheximide, which inhibits RNA synthesis, may be able to cure the fungi carrying ds-RNA mycoviruses. *F. graminearum* hyphae containing ds-RNA were transferred to potato dextrose agar (PDA) medium amended with cycloheximide (5–70 ppm) and incubated for 10 days at 25°C. The hyphal tips were transferred to fresh PDA medium and incubated again for 10 days at 25°C. The hyphal tips from these colonies were found to be free of viral ds-RNA as revealed by electrophoresis technique, indicating the ability of cycloheximide to inhibit the ds-RNA virus synthesis in fungal pathogens (Aminian et al. 2011).

The presence of the ds-RNA mycovirus FgV-ch9 in *Fusarium graminearum* (Fg) isolate China-9 infecting wheat and maize was detected and hypovirulence characteristics were studied. Single conidia-originating cultures of Fg China-9 contained high, medium, or low amounts of viral ds-RNAs. At high and medium ds-RNA concentrations, the Fg China-9 isolates showed reduced mycelial growth rate and conidial production, abnormal colony morphology, and disorganized cytoplasm. Virulence of the infected Fg isolates showed significant reduction when inoculated onto wheat and maize plants. No recognizable symptoms could be seen when the pathogen isolates containing low titers of ds-RNAs were used for inoculation. However, the presence of the RNA segments of FgV-ch9 could be detected by RT-PCR technique. Transfection of the virulent

F. graminearum PH-1 isolate with purified virus-like particles (VLPs) of FgV-ch9 resulted in reduction in sporulation, perithecial production, and pathogenicity to wheat and maize by many folds. The results showed that FgV-ch9 induced hypovirulence in *F. graminearum* (Darissa et al. 2012).

Alternaria alternata infects many crops such as bean, soybean, and sunflower, causing substantial losses in yields as well as in quality. The presence of ds-RNA-1, ds-RNA-2, ds-RNA-3, and ds-RNA-4 was detected in the *A. alternata* strain EG35-193 which showed impaired growth phenotype. Cycloheximide inhibited ds-RNA synthesis, resulting in the elimination of mycovirus from hyphal tips taken from infected colony. The ds-RNA mycovirus might be involved in modulating traits of *A. alternata*. The ds-RNA encoded a single open reading frame (ORF) (3447-nt) containing conserved motifs of viral RNA-dependent RNA polymerase (RdRp) which was related to the ORF encoded by ds-RNA-1 of *Aspergillus* mycovirus 341. All of the encoding strands of the four ds-RNA genomes had 3'-poly (A) tails ranging from 33 to 50-nt in length. The virus infecting *A. alternata* was named *Alternaria alternata virus-1* (AaV-1) (Aoki et al. 2009). A simple and rapid method of detecting the mycovirus pathogenic to *Magnaporthe grisea* (*oryzae*), causal agent of rice blast disease, was developed. A sterilized toothpick was used to obtain the *Magnaporthe chrysovirus 1-A* colonies growing on the growth media in plates. The virus was detected and identified by employing direct one-step RT-PCR assay. This procedure could be employed for direct detection of the virus in the lesions in rice leaves induced by *M. grisea* (Urayama et al. 2015).

Bacterial viruses, designated bacteriophages, are capable of infecting some of the economically important bacterial pathogens. Bacteriophages (phages) have been evaluated for their efficacy in suppressing the development of plant bacterial diseases. An extract from decomposed cabbage tissues was demonstrated to have inhibitory principle (filterable virus) arresting the growth of *Xanthomonas campestris* pv. *campestris*, causing cabbage black rot disease (Mallmann and Hemstreet 1924). Several studies have indicated the possibility of employing bacteriophages as biocontrol agents against bacterial pathogens as an alternative disease management strategy, either to replace or reduce the use of chemicals. The relative ease of preparing phages and low cost of production of the phages are the desirable factors for considering bacteriophages as candidates for wider application. Phages are abundant in most natural ecosystems so a high genetic diversity of phages may be expected (Rowher 2003). Phages are likely to be present in large populations in environments where there is a high density of metabolically active bacteria. A metagenomic approach has been applied for assessing the diversity of phages that may be antagonistic to bacterial pathogens present in the soil and plant rhizosphere (Fierer et al. 2007; Kim et al. 2008).

As the phages are natural components of the biosphere, their use as biocontrol agents against bacterial pathogens is ecologically acceptable. Phages are nontoxic to plants and animals, so may be preferable for application in situations where chemical control is not permissible due to legal regulations. The specificity of phages may allow their application in combination with antagonistic bacteria that have been already employed as biocontrol agents. The development of bacterial strains resistant to phages may become a major limiting factor for successful management of bacterial diseases. The process to employ a mixture of host range mutant phages (h-mutants) for disease control was developed and patented. The h-mutants could lyse all bacterial strains that were resistant to the mild-type strains, while still being capable of lysing

the wild-type bacterial pathogen strain. The phage mutants had an extended host range compared to the parent phage alone. A mixture of four phages, including wild-type and h-mutant phages, were applied twice weekly in the early morning prior to sunrise. The combination of phages suppressed the development of tomato bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria* more effectively (Jackson 1989). Phages are specific to host bacterial species/strains, destroying only target bacterial species without affecting other organisms that may be beneficial to plant growth. Bacteriophages were rapidly degraded under field conditions and practically disappeared from tomato canopy at 2 days after application for the control of bacterial spot disease caused by *X. campestris* pv. *vesicatoria*. In order to improve the efficacy of the phage three formulations were tested: (i) 0.5% pregelatinized corn flour (PCF); (ii) 0.5% casecrete NH-400 + 0.5% sucrose + 25% PCF; and (iii) 0.75% powdered skim milk + 0.5% sucrose. These formulations significantly increased the phage longevity on tomato plant surface under greenhouse conditions. The nonformulated PCF, casecrete-, and skim milk-formulated phage mixtures reduced the disease severity on plants compared with the controls by 1, 30, 51, and 62%, respectively. These three formulations increased the concentrations of the phage populations by a factor of 4700, 38,500, and 100,000, respectively, at 2 days after application. The protective formulations increased the efficiency of phage treatments in suppressing the development of tomato bacterial spot disease (Balogh et al. 2003).

Bacteriophages specific to *Ralstonia solanacearum* which is pathogenic to many crops such as pepper, tomato, and potato were isolated from rhizospheres of plants infected with *R. solanacearum*. A host specificity analysis of 15 phage isolates using 9 strains of *R. solanacearum*, including race 1 (biovars 3 and 4) and race 3, indicated great phage diversity even in a single soil. A filamentous phage PE226 had a relatively wide range of host bacterial infection. The phage isolates PE226 and TM227, isolated from pepper and tomato, respectively, were pathogenic to all 9 strains of *R. solanacearum* tested by producing clear plaques (clear spots in a lawn of bacterial pathogen grown in petridish). These two strains might be closely related, as indicated by transmission electron microscope (TEM) observations and genomic characteristics. The isolate PE226, an *Inovirus*, had a long flexible filamentous shape, carrying a circular (+) sense single-stranded DNA genome of 5475 nucleotides. DNA sequences of PE226 exhibited 9 open reading frames (ORFs) that were not highly similar to those of other phages infecting *R. solanacearum*. The phage PE226 carried unique ORF encoding zot-like protein with toxic activity and was also highly virulent to *R. solanacearum* strain SL341. The phage PE226 might be a typical temperate phage carrying both lytic and lysogenic activities (Murugaiyan et al. 2010).

The effectiveness of bacteriophages to suppress the development of grapevine Pierce's disease pathogen *Xylella fastidiosa* (*Xf*) was assessed. A functional *Xylella* phage named Xfas53 was evaluated. The plate overlay method was employed to screen a 30 × 30 matrix, using each isolate as an indicator to test supernatant of isolates grown on PW-M broth. Plaque production due to dissolution of *Xf* cells was observed on plates seeded with pathogen cells. Individual plaques were excised from the overlay, suspended in phage buffer and titered. High titer lysates (10^{10} PFU mL⁻¹) were obtained by harvesting overlays of plates exhibiting confluent lysis. Observations under transmission electron microscope (TEM) revealed that the phage Xfas53 belonged to the family Podoviridae with a head diameter of 55 nm and a short noncontractile tail with a diameter of 12 nm. The isolates of *X. fastidiosa* from different locations were

sensitive to phage Xfas53. The results indicated the effectiveness of phage Xfas53 in suppressing the development of the pathogen *X. fastidiosa* and Pierce's disease in grapevine effectively (Gonzalez et al. 2008).

Bacteriophages specific to *Streptomyces scabiei*, causal agent of potato scab disease, isolated from soil samples were investigated by analyzing their genomes. The DNA fragment profiles obtained from restriction enzyme digestion were compared. Two genetically distinct phages infecting *S. scabiei* were designated Stsc1 and Stsc3. Phage morphology was recorded using electron microscopy. The phages Stsc1 and Stsc3 had a binal symmetry with an icosahedral head and a long striate tail. The phages Stsc1 and Stsc3 infected 81% and 75% of the pathogenic strains of *S. scabiei* strains tested. The phages suppressed the development of symptoms of infection by *S. scabiei* under in vitro conditions, indicating the potential of the phages which have to be tested under field conditions (Goyer 2005).

Two bacteriophages were able to lyse the cells of *Burkholderia plantarii* and *B. glumae* and to suppress the development of rice seed rot and seedling blight diseases caused by these bacterial pathogens. Phage BGPP-Ar more effectively suppressed disease development than the bactericides available in the market. The ratio of seedlings exhibiting infection to the total number of seedlings examined after treatment with BGPP-Ar (1×10^8 PFU mL⁻¹) were 0.0 for seed rot and 2.0 for seedling blight, compared to the ratios of 14.3 and 15.0 for chemical treatments (ipconazole and copper II hydroxide) for seed rot and seedling blight, respectively. BGPP-Ar treatment was highly effective in suppressing seedling rot of rice, even at a low concentration of 1.0×10^5 PFU mL⁻¹. The best BGPP-Ar phage treatment effect for sterilizing seed was achieved indoors to avoid phage inactivation by UV irradiation. Seed treatment with phage was effective in reducing seedborne infection by *B. glumae* and *B. plantarii* (Adachi et al. 2012).

Pantoea ananatis, incitant of bacterial palea browning disease of rice infects during flowering and degrades the quality of rice appreciably in Japan. Phages lytic to both pathogenic and nonpathogenic isolates of *P. ananatis* were isolated from the inflorescence of eulalia (*Miscanthus sinensis*), a graminaceous weed. One of the phage isolates was sprayed with and without a nonpathogenic isolate of *P. ananatis* on rice plants at the flowering stage. Coapplication of the phage and nonpathogenic *P. ananatis* suppressed the disease in sunlight. Application of the light-labile phage itself was able to suppress development of bacterial palea browning disease. The phage retarded the growth of the pathogen on rice plants and also on LB medium. The nonpathogenic *Pantoea* strains were present abundantly on rice panicles at flowering stage and could be hosts of the phage. The optimum infection period of rice with *P. ananatis* was found to be the flowering stage. The disease suppression was therefore considered to be due to the combined activities of the phage and naturally inhabiting nonpathogenic bacteria during the short period of susceptibility of the inflorescence to bacterial infection (Azegami 2013).

13.1.3.1 Mild Strains as Biological Control Agents

Mild strains of a virus have been demonstrated to protect plants against infection by severe strains of the same virus or related virus(es). This phenomenon of cross-protection has been effectively exploited as a practical disease management tactic in the case of diseases caused by *Citrus tristeza virus* (CTV) and *Tomato mosaic virus* (TMoV). The mild strains may be selected from naturally occurring strains or may be generated

artificially by exposure to chemicals, UV-irradiation, or unusual temperature (high or low) regimes. Systemic infection of a plant with a mild or attenuated strain inhibits or restricts secondary infection by a related or more virulent strain of the same virus. The absence of additional symptoms due to the challenging virus was considered to indicate that the protecting and challenging virus isolates are related, whereas the production of symptoms due to the challenging virus isolate is indicative of a lack of relationship with the protecting (challenged) strain of the virus (Narayanasamy 2002). Cross-protection as a management strategy was most successfully applied by inoculating citrus plants with a mild strain of *Citrus tristeza virus* (CTV). In Brazil, 3 of 45 mild strains of CTV were able to offer satisfactory levels of protection against severe strains of CTV in Pera sweet orange, two strains in Galigo lime, and one strain in Ruby grapefruit cultivars. No evidence of breakdown of cross-protection was observed in about 5,000,000 preimmunized citrus planted during a period of 10 years (Costa and Muller 1980). Cross-protection of citrus plants employing mild strains of CTV was adopted on a commercial basis in several countries such as Australia, Japan, and South Africa. However, the need to carefully adopt the cross-protection as a disease management strategy was highlighted by later investigations. Constant monitoring of the strain composition in a geographical location has to be carried out. Sensitive and precise molecular techniques have to be employed for detection and identification of CTV strains occurring in different locations, as the traditional biological indexing requires a long time to provide results. The limited sensitivity/specificity of detection procedures has hampered their usefulness to determine the pathogenic potential of unknown isolates of *Citrus tristeza virus* (Garnsey et al. 2005; Sideburth et al. 2005).

Preimmunization of Marsh grapefruit trees with the mild strain (PB61) of *Citrus tristeza virus* (CTV) protected the plants for 35 years against the severe strains, causing stem pitting (Broadbent et al. 1995). The efficacy of the mild strains PB61 to protect seedlings against superinfection with a severe grapefruit stem-pitting isolate PB219 or two orange stem-pitting (OSP) isolates PB155 or PB235 was assessed in the glasshouse. Symptom development was monitored by visual observations and the presence of each isolate was determined using isolate-specific restriction fragment length polymorphism (RFLP) analysis of amplicons generated by RT-PCR assays. Preimmunization with PB61 provided partial protection against superinfection using aphid-inoculation and delayed superinfection through graft inoculation. Preimmunization with PB61 did not ameliorate the expression of OSP symptoms once superinfection with OSP-inducing isolates was initiated. The results suggested that mild strain cross-protection (MSCP) did not prevent movement of the challenge virus since, once superinfection has occurred, the challenge virus could be detected both in the shoots and feeder roots, indicating systemic movement of the challenge virus (Zhou et al. 2002).

Bark chips from six container-grown citrus trees infected with nondecline-inducing *Citrus tristeza virus* (CTV) isolates and maintained in a vector-free greenhouse for 10 years were used as sources of virus inoculum. A total of 15 commercial grapefruit (*Citrus paradisi*) trees and 16 commercial sweet orange (*C. sinensis*) trees were used to inoculate 3 indicator plants each of sweet orange cultivar Madam Vinous, sour orange (*C. aurantium*), grapefruit cultivar Duncan, Mexican lime (*C. aurantifolia*), Swingle citrumelo, and sour orange grafted with sweet orange cultivar Hamlin. All source plants tested positive by ELISA tests for Florida decline-inducing isolates of CTV, using MAB MCA13. All inoculated trees (except Swingle citrumelo resistant to CTV) were positive for CTV by ELISA after 6 months in vector-free greenhouses. Some indicator plants

were inoculated with the inoculum of decline-inducing strain from 9 of the 37 bark chip source plants. Some of the ELISA-positive indicator trees exhibited vein clearing symptoms, characteristic of severe isolate of CTV. None of the control, noninoculated indicator trees in the same greenhouse was infected by mild or severe strain of CTV. The results indicated that decline-inducing isolates of CTV might be present as a minor component of a mixture at levels undetectable by ELISA and the decline-inducing strains could be detectable later by ELISA or by symptom expression in indicator plants (Powell et al. 2003).

The mild strain of CTV PB61 protected preimmunized seedlings more effectively against OSP isolate PB235 than PB155. The nucleotide sequence identity between PB61 and PB235 (97.6%) was much greater than between PB61 and PB155 (91.2%). Cross-protection seemed to be more effective if the nucleotide sequence identity is greater between the interacting CTV isolates. RNA-mediated defense was suggested to be involved in the cross-protection between CTV isolates (Zhou et al. 2002). The mild isolates of CTV had to be accurately differentiated from the isolates inducing severe symptoms of seedling yellows (SY) and or stem-pitting (SP) in grapefruit or sweet orange. A quantitative real-time PCR assay was developed to identify 56 biologically characterized CTV isolates from 20 countries. This procedure, employing a general primer set and three TaqMan-locked nucleic acid (LNA) probes, was found to be effective in estimating the ratio of different genotypes of CTV present in virus populations in samples from different geographic locations (Ruiz-Ruiz et al. 2009).

Grapevine crops are seriously affected by *Grapevine fan leaf virus* (GFLV) transmitted by the nematode vector *Xiphinema index*. Mild isolates of GFLV and the closely related *Arabidopsis mosaic virus* (ArMV) were identified by comparative performance analysis of grapevines infected with different strains and comparative symptom severity on assay host (*Chenopodium quinoa*) of GFLV. Healthy scions were grafted onto rootstocks that were healthy or infected with mild protective strains of GFLV-GHu or ArMV-Ta. Challenge inoculation using the nematode vector was monitored over 9 consecutive years in control and ArMV-Ta-cross-protected vines using DAS-ELISA format employing GFLV-specific antibodies. Immunocapture (IC-) RT-PCR-RFLP technique was applied to detect GFLV infection in GFLV-GHu-cross-protected grapevines. Cross-protected vines showed a significantly low challenge infection rate consistently. However, both mild strains reduced fruit yield by 9% (ArMV-Ta) and 17% (GFLV-GHu) over 8 years and had limited adverse effect on fruit quality. Use of mild strains for the protection of grapevines against GFLV therefore did not appear to be an acceptable alternative for the management of grapevine fan leaf virus disease (Komar et al. 2008).

Sweet potato feathery mottle virus strains cause russet crack and skin discoloration of storage roots of sweet potato (*Ipomoea batatas*), making the tubers unacceptable to consumers. Strains of SPFMV were distinguished based on symptoms, amino acid sequence identities, and serological properties. A mild isolate 10-0 of SPFMV rarely caused skin discoloration of storage roots, and russet crack symptoms were not observed as in the case of infection by severe strains of SPFMV-S. Sweet potato plants inoculated with the mild strains 10-0 did not contract latent infection by SPFMV-S as indicated by RT-PCR-RFLP analysis. The mild strain 10-0 did not reduce the yield of storage roots compared with that of healthy sweet potato plants. The coat protein (CP) gene of 10-0 encoded 315 amino acids and showed sequence identities of 91.1% at the nucleotide level and 95.6% at the amino acid sequence level with the corresponding region of SPFMV-S. Based on the nucleotide sequence of its 3' noncoding region and CP gene,

the strain 10-0 was genetically shown to have greater similarity to SPFMV-S. Sweet potato was rarely infected with 10-0 strain under natural conditions. Hence, *Ipomoea nil* plants were graft-inoculated with 10-0 to prepare cuttings infected by the strain 10-0. The mild strain 10-0 could be maintained in sweet potato in subsequent generations and the characteristics of infection by the mild strains were stable in infected plants. The results indicated that the mild strain had the potential for wide application for the protection of sweet potato plants against SPFMV-S strain (Yamasaki et al. 2009).

Plant virus strains with reduced virulence may be generated by exposing infected plants to high or low temperatures for predetermined duration or to treatments with chemicals such as nitrous acid or UV-irradiation. The variants with low virulence are evaluated for their ability to protect plants against severe strains of the same virus. The mutant MII-16 of *Tomato mosaic virus* (TMoV) was generated by artificially exposing the purified virus preparation to nitrous acid. The mutant MII-16 was mechanically inoculated onto tomato seedlings using a spray gun with adequate pressure (Fletcher and Rowe 1975). The mutant strain has been extensively used to protect tomatoes grown in glasshouses in England and Netherlands. An increase in yield of tomatoes up to 10% was obtained by protecting the tomato plants with the mutant strain MII-16 (Channon et al. 1978). An attenuated strain LIIA of TMoV was found to be effective in protecting tomatoes against severe strains (Motoyoshi and Nishiguchi 1988). Attenuated mutants of *Potato virus Y* (PVY) necrotic strain were generated by nitrous acid treatment and also by a novel method of mutagenesis-in-tissue culture. The procedure relied on the natural or experimental generation of virus sequence variants within an infected plant followed by isolation of the mutants by serially cloning them in plants. A total of 15 attenuated mutants was generated. Nucleotide and amino acid sequences of the genomes of the attenuated mutant populations were compared with the wild-type isolates and the amino acid changes in relevant genomic regions for viral attenuation were inferred. Many of the mutations were located in the protein 1 (P1) and helper component proteinase (HC-Pro) encoding regions. Amino acid changes mostly involved simultaneous changes in two or more protein encoding regions, one of which was often in the HC-Pro encoding region. The attenuated mutants M-MY10 and N-NA10 were effective in providing protection against the wild-type parent isolate NTND6 of *Potato virus Y* (Ogawa et al. 2013).

Attenuated strains of *Soybean mosaic virus* (SbMV) were generated by exposing infected soybean plants to low temperature. The soybean seedlings inoculated with SbMV were maintained at 15°C for 14 days or alternatively for 30 days. Two attenuated strains of SbMV, Aa15-M1, and Aa15-M2 generated in this way showed similar serological and biochemical properties as the parent strain Aa of SbMV. However, the attenuated strains induced different types of symptoms and varied in aphid- and seed-transmissibility and accumulation in soybean plants. When the soybean plants preinoculated with attenuated virus isolates were challenged with wild-type parent strain, they produced mild mottle symptoms on seeds with a normal colored seed coat (Kosaka and Fukunishi 1993). The effectiveness of attenuated *Soybean mosaic virus* (SbMV) Aa15-M2 in protecting black soybean seedlings under field conditions was assessed. After protective inoculation with SbMV Aa15-M2 strain, about 20,000 and 78,000 seedlings of black soybean cultivar Shin Tambaguro were planted in 1990 and 1991, respectively, in seed farms in Japan. The incidence of virulent strains of SbMV was effectively suppressed by the attenuated strain. Seeds virtually free of SbMV were produced in both years. Black soybean seedlings grown from these seeds were widely

transplanted in growers fields in 1991 and 1992. The incidence of virulent strains of SbMV in late July (preflowering stage about 40 days after transplanting) was 1.3% in 1991 and 0.8% in 1992, whereas the rates of SbMV incidence in earlier years was over 60%. The attenuated SbMV strain Aa15-M2 was found to be very effective in suppressing the infection of soybean plants by severe strains and also in reducing seed transmission of SbMV to subsequent generation (Kosaka and Fukunishi 1994).

A similar approach was adopted for protecting gladiolus plants against *Bean yellow mosaic virus* (BYMV) using a low-temperature treatment procedure to generate attenuated strain(s). A promising attenuated isolate M11 of BYMV was obtained after the low-temperature treatment of severe isolate originally obtained from gladiolus. An efficient method for inoculating mericlone gladiolus seedlings with M11 isolate was developed to prevent severe symptoms on gladiolus (Nakazono-Nagaoka et al. 2004). The effects of cross-protection by *Bean yellow mosaic virus* (BYMV)-M11 obtained after low-temperature treatment against six BYMV isolates derived from gladiolus, broad bean (*Vicia faba*), and white clover (*Trifolium repens*) were determined by employing Western blotting and RT-PCR assay. The levels of cross-protection differed based on the challenge virus isolates. The attenuated strain provided complete protection against BYMV isolates from gladiolus, whereas only incomplete protection was conferred against BYMV isolates from other hosts. Infection by an isolate of *Clover yellow vein virus* was partially suppressed by BYMV-M11 strain. Higher homology of nongladiolus isolates appeared to be the crucial factor determining the extent of cross-protection by the attenuated strain. Phylogenetic analyses indicated that the five gladiolus isolates clustered together, completely separated from three BYMV isolates from other hosts. A comparison of the amino acid sequences between M11 and its parental isolate IbG and analysis of recombinant infectious clones between M11 and IbG showed that an amino acid at position 314 was involved in the alteration of *Bean yellow mosaic virus* (Nakazono-Nagaoka et al. 2009).

The attenuated strains obtained from severe parental strains of *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), and *Watermelon mosaic virus 2* (WMV-2) were evaluated for their effectiveness in cross-protecting plants against the respective viruses. No significant difference could be seen between single and multiple inoculations in the cross-protection by the attenuated virus isolates against their respective virulent strains, in spite of virus accumulation. This indicated that the cross-protection effect of an attenuated virus isolate was not affected by the presence of unrelated attenuated virus particles in the same plant. In addition, the protecting strain exhibited its efficacy when it had reached a minimum concentration or before it reached its highest concentration. The degree of cross-protection was correlated with the concentration of the protecting strain in the plants. The cross-protection effectiveness of CMV-mild and ZYMV-mild was not as good as that of WMV-mild which provided complete protection against severe strain under greenhouse conditions. Multiple inoculation of cucumber with mild isolates might be effective for reducing losses due to concurrent infection by CMV, WMV-2, and ZYMV, which might lead to greater losses than would occur due to multiple inoculations of mild isolates (Kosaka and Fukunishi 1997). Another attenuated strain ZYMV-2002 was isolated from a virulent isolate of ZYMV after repeated exposure to low-temperature treatments at 12.5–15°C for about 2 months, followed by five cycles of single plant transfer. This isolate ZYMV-2002 differed from the wild-type isolate in its inability to produce local lesions on *Chenopodium quinoa* and its ability to induce very mild or no symptoms on

cucurbit plants. Cucumber plants preinoculated with ZYMV-2002 isolate showed delayed and suppressed expression of mosaic symptoms and/or deformed leaves characteristic of challenge isolates. Plants preimmunized with ZYMV-2002 had fruit productivity similar to that of healthy control plants under field conditions. Poor aphid transmissibility of ZYMV-2002 isolate was considered as a desirable attribute of attenuated viruses to minimize the risk of economic losses due to natural spread of the mild isolate to other crops in which it might become virulent (Kosaka et al. 2006).

The attenuated strain CM95 of *Cucumber mosaic virus* (CMV) was evaluated for practical application to protect cucumber plants against severe strain of CMV under field conditions. The molecular technique RT-PCR-RFLP was employed to differentiate strain CM95 from severe isolates of CMV rapidly. In cross-protection tests, the effectiveness of protection by CM95 was evaluated by RT-PCR-RFLP procedure on challenge-inoculated leaves at 14 days after preimmunization. The results were corroborated by those of a concomitant experiment conducted to assess symptom expression on upper leaves at 1 month after inoculation. The RT-PCR-RFLP protocol provided reliable results on the cross-protective ability of CM95 in 15 days, whereas the traditional bioassay required 30 days to obtain the results (Nakazono-Nagaoka et al. 2005). *Cucumber mosaic virus* (CMV) encodes the 2b protein, a suppressor of RNA silencing, triggered following infection of susceptible host plants. The 2b protein could also act as a symptom determinant and induces disease symptoms by interfering with microRNAs-mediated gene regulation (Lewsey et al. 2007).

Genetic engineering of viruses may provide a means of designing and generating mild, potentially cross-protective, virus strains. If RNA silencing is the mechanism operating in plants cross-protected by mild strains, then a mutant virus strain lacking the ability to express a silencing suppressor would be a potent cross-protecting agent which might act as a trigger of silencing against its own RNA and homologous viral RNA sequences. The ability of the CMV mutant CMV Δ 2b which could not express 2b silencing suppressor protein (2b) to protect plants against CMV strains was assessed. A CMV mutant of the subgroup IA strain Fny (Fny-CMV Δ 2b) could not express the 2b silence-suppressing protein cross-protected tobacco and *Nicotiana benthamiana* plants against disease induction by wild-type Fny-CMV strain. However, protection was most effective only if inoculation with Fny-CMV Δ 2b and challenge inoculation with wild-type CMV was performed in the same leaf. In addition, Fny Δ 2b mutant also protected plants against infection by TC-CMV, a subgroup II strain not closely related to Fny-CMV. In situ hybridization analysis showed that Fny-CMV Δ 2b and Fny-CMV wild-type isolate could coexist in the same tissues, but tissues contained zones of Fny- Δ 2b-infected host cells from which Fny-CMV, the wild-type strain, appeared to be excluded. The results suggested that protection by mutant isolate lacking CMV-2b protein expression was operated through either induction of very highly localized RNA silencing or by competition between strains for host cells or resources (Ziebell et al. 2007).

Attenuated strains generated by genetic modification of a locally prevalent severe strain may result in greater level of cross-protection. Three amino acid changes, Arg₁₈₀ $\rightarrow$ Ile₁₈₀ (GA mutation), Phe₂₀₅ $\rightarrow$ Leu₂₀₅ (GB mutation), and Glu₃₉₆ $\rightarrow$ Asn₃₉₆ (GC mutation) of the conserved motifs of the helper component, protease (HC-Pro) of a severe strain TW-TN3 of *Zucchini yellow mosaic virus* (ZYMV), were generated from an infectious cDNA clone that carried a green fluorescent protein (GFP) reporter. These three mutants induced milder symptoms on squash plants. Single substitution in these three amino acids apparently affected the virulence of the mutants and also significantly

reduced accumulation of GFP. The GAC mutant conferred complete cross-protection against the parental virus at 5 days after protective inoculation. Accumulation of challenge virus (parental virus) was inversely related to the increase in the length of protection, as shown by the absorbance values of ELISA tests. The GAC mutant was stable, as reflected by the observation that no revertants with severe strain sequences were found after nine passages through squash plants after a period of 9 months. The results indicated the potential of the GAC mutant for effective protection of squash plants against severe strain of ZYMV (Lin et al. 2007).

An attenuated mutant strain of *Zucchini yellow mosaic virus* (ZYMV) without the GFP reporter derived from GAC mutant was evaluated for its aphid transmissibility and effectiveness of cross-protection in cucurbits. Apterous aphids *Myzus persicae* were provided a 10 min acquisition access on plants inoculated with a severe strain of TN3 or attenuated ZAC strain. The mutant ZAC isolate was not transmitted by the aphid vector, even though the level of virus acquisition was equally high as that of the severe strain. Squash plants protected with ZAC strain remained symptomless and appeared normal. The presence of the severe strain T3 could not be detected in plants protected by ZAC strain at 5 and 10 days after challenge inoculation with the severe strain T3 using ELISA tests, indicating that protection provided by the attenuated strain was complete. The results of ELISA tests also revealed that replication of the challenging strain T3 was entirely inhibited in protected plants. The ZAC mutant could protect other cucurbit crops, watermelon (*Citrullus lunatus* cultivar Fu-Bo No.2), cucumber (*Cucumis sativus* cultivar Da-fong No. 2), and oriental melon (*Cucumis melo* cultivar Silver Light) grown in Taiwan. The lack of aphid transmissibility of the mutant could be an additional desirable factor for its use to cross-protect the cucurbit crops (Chao et al. 2010).

The attenuated strain KM3 of *Chinese yam necrotic mosaic virus* (CYNMV) selected from among CYNMV field isolates induced a mild mosaic symptom on leaves of Chinese yam (*Discorea opposita*) cultivar Nagaimo during the early stages of growth and the severity of symptoms decreased with plant growth. Aphids were used to inoculate KM3 to protect yam plants against severe strains of CYNMV YS117 and IW5. The RT-PCR-RFLP procedure was applied to differentiate KM3 from other field isolates of CYNMV. The nucleotide sequence of attenuated strain KM3 offering effective protection against severe strain of CYNMV under field conditions was determined for use in the identification of the mild strain (Kondo et al. 2007). Another attenuated strain T-3 of *Japanese yam mosaic virus* (JYMV) was evaluated for its cross-protection potential against virulent (native) strains in Chinese yam (*D. opposita*) grown in farmers' fields in Japan. The presence of native strains of JYMV could be detected by PCR-based assay in all Chinese yam plants grown from virus-free tubers in the first growing season in the fields, but the native strains were detectable in only one of 50 plants grown from tubers preimmunized with T-3 strain during 6 years of experimentation. The preimmunized plants yielded significantly more than the unprotected plants, indicating the effectiveness of the attenuated strain T-3 of JYMV not only in protecting the Chinese yam plants but also in increasing the yield from protected plants (Kajihara et al. 2008). The effectiveness of an attenuated strain in protecting Chinese yam (*Dioscorea opposita*) against infection by *Chinese yam necrotic mosaic virus* (CYNMV) was assessed under field conditions. The attenuated strain protected the Chinese yam plants against CYNMV strain effectively for 5 years under field conditions. In addition, yields of total tubers and grade A tubers were increased by 25 and 57% in protected crops compared with unprotected crops after 5 years of cultivation. Plants protected by KM3 strain produced

greater yield (31%) over that of unprotected plants grown from virus-free seed tubers. The results showed that the attenuated strain KM3 could be employed to reduce the infection by CYNMV in Chinese yam (Kondo et al. 2015).

13.1.4 Mechanisms of Action of Biological Control Activities of Biotic Agents

Biotic biological agents (BCAs) may act directly or indirectly on plant pathogens/host plants by one or more mechanisms: (i) the ability to inhibit or destroy the pathogen by producing toxic metabolites (antibiosis); (ii) competition between the antagonist and the pathogen for essential food materials such as carbohydrates, nitrogen, growth factors and/or space; (iii) parasitism of the pathogen by the antagonist, resulting in necrosis or death of the pathogen; (iv) prevention of pathogen colonization of host tissues; and (v) induction of resistance in host plants.

13.1.4.1 Mechanisms of Action of Fungal Biological Control Agents

The biocontrol activity of *Trichoderma harzianum* against *Phytophthora capsici* was investigated. *T. harzianum* inhibited the spread of mycelial growth of *P. capsici* in petriplates. It also invaded the whole surface of the pathogen colony later and sporulated over the pathogen. The hyphae of *P. capsici* were surrounded by those of *T. harzianum*, resulting in their subsequent disintegration and eventual suppression of the growth of *P. capsici*, as observed under the scanning electron microscope (SEM) (Ezziyyani et al. 2007). In a later investigation, the interaction between *T. harzianum* T472 strain and *Gibberella zeae* (anamorph *Fusarium graminearum*) was studied using scanning electron microscope (SEM). In the presence of T472 strain in the inoculated wheat straw, *G. zeae* produced only an average of 15 perithecia as against an average of 167 perithecia in the absence of T472. At 15 days after inoculation (dai), mycelia and numerous spores of T42 could be visualized covering the surface of the perithecia of *G. zeae* and colonization of the perithecia by T472 could be clearly viewed under SEM. The BCA colonized the substrate rapidly and out-competed the pathogen. The results revealed that the suppression of *G. zeae* development was effected through parasitism (Inch and Gilbert 2011).

Fusarium head blight (FHB) disease of wheat and other cereals caused by different *Fusarium* spp. has been a difficult problem without effective solution because of the complexities involved. The usefulness of the biotrophic mycopathogenic *Sphaerodes mycoparasitica* was assessed for the control of *Fusarium* pathogens. Germination of ascospores of *S. mycoparasitica* was greatly increased in the presence of *F. graminearum* strains, indicating a compatible interaction between the BCA and pathogen strains. A quantitative real-time PCR was developed employing the *Fusarium*-specific (Fg16N) and trichothecene *Tri5* (TOX-1/2) -specific primer sets. The amounts of DNA of *F. graminearum*, 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON) strains were reduced in the presence of *S. mycoparasitica*, indicating a significant reduction in pathogen population, when the BCA and the pathogen were coinoculated. *S. mycoparasitica* was able to germinate in the presence of *F. graminearum* culture filtrates and also establish biographic mycoparasitic relations with two *F. graminearum* chemotypes, suppressing their growth in vitro (Vujanovic and Goh 2011).

Alcohol percentage test (APT) and real-time PCR quantification of DNA were employed to evaluate the efficiency of *S. mycoparasitica* to replace an array of pathogenic/toxigenic *Fusarium* in cultures. Shifts in *Fusarium* mycelial growth and

hydrophobicity measured (APT) at the interaction zone with *S. mycoparasitica* indicated the degree of *Fusarium* mycelial replacement by the mycoparasite ($P < 0.05$), while qRT-PCR estimated changes in DNA quantities of *Fusarium graminearum*. The 3-ADON and 15-ADON chemotypes were challenged with *S. mycoparasitica*. HPLC analysis also revealed a considerable reduction in mycotoxin (DON, 3-ADON, 15-ADON, and zearalenone) concentrations at the contact zone. Estimated shifts in the DNA quantity and mycotoxin production, assessed by qRT-PCR and HPLC respectively, correlated with an alteration in APT values, demonstrating the effectiveness of the mycoparasite under natural conditions (Vujanovic and Chau 2012). *Gibberella fujikuroi* (anamorph *Fusarium moniliforme*), causing bakanae disease of rice, is seedborne. *Talaromyces* sp. KNB-422 isolate was evaluated for its biocontrol potential against *G. fujikuroi*. The fluorescent protein marker GFP and RFP were used to visualize cell-to-cell interactions between the BCA and the pathogen. In slide culture, the hyphal cell wall of *G. fujikuroi* collapsed, and fluorescence of its cytoplasm disappeared at 3 days after contact with the hyphae of *Talaromyces* sp. On inoculated plants, both fungi occupied the same regions of coleoptiles and roots, where the mycoparasite activity might be exerted on the pathogen (Kato et al. 2012).

Sclerotinia sclerotiorum is an economically important pathogen infecting oilseed rape and many other crops, accounting for substantial losses. The process of mycoparasitism of *S. sclerotiorum* infecting *Brassica* by green fluorescent protein (GFP-) transformed *Trichoderma harzianum* ThzID1-M3 strain was studied. A specific PCR primer/probe set for detecting the GFP-transformed isolate was generated. Quantitative real-time PCR was evaluated along with epifluorescence microscopy and image analysis to investigate the dynamics of colonization of sclerotia in nonsterile soil. It was possible to quantify the amounts of ThzD1-M3 DNA and *S. sclerotiorum* DNA from individual sclerotia using the real-time PCR assay. Epifluorescence from the transformant was quantified using computer image analysis to estimate colonization on a per-sclerotium basis. Colonization of sclerotia of *S. sclerotiorum* by *T. harzianum* on agar plates was observed using confocal laser scanning microscopy. The GFP-fluorescing hyphae of *T. harzianum* could be clearly seen. This procedure, although highly labor-intensive, provided high spatial resolution of colonization dynamics. Both methods quantified colonized sclerotia by the BCA over a period of time. The real-time PCR assay provided a more precise assessment of the extent of sclerotial colonization and could be more easily applied to sample entire sclerotia (Kim and Knudsen 2011).

Aspergillus strain ASP-4 belonging to the *Aspergillus aculeatus* clade of the black aspergilli was isolated from a field plot where oilseed rape had been planted for the past 20 years. The mycoparasitic activity of the strain ASP-4 on *S. sclerotiorum* was evaluated both in the laboratory and field plots over a 3 year period. The results clearly revealed the highly significant effect of the mycoparasite in reducing the numbers of germinating sclerotia and subsequent spread of the pathogen. Since *Aspergillus* sp. ASP-4 infected the sclerotia of the pathogen, it might be possible to fix the critical time and method of application of the BCA for the suppression of *S. sclerotiorum*. The results showed that the spore suspension of the BCA could be sprayed at early-mid-flowering season on the soil and after harvest (Hu et al. 2013b).

Rice seeds were found to be contaminated with mycotoxin citrinin produced by several fungi associated with rice seed. An average of 6.79×10^4 fungal spores per gram seeds was present, belonging to 47 species in 28 genera of which *Aspergillus*, *Cladosporium*, and *Penicillium* were predominantly found. *A. niger* and *A. flavus*

occurred more frequently than other fungi associated with rice seeds. Of the 30 rice seed samples, 10 samples were contaminated with mycotoxin citrinin. *Penicillium viridicatum* produced the highest amount of citrinin on glucose ammonium nitrate salts broth and rice grains. The fungal biocontrol agents *Trichoderma hamatum* reduced the amount of citrinin to the maximum extent. The growth of *T. hamatum* infecting *P. viridicatum* increased with lapse of time. The viability of *P. viridicatum* conidia was decreased by *T. hamatum* with an increase in the incubation period. Chitinases and β -1,3-glucanase activities of *T. hamatum* also increased with increased incubation period on *P. viridicatum* mycelia up to maximum values for 72 h and 84 h, respectively. Treatment of rice grains with *T. hamatum* infested by *P. viridicatum* reduced production of citrinin in rice grains relative to the control seed sample (Abd-Allah and Ezzat 2005).

Fusarium head blight (FHB) disease pathogens *Fusarium graminearum* and *F. culmorum* infecting wheat, maize, and barley produce the trichothecene mycotoxin, deoxynivalenol (DON). *Trichoderma viride* P1 is antagonistic to several plant pathogens as it produces an array of cell-wall-degrading enzymes (CWDEs) that act synergistically to advance parasitism. The endochitinase ECH42 is the most important among the CWDEs produced by the BCA and is encoded by *ech42* gene, whereas an N-acetyl- β -D-glucosaminidase was encoded by *nag1* gene. Disruption of the *ech42* gene reduced the biocontrol activity of the strain P1. The *nag1* gene was induced by low-molecular-weight chito-oligosaccharides and its own catabolic products, while *ech42* expression was indirectly induced by carbon starvation and other stress conditions (Mach et al. 1999; Woo et al. 1999).

The expression of these genes contributing to biocontrol activity was monitored in vitro and on crop residues of two maize cultivars by using *goxA* reporter gene fusions. The pathogen toxin DON repressed expression of *nag1* gene in *T. viride* P1. Expression of this gene was reduced by 50% in maize residues when the antagonist was placed in competition with DON-producing strains of *F. culmorum* and *F. graminearum*. By adding synthetic DON to assay mixtures with *Fusarium* strains that otherwise had no effect on the BCA, DON-induced repression could be reproduced, confirming the adverse effects of the pathogen on the expression of key enzyme involved in the biocontrol activity of strain P1. On the other hand, expression of *ech42* gene was neither positively nor negatively affected by DON or contact with *Fusarium* spp. The negative signaling could be an additional factor that might contribute to the inconsistent performance often observed with biocontrol agents (Lutz et al. 2003).

The interaction between the fungal pathogen *Fusarium moniliforme*, infecting maize kernels, and two antagonistic isolates of *Trichoderma harzianum* was investigated in respect of their secondary metabolites fusaric acid (FA) and 6-pentyl- α -pyrone (6PAP). Among 10 isolates of *F. moniliforme* screened for production of FA, isolate 8 produced a higher amount of FA. The mycelial growth and conidial production of both *Trichoderma* isolates T16 and T23 were retarded by increasing concentrations of FA in agar medium. Both *T. harzianum* isolates were able to degrade FA in potato dextrose medium and the isolates T23 and T16 reduced FA production by 51.4% and 88.4%, respectively, at 9 days postinoculation. The antifungal metabolite 6PAP isolated from *T. harzianum* T23 cultures reduced FA contents per gram mycelial dry weight significantly, compared to control. Dosages of 300 and 400 mg L⁻¹ of 6PAP in cultures retarded FA accumulations by 62.5% and 77.2%, respectively, providing evidence that the antifungal activity of *T. harzianum* was derived from the presence of 6PAP which might degrade FA, resulting in the reduction in the contents of *Fusarium* toxin FA (El-Hasan et al. 2008).

The biocontrol activity of *Clonostachys rosea* against *Fusarium culmorum* was shown to be associated with the secretion of cell-wall-degrading enzymes (CWDEs), including chitinases and expression of *N*-acetyl- β -glucosaminidase-encoding gene *cr-nag1* in *C. rosea*. Enzymatic assays and RT-PCR showed that the NAGase activity of *C. rosea* was specifically repressed in medium containing high glucose contents and was expressed in media containing chitin or *F. culmorum* cell walls, as sole carbon sources. *C. rosea* inhibited the mycelial growth of *F. culmorum* and the high expression of *cr-nag1* occurred only in the interaction between *C. rosea* and *F. culmorum*, but not with another fungal pathogen *Pythium ultimum*. The results indicated that the chitin-hydrolyzing agents targeted the cell walls of *F. culmorum*, resulting in the suppression of the mycelial growth of the pathogen (Mamarabadi et al. 2009).

A strain of *C. rosea* ACM 941 (American Type Culture Collection ATCC 7447) was evaluated for antibiosis against *Gibberella zeae* in vitro and for the control of Fusarium head blight (FHB) disease of wheat under greenhouse and field conditions, in comparison to the registered fungicide Folicur (tebuconazole). The strain ACM941 reduced mycelial growth of the pathogen by 52.6% in dual culture after 6 days and completely suppressed spore germination for 6h when cocultured with a macroconidial suspension of *G. zeae*. Strain ACM941 reduced *G. zeae* perithecial production by more than 99% in the leaf disk assay, 60–77% on infected corn kernels, and 32–57% on spikelet debris in the field. These effects of *C. rosea* strain ACM941 on *G. zeae* were similar to those induced by the fungicide. The mechanism of action of *C. rosea* on *G. zeae* was through antibiosis, resulting in the suppression of development of *G. zeae* infecting wheat and maize (Hue et al. 2009). In another investigation, the extracts from 11 isolates of *T. harzianum* and cell-free filtrates were screened for antifungal activity against perithecial development. Isolates T472 and T22 significantly reduced numbers of perithecia produced by more than 70%. Several compounds unique to T472 and T22 capable of suppressing perithecial development were isolated, indicating that these compounds might have a role in suppressing the development of *G. zeae* causing FHB disease of wheat (Inch et al. 2011).

A nonpathogenic *Fusarium oxysporum* antagonistic to *Sclerotinia sclerotiorum* was isolated from the suppressive soil. The antagonistic activity of *F. oxysporum* strain S6 was observed in dual culture plates. The toxic nonvolatile metabolites from *F. oxysporum* S6 strain were detected and differentiated using chromatographic techniques. They were purified and identified as cyclosporine A by spectroscopic methods. The antifungal activity against *S. sclerotiorum* was correlated with the presence of cyclosporine A by a dilution plate assay. The BCA also induced a similar adverse effect on mycelial growth and sclerotial production by *S. sclerotiorum*. Germination percentage of sclerotia was significantly reduced due to infection of sclerotia by the BCA. In the greenhouse experiment, the number of surviving soybean plants significantly increased when the BCA and pathogen were coinoculated. The results indicated that the fungal activity of *F. oxysporum* S6 against *S. sclerotiorum* was primarily due to secretion of cyclosporine A by the BCA (Rodriguez et al. 2006). In another investigation, inhibition of mycelial growth by *Trichoderma* spp. was attributed to production of volatile and nonvolatile inhibitors under greenhouse and field conditions. *T. koningii*, *T. virens*, *T. ceramicum*, and *T. viridiscens* provided maximum protection to potatoes against stem rot caused by *S. sclerotiorum* (Ojaghian 2011). Another fungal biocontrol agent *Clonostachys rosea* BAFC3874 strain isolated from soil suppressive to *S. sclerotiorum* effectively inhibited the infection of the pathogen in pot-grown lettuce and soybean plants. The production

of antifungal compounds against *S. sclerotiorum* with secondary metabolism by the BCA strain was observed. *C. rosea* BAFC3874 produced a microheterogenous mixture of peptides belonging to the peptaibols family. In addition to its ability to secrete the antifungal compounds, the strain BAFC3874 also showed mycoparasitic activity against *S. sclerotiorum* in the dual culture assays (Rodriguez et al. 2011).

The antagonists *Trichoderma* spp., nonpathogenic *Fusarium* spp., and isolates of *Clonostachys rosea* consistently suppressed sporulation of the pathogens *F. culmorum*, *F. graminearum*, *F. proliferatum*, and *F. verticillioides* causing wheat head blight or maize ear rot diseases. These biocontrol agents (BCAs) significantly reduced the colonization of stalk pieces by pathogen *Fusarium* spp. at several sampling dates. The results indicated that wheat head blight/maize ear rot pathogens might be suppressed by the competition with BCAs for nutrients available in plant tissues (Luongo et al. 2005). The colonization pattern of nonpathogenic *Fusarium oxysporum* endophytes was investigated by using *F. oxysporum* isolates transformed with green fluorescent protein (GFP) and red fluorescent (DsRed) genes. Root and rhizome tissue colonization by transformants were similar to that of wild-type isolates. DsRed transformants were difficult to visualize in tissues colonized by them. On the other hand, GFP-transformation was useful to differentiate the transformants from other saprophytic fungi present in the rhizosphere (Paparou et al. 2009).

The contamination of maize (corn) grains with *Aspergillus flavus* is of great concern due to the harmful effects of the mycotoxins secreted by the pathogen. The efficacy of two nonaflatoxigenic isolates of *A. flavus* CT3 and K49 in reducing aflatoxin levels in maize grains was assessed. These two nonaflatoxigenic isolates decreased the aflatoxin levels by 61% and 76%, respectively. The strain K49 capable of producing sclerotia grew rapidly and had greater ability to colonize maize grains than nonsclerotia producing CT3 strain following inoculation of maize grains, indicating its greater ecological (sphere) competence. The native strain K49 exhibited potential for application as a biocontrol agent for reducing aflatoxin levels in maize grains (Abbas et al. 2006).

Biofumigation with *Muscodor albus*, which produces volatile compounds inhibitory to the development of fungal pathogens, was evaluated for the suppression of development of smut pathogens. The effects of these volatiles on dormant and physiologically active teliospores of smut fungi *Tilletia horrida*, *T. indica*, and *T. tritici*, which cause kernel smut of rice, Karnal bunt of wheat, and common bunt of wheat, respectively, were assessed. Dormant and physiologically active teliospores were fumigated in dry petriplates using reactivated culture of *M. albus* for up to 5 days at 22°C. The germination of all three *Tilletia* spp. was totally inhibited when fumigated for 5 days. Biofumigation of smut fungi affecting rice and wheat using *M. albus* resulted in the inhibition of spore germination to a different extent when dry loose teliospores were exposed to the volatiles produced by the BCA. Teliospores within intact sori were not affected by the volatiles produced by *M. albus*. The results suggested that *M. albus* might be effective as seed or soil treatment for controlling seedling-infecting smuts whose infection is initiated by germinating teliospores prior to seedling emergence (Goates and Mercier 2009).

Helminthosporium solani, incitant of potato silver scurf disease, causes significant widespread economic losses. *Acremonium strictum* present along with *H. solani* in infected tubers showed distinct parasitic potential by reducing sporulation, spore germination, and mycelial growth of the pathogen, compared with control culture without *A. strictum*. The mycoparasite reduced conidial production of *H. solani* on minitubers,

resulting in a decrease in inoculum for further infection. *A. strictum* was able to protect healthy minitubers, but not the tubers already infected with *H. solani*. Scanning electron microscopic observations revealed shriveled and shrunken conidia of *H. solani* when contaminated with *A. strictum*, indicating that the mechanism of action of *A. strictum* on *H. solani* might be either direct parasitism or antibiosis by production of toxic metabolites (Rivera-Varas et al. 2007).

Microorganisms (fungi, bacteria, and actinomycetes) were isolated from soil using vegetable tissues as baits. Of the 70 microorganisms tested, 16 were able to produce substances in culture fluids with the capacity to suppress the development of rice blast pathogen *Magnaporthe grisea*. *Fusarium solani* KS-F14 secreted compounds with the potential to activate resistance in rice leaves and was selected for further testing. The resistance-inducing substances were effective at a range of pH values from 5 to 10 and were stable under high temperatures, maintaining the same level of activity even after autoclaving for 20 min. After application, the resistance induced in rice leaves persisted for 14 days. The majority of the secreted resistance-inducing compounds were hydrophilic and some were hydrophobic. The compounds inducing resistance produced by *F. solani* possessed the potential to be developed as a commercial product for the management of rice blast disease (Chern et al. 2014).

13.1.4.2 Mechanisms of Action of Bacterial Biological Control Agents

The bacterial biocontrol agents (BCAs) may act on the plant pathogens directly or indirectly by different mechanisms such as antibiosis, competition for nutrients, colonization of specific sites required for establishing infection, and inducing resistance to pathogens by activating host defense systems. Antagonism exhibited by bacterial BCAs depends predominantly on the ability to produce compounds inhibitory to the development of bacterial and fungal plant pathogens infecting various crops, or adversely affect pathogen development in other ways. Many bacterial BCAs belonging to different genera exhibit metabolites-mediated antagonism, resulting in the suppression of development of fungal and bacterial pathogens and consequently expression of disease symptoms. Root colonization is a process by which bacterial BCAs introduced through seeds or propagules or into the soil are distributed along roots. The plant growth-promoting rhizobacteria (PGPR) have dual roles in promoting plant growth and also in suppressing the development of phytopathogens, resulting in healthy and vigorously growing plants. The PGPR species belong to the genera *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Streptomyces*, and *Achromobacter*.

Among the antibiotics produced by the bacterial BCAs, the presence of phloroglucinols has been more commonly detected in the substrates. The antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), among phloroglucinols, has been investigated intensively. Two major phenotypic groups of 2,4-DAPG producers have been differentiated based on the production of antifungal compounds. One group produces 2,4-DAPG, HCN, and pyoluteorin and another group synthesizes only 2,4-DAPG and HCN (Raaijmakers and Weller 1998). The genes required for the biosynthesis of 2,4-DAPG by *Pseudomonas fluorescens* Q2-87 have been cloned and the biosynthetic locus includes *phlA*, *phlB*, *phlC*, and *phlD*. The limited distribution of *phlD* among bacteria facilitated its use as marker gene for 2,4-DAPG-producing *Pseudomonas* spp. present in the soil and other environmental samples to be detected by polymerase chain reaction (PCR) assay (McSpadden Gardener et al. 2001). Strains of *P. fluorescens* were screened for the production of 2,4-DAPG by using PCR-based screening procedure employing primers

Phl2a and Phl2b. A fragment of 745 bp characteristic of the biosynthetic genes was amplified. The purified antibiotic 2,4-DAPG from the BCA culture suppressed the development of *Xanthomonas oryzae* pv. *oryzae* (Xoo), causing rice bacterial leaf blight (BLB) disease. This antibiotic suppressed the BLB pathogen development up to 59–64% in the greenhouse and field trials (Velusamy et al. 2006).

The biocontrol potential of *Bacillus subtilis* NSR589-24 strain to suppress the development of two important rice pathogens *Magnaporthe grisea* and *Rhizoctonia solani*, causing rice blast and sheath blight diseases, respectively, was assessed. Antifungal compounds in the culture filtrates of the BCA strain inhibited the mycelial growth of *M. grisea* and *R. solani* and they remained stable after autoclaving, indicating their heat stability. In addition, heat labile β -1,3-glucanase was also purified from the culture filtrates of the strain NSR589-24. Both the purified enzyme and the antibiotic extract from the culture filtrate of the strain NSR589-24 separately inhibited *R. solani* and *M. grisea* with minimum inhibitory concentration (MIC) values of 12.5 and 6.25 mU mL⁻¹ and 3.13 and 1.56 μ g mL⁻¹, respectively. The glucanase enzyme in combination with antibiotic showed a strong synergistic inhibitory effect on the hyphal growth of both fungal pathogens. The results indicated that NSR589-24 could effectively restrict the development of rice blast and sheath blight diseases because of the synergistic activity of the bioactive compounds produced by the biocontrol agent (Leelasuphakul et al. 2006).

A bacterial strain GC-B26 showing strong antifungal and antioomycete activity was isolated from a grassland soil in Korea. Based on the morphological, physiological, and biochemical properties, the strain GC-B26 was identified as *Pseudomonas aeruginosa*. The antibiotic G26A capable of suppressing the development of *Phytophthora capsici* was isolated from the culture filtrates of the strain GC-B26 using different chromatographic techniques. The EI mass and UV spectral analyses indicated that G26A was an analogue of phenazines. On the basis of NMR spectral data, G26A was confirmed as phenazine-1-carboxylic acid. *P. capsici* was highly sensitive to G26A, with mean inhibitory concentration (MIC) value of c. 5.0 μ g mL⁻¹. Treatment with the antibiotic effectively protected pepper plants against infection by *P. capsici* and also by anthracnose disease of cucumber caused by *Colletotrichum orbiculare*. The efficacy of the antibiotic produced by *P. aeruginosa* compared well with the fungicides metalaxyl and chlorothalonil (Lee et al. 2003).

The bacterial BCAs *Paenibacillus polymyxa* and *Bacillus subtilis* from commercial products were assessed for their antagonistic activity against *P. capsici*. *B. subtilis* inhibited the mycelial growth of *P. capsici*, whereas *P. polymyxa* suppressed formation of zoosporangium by *P. capsici*. The release of zoospores from zoosporangium was significantly reduced by both BCAs. The secondary metabolites of the BCAs targeted the pathogen at different stages of its life cycle (Kim et al. 2009). *Paenibacillus polymyxa* E681 strain suppressed the development of *Phytophthora capsici*, causing *Phytophthora* blight of pepper. Fusaricidin, a cyclic depsipeptide isolated from *P. polymyxa* E681 inhibited the development of *P. capsici*, with a minimum inhibitory concentration (MIC) of 16 ppm. The disease severity induced by *P. capsici* in plants treated with 0.1 ppm fusaricidin was 40%, whereas the severity of disease in water-treated control plants was 81.7% on post-treatment. Significant disease suppression ($P < 0.05$) was observed on treatment with fusaricidin (0.1 ppm) by foliar spray and soil drench. The disease severity was drastically reduced to 3.3% by soil drench of fusaricidin (0.1 ppm), while disease severity was as high as 83.3% in water-treated control in one of the two experiments conducted. In the second experiment, the effectiveness of fusaricidin was

considerably reduced in treated plants (Lee et al. 2013). The effectiveness of *Paenibacillus ehimensis* KWN38 in suppressing the development of *P. capsici* was assessed in a grass medium. High antifungal and lytic enzyme activities of *P. ehimensis* KWN38 on *P. capsici* were observed. The BCA strain consistently suppressed the development of Phytophthora blight disease under pot trial conditions (Naing et al. 2014).

The efficacy of *Pseudomonas chlororaphis* strain PCL1391 and *P. fluorescens* WCS365 in suppressing the development of three races of *Colletotrichum lindemuthianum* infecting bean plants was assessed. The strain PCL1391 was more effective in suppressing the development of bean anthracnose disease, while the strain WCS365 did not show any improvement over positive control. Further, combination of the two strains of the biocontrol agents (BCAs) was not more effective than *P. chlororaphis* alone. *P. chlororaphis* inhibited the growth, sporulation, and conidial germination of all three races of *C. lindemuthianum*. The antibiotic activity of *P. chlororaphis* was considered to be due to its ability to produce phenazine-1-carboxamide which was detected using the thin-layer chromatography (TLC) technique. The results revealed the effectiveness and the mechanism of action of *P. chlororaphis* in suppressing the development of the pathogen and the anthracnose disease induced by *C. lindemuthianum* in bean (Bardas et al. 2009). The strain PA23 of *P. chlororaphis* effectively protected canola and sunflower against *Sclerotinia sclerotiorum*. The BCA strain produced nonvolatile antibiotics phenazine and pyrrolnitrin as well as the volatile antibiotics nonanal, benzothiazole, and 2-ethyl-1-hexanol. In the greenhouse, the role of the nonvolatile antibiotics on root colonization and biocontrol potential of the strain PA23 against *S. sclerotiorum* on sunflower was investigated. Application of the strain PA23 alone or in combination with phenazine- and pyrrolnitrin-deficient *Tn* mutants resulted in significantly higher ($P=0.05$) root bacterial number and suppression of *Sclerotinia* wilt disease. The bacterial population decreased considerably and appeared to be negatively correlated with the number of antibiotics produced by PA23 strain. The results did not indicate any clear role of phenazine or pyrrolnitrin for the biocontrol activity of *P. chlororaphis* strain PA23 against *S. sclerotiorum* (Athurkoral et al. 2010).

Six endobacteria capable of producing a variety of volatile compounds were isolated from different plant species. Two isolates LCB01 and AVA02 produced volatile compounds inhibiting the mycelial growth of *Fusarium oxysporum* f.sp. *cubense* race 4 (Foc R4). The volatile compounds secreted by LCB01 (*Herbaspirillum* spp.) were the most effective with 20.3% inhibition of banana wilt pathogen. Volatile compound profiles indicated that inhibition of Foc R4 was due to single compounds such as 2-pentane-3-methyl, methanethiol, and 3-undecane or their synergistic action. The isolate AVA02 produced methanethiol and 3-undecene only and not 2-pentane-3-methyl, the absence of which might have resulted in lower level of inhibition (1.4%). Likewise, the absence or low levels of the three compounds in other four isolates of endobacteria resulted in their inability to inhibit the growth of Foc R4. The results suggested that the volatile compounds might be required to be produced in concentrations sufficient to inhibit pathogen growth effectively (Ting et al. 2011).

Pseudomonas aeruginosa LN strain, capable of producing bioactive compounds, was evaluated for its inhibitory effects of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), *X. axonopodis* pv. *Phaseoli* (*Xap*), and *X. axonopodis* pv. *citri* (*Xac*). The antibiotic activity of the compounds produced by *P. aeruginosa* was assessed under greenhouse conditions and by visualizing the effects under electron microscope. Chromatographic fractions EAP, VLC3, VLC4, VLC3d, and VLC4f were tested by disc diffusion assay.

Electron microscopic observations indicated that VLC4f affected the biofilm formation by the pathogen and cell morphology. The partially purified fractions of the compounds secreted by LN strain, when sprayed on the plants under greenhouse conditions, suppressed the development of disease symptoms. VLC4f fraction was the most effective and reduced the number of lesions induced by *Xam* and *Xac* on cotton and orange leaves, respectively, by 94% and reduced the disease severity caused by *Xap* in bean leaves by 73%. The results showed that fractions purified from culture filtrates had potential for use as an alternative to conventional bactericides applied for the management of diseases caused by *Xanthomonas* spp. (Spago et al. 2014).

The biocontrol efficacy of *Pseudomonas* strain LBUM223 in suppressing the development of potato scab disease caused by *Streptomyces scabies* was assessed. The pathogen produced the toxin thaxtomins involved in the induction of disease symptoms on tubers. The involvement of phenazine-1-carboxylic acid (PCA) in repressing thaxtomin biosynthetic genes *txtA* and *txtC* and suppression of potato common scab disease were investigated by employing a mutant deficient in PCA production (LBUM223 *phzC*⁻). Treatment of seed tuber treatment with strain LBUM223 prior to planting in pathogen-infested soil suppressed the disease development. A relationship was observed between PCA-producing ability of strain LBUM223 and its capacity to inhibit the growth of *S. scabies* and its ability to repress the biosynthetic genes *txtA* and *txtC*, and consequently in limiting the surface area of progeny tubers covered with common scab lesions (St-Onge et al. 2011).

The strain LBUM 300 was evaluated for its antagonistic efficacy to suppress the development of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), incitant of tomato bacterial canker disease. The strain LBUM300 produced both DAPG and HCN on *Cmm* under in vitro and in planta conditions. Nonsynthesizing isogenic mutants of producer strains were generated to dissect the role of individual metabolite on biological control of *Cmm*. *Pseudomonas* sp. strains LBUM223 and LBUM300 significantly repressed the growth of *Cmm* in vitro, while their respective nonproducing mutants showed less or no significant antagonistic activity. In planta, only the strain LBUM300 reduced disease development and *Cmm* rhizospheric populations. The results suggested that the production of DAPG and HCN contributed to the antagonistic potential of the strain LBUM300. Simultaneous production of DAPG and HCN by LBUM300 was considered as a desirable factor for commercialization of this strain for large-scale application for the control of tomato bacterial canker disease (Lanteigne et al. 2012).

Antibiotics-producing strains DF190 and PA23 of *Pseudomonas chlororaphis*, strain DFE4 of *Bacillus cereus*, and strain DFE of *B. amyloliquefaciens* were evaluated for their ability to suppress the development of canola black leg disease caused by *Leptosphaeria maculans* (anamorph *Phoma lingam*). The strains PA23 and DF190 produced phenazines (phenazine-1-carboxylic acid, 2 hydroxyphenazine) and pyrrolnitrin, while the strains DFE4 and DFE16 produced the lipopeptide antibiotics iturin A, bacillomycin D, and surfactin. The tests with representative strains PA23 and DFE4 showed a small but significant reduction in disease severity via a systemic response. The local split-inoculation (SPI) of the extract (for direct antagonism) indicated a significantly higher level of reduction of disease severity which was also consistent with the SPI of bacterial cells, establishing a more important role for the antifungal metabolites present in the culture filtrates for the direct suppressive activity on black leg symptoms. The BCAs could be effective due to successful colonization of the infection site which, in turn, most possibly

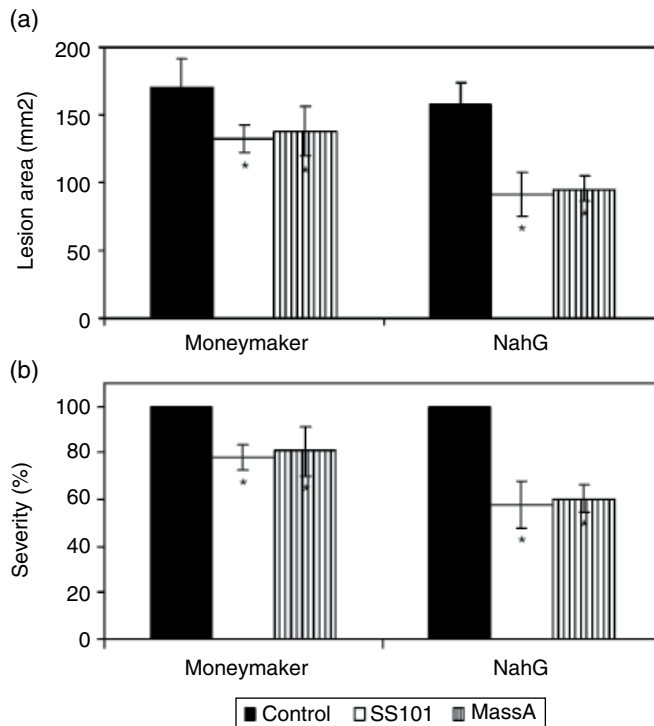


Figure 13.1 Induction of resistance to tomato late blight by application of *Pseudomonas fluorescens* SS101 or massetolide A to leaves of tomato cultivar Moneymaker and its transgenic derivative *nahG*. Effect on (a) lesion area (mm²) and (b) disease severity (%) determined at 7 days postinoculation with *Phytophthora infestans*; asterisk (*) indicates significant reduction in disease severity relative to control ($P < 0.05$); filled columns: control; blank columns: SS101 strain; lined columns: massetolide A. Source: Tran et al. (2007). Reproduced with permission of John Wiley & Sons.

acts as a suitable delivery system for the antifungal metabolites. Direct antifungal activity at the site of infection appeared to be the dominant mechanism mediating control of canola black leg disease (Ramarathnam et al. 2011).

Pseudomonas fluorescens SS101 strain produced a surfactant identified as massetolide A which suppressed the development of *Phytophthora infestans*, incitant of late blight disease of potato and tomato. *P. fluorescens* SS101 effectively prevented infection of tomato leaves by *P. infestans* and also restricted the expansion of lesions already formed and sporangial production. A massetolide A-deficient mutant 10.24 had significantly reduced biocontrol activity compared with wild-type strain (Fig. 13.1). When applied to the lower leaf, the mutant was significantly less effective in reducing the lesion area than the wild-type SS101 strain or massetolide A. Further, the lesion area was significantly reduced in lower leaves of *nahG* transgene treated with SS101 or massetolide A, demonstrating values similar to that obtained in the wild-type progenitor tomato cultivar Moneymaker. The results suggested that *P. fluorescens* SS101 or massetolide A might induce resistance in tomato plants, independent of SA signaling. Application of massetolide A on tomato leaves and roots effectively protected the treated plants against infection by *P. infestans*, indicating the important role of massetolide A in the biocontrol potential of *P. fluorescens* SS101 (Tran et al. 2007).

In a later investigation, the efficacy of the biosurfactant produced by *Pseudomonas koreensis* strain 2.74 in protecting potato plants against infection by *P. infestans* was assessed using the detached-leaf assay. Development of disease on treated leaves challenged with the zoospores of *P. infestans* was reduced significantly. The biosurfactant inhibited the motility of the zoospores, but caused only a minor reduction in mycelial growth rate. No adverse effect on the rate of sporangia production in pure culture was evident following treatment with bacterial surfactant (Hultberg et al. 2012a, b).

The antimicrobial activities of culture filtrates and selected metabolites of *Bacillus* spp. were assessed in vitro against *Xanthomonas campestris* pv. *campestris* (Xcc), causing cabbage black rot disease. All three species, *B. amyloliquefaciens*, *B. subtilis*, and *B. pumilus*, were found to be efficient producers of metabolites toxic to Xcc when they were grown at half-strength of trypticase soy broth (TSB) or cabbage broth (CB). TSB stimulated greater synthesis of metabolites compared with the control. *B. amyloliquefaciens* isolates produced surfactin, iturin, bacillomycin and/or azalomycin F, whereas *B. subtilis* isolates produced mostly surfactin and arthrobactin. The culture filtrates of *B. pumilus* contained surfactin, amphomycin, arthrobactin, and valinomycin. All three species of *Bacillus* produced surfactin in their cultures (Wulff et al. 2002). The ability of *B. subtilis* to inhibit the mycelial growth of *Fusarium verticillioides*, causative agent of Fusarium head blight (FHB) disease of wheat, and to reduce the accumulation of the mycotoxin fumonisin B1 was assessed in vitro. *B. subtilis* strain CE1 was the most efficient in inhibiting the mycelial growth of the pathogen as well as production of mycotoxin. The antibiotic activity of CE1 strain was negatively correlated with the population of *F. verticillioides* in the greenhouse tests (Cavaglieri et al. 2005). *Bacillus amyloliquefaciens* Lx-11 strain exhibited biocontrol activity against rice bacterial leaf streak (BLS) pathogen *Xanthomonas oryzae* pv. *oryzicola*, and secreted three kinds of lipopeptides including surfactin, bacillomycin D, and fengycin which inhibited the development of the pathogen. The antibacterial activity could be associated with surfactin and lipopeptides. The surfactin-deficient mutants lost their biocontrol activity, indicating that the surfactin was the principal compound involved in the suppression of the BLS disease development. Further, the defense-related genes *PR-1a*, *PR-1b*, *NPR1*, and phenylalanine-ammonia-lyase (PAL) were concurrently expressed in rice leaves following treatment with Lx-11 strain. Results suggested that Lx-11 might trigger systemic resistance in treated rice plants. *B. amyloliquefaciens* Lx-11 strain significantly reduced the incidence of rice bacterial leaf streak disease, the efficacy of disease suppression ranging over 60.2–70.6% which was better than what could be achieved by applying bismethiazol under field conditions (Zhang et al. 2012).

Bacteriocins are proteinaceous compounds secreted by bacterial species and exert harmful effects on closely related bacterial competitors present in the same substrate/environment. These toxic metabolites form a structurally and functionally diverse group with antimicrobial properties. Bacteriocin production by biocontrol agents such as *Pseudomonas* spp. may contribute to their rhizosphere competence (Parret et al. 2005). A bacteriocin-like substance (BLS) produced by *Bacillus licheniformis* P40 inhibited the soft rot bacterium *Erwinia carotovora*. The BLS exhibited a bactericidal effect on *E. carotovora* cells at $30\mu\text{g mL}^{-1}$. Observations under transmission electron microscope revealed wrinkled bacterial surfaces and shrinkage of whole cells due to treatment with BLS, indicating plasmolysis. BLS prevented spoilage of potato tubers by *E. carotovora* when tubers were treated at $240\mu\text{g mL}^{-1}$ or above concentrations. Development of soft rot was totally blocked when BLS at 3.7 mg mL^{-1} was used to treat the tubers. The results

indicated that *B. licheniformis* suppressed the soft rot of tubers induced by *E. carotovora* by producing bacteriocin-like compound (Cladera-Olivera et al. 2006).

Different bacterial species with biocontrol potential have been reported to produce antibiotics, lytic enzymes, and siderophores which are involved in the suppression of microbial plant pathogens to a different extent. Enzyme-mediated antagonism has been shown to be the principal mechanism of pathogen suppression in some crop–pathogen combinations. *Pseudomonas chlororaphis* strain PA23 was effective in suppressing the development of canola stem rot disease caused by *Sclerotinia sclerotiorum*. The pathogen produces ascospores from germinating sclerotia and they infect the flowers of canola. The ability of the strain PA23 to induce activities of chitinase and β -1,3-glucanase was assessed by spraying BCA suspensions on plants twice at 30 and 50% bloom, followed by challenge inoculation with *S. sclerotiorum*. The BCA-treated canola plants had significantly higher chitinase and β -1,3-glucanase activities. On the other hand, the activities of these enzymes were less in healthy control, ascospore-inoculated control, and strain PA23-treated plants. The activities of β -1,3-glucanase reached the peak at 6 days after inoculation (dai) and at 4 dai for chitinase. The activities of these enzymes declined slowly with time after ascospore inoculation (Fernando et al. 2007a).

The possible involvement of enzyme-mediated antagonism of *Phytophthora capsici* and *Rhizoctonia solani* by fluorescent *Pseudomonas* isolates GRC3 and GRC4 was examined. The antifungal potential of *Pseudomonas* was assessed by using dual culture technique on solid and liquid media. The activities of chitinases and β -1,3-glucanases and antifungal metabolites of nonenzymatic nature were negatively correlated with the inhibition of *P. capsici* and *R. solani*, indicating that the isolates of *Pseudomonas* might suppress the fungal pathogens through enzyme-mediated antagonism (Arora et al. 2007). *Bacillus pumilus* SG2 inhibited the mycelial growth of *Fusarium graminearum* and *Bipolaris sorokiniana*. The role of chitinases produced by *B. pumilus* in its biocontrol potential against the wheat pathogens was investigated. When grown in salt medium with colloidal chitin, *B. pumilus* SG2 strain secreted two chitinases into the medium, resulting in the inhibition of mycelial growth of *F. graminearum* and abolition hyphal elongation of *B. sorokiniana*. In the presence of glucose as carbon source however, the chitinases were not produced and the antifungal activity of *B. pumilus* SG2 was entirely abolished. The results showed that the expression of the chitinases in *B. pumilus* was under the control of two types of regulation: special regulation by chitin and global regulation by glucose. Chitinases were the principal compounds involved in the biocontrol activity of *B. pumilus* SG2 strain (Shali et al. 2010).

The rice blast pathogen *Magnaporthe grisea* was strongly inhibited by *Bacillus subtilis* KB-1122 strain in agar diffusion assays. The suppressive activity of *B. subtilis* was investigated using comparative proteome analysis. The cellular and culture supernatant (CSN) proteins were prepared from the cocultures of the pathogen and the biocontrol agent (BCA) and subjected to two-dimensional PAGE analysis. Proteome analysis revealed the presence of 33 cellular and 18 CSN proteins. The cellular proteins from *M. grisea* were downregulated, whereas upregulated proteins were derived from *B. subtilis*. The results suggested that glyceraldehyde-3-phosphate dehydrogenase and serine protein kinase might contribute to antifungal activity of *B. subtilis* KB-1122. The endo-1,4- β -glucanase involved in the degradation of polysaccharides was identified among the CSN proteins and was upregulated consistently at different times of incubation. This enzyme might play an important role in the interaction between *B. subtilis* KB-1122 with *M. grisea* P131 (Zhang et al. 2014).

The ability of *Achromobacter xylosoxidans* AUM54 strain was evaluated for the suppressive activity against rice blast pathogen *Magnaporthe grisea*. The antagonistic activity of AUM54 strain was revealed by the inhibition of mycelial growth of the pathogen under in vitro conditions. The BCA strain was able to survive in the spermosphere and spermaplane and was able to move systemically through roots and stems. Furthermore, plants treated with *A. xylosoxidans* AUM54 followed by inoculation with *M. grisea* showed a significant increase in the activities of defense-related enzymes such as polyphenoloxidase (PPO), peroxidase (POD), and phenylalanine ammonia lyase (PAL) and chitinase. Treatment of rice plants with *A. xylosoxidans* AUM54 significantly enhanced the growth of the plants (3–10% plant height) and yield (11–13%) of inoculated rice plants under conditions of no disease and disease in the greenhouse (Joe et al. 2012).

Paenibacillus kribbensis PS04 strain was evaluated for its biocontrol activity against the rice sheath blight pathogen *Rhizoctonia solani*. Microscopic observations on hyphal structures revealed that the morphology of the mycelium was strongly affected by *P. kribbensis* PS04 strain. Proteomic approach was applied to understand the inhibitor mechanisms. Using the two-dimensional gel electrophoresis technique, the differentially expressed proteins from *R. solani* GD118 in response to the association with *P. kribbensis* PS04 were identified. Protein profiling was employed to identify 13 differentially expressed proteins. Of these proteins, 10 were found to be downregulated while 3 were upregulated. These proteins were involved in material and energy metabolism, antioxidant activity, protein folding and degradation, and cytoskeleton regulation. Among them, material and energy metabolism was differentially regulated by the BCA. Protein expression was separately inhibited by *P. kribbensis* in oxidation resistance, protein folding and degradation, and cytoskeleton regulation. Assessment of proteome changes in the mycelium facilitated a better understanding of the direct suppression of the fungal pathogen by the bacterial biocontrol agents (Wang et al. 2014).

The antagonistic activity of *Streptomyces philanthi* RM1-138 strain isolated from the rhizosphere soil of pepper (chilli) in Thailand was assessed using the dual culture method against the rice sheath blight pathogen *Rhizoctonia solani* and other rice pathogens. The RM1-138 strain suppressed the mycelial growth of all pathogens tested with inhibition percentages varying over 82–89.2%, the inhibitory effect being most pronounced on *R. solani*. Heat treatments ranging over 40–100°C for 30 min and 121°C for 15 min did not affect the inhibitory effect of the culture filtrate of *S. philanthi* on *R. solani*, indicating the heat stability of the culture filtrate. The effective dose (>80% inhibition) of culture filtrate in liquid culture was at 5.0% (v/v), while it was 10% (v/v) on solid medium. The suppressive effect of the culture filtrate of *S. philanthi* on *R. solani* was similar to those of four fungicides tested. Observations under scanning electron microscope revealed that the metabolites of *S. philanthi* induced alterations in the cell-wall structure of *R. solani*, resulting in the loss of cytoplasmic materials by partial lysis. The greenhouse tests showed that culture filtrate or autoclaved culture filtrate of *S. philanthi* could be applied for effective suppression of development of rice sheath blight disease (Boukaew and Prasertsan 2014).

Resistance to crop diseases may be induced in plants by applying appropriate biotic or abiotic agents alone or in combination. Induced resistance may be broadly differentiated into two types: systemic acquired resistance (SAR) and induced systemic resistance (ISR). The SAR process is initiated in response to pathogen infection or chemical application, whereas colonization of plant roots by nonpathogenic plant growth-promoting

rhizobacteria (PGPR) leads to ISR. The PGPRs are capable of suppressing the disease development in aerial plant parts which are separated from the roots that are treated with BCA by eliciting ISR in several plant species (van Loon et al. 1998; Kloepper et al. 2004). The potential of *Pseudomonas fluorescens* 89B61 to induce ISR in tomato against late blight disease caused by *Phytophthora infestans* was evaluated. This strain elicited systemic protection against the late blight pathogen and reduced disease severity to a level equivalent to the SAR induced by the chemical inducer β -aminobutyric acid (BABA) in greenhouse experiments. Lack of colonization of tomato leaves by the BCA strain suggested that the ISR was due to systemic protection by this PGPR strain and not attributable to a direct interaction between the pathogen and the BCA strain (Yan et al. 2002). Treatment with *Paenibacillus illinoisensis* KJA-424 strain reduced root mortality by about 84% in pepper plants inoculated with *Phytophthora capsici*, incitant of Phytophthora blight disease. In pepper plants inoculated with *P. capsici*, peroxidase (POD) and superoxide dismutase (SOD) were significantly increased by 36.7% and 27.7%, respectively, at 7 days after inoculation. Treatment of pepper plants with KJA-424 strain inoculated with the pathogen resulted in an increase in the activities of POD and SOD at 5 days, but returned to the original level as in noninoculated plants. In addition, catalase activity fluctuated but again increased over a 7 day period. The results indicated that the BCA KJA-424 alleviated root mortality and suppressed the elevated activities of POD and SOD in pepper roots (Jung et al. 2006).

Fluorescent *Pseudomonas* strains (FPs) have been reported to be effective in suppressing the development of plant diseases via different mechanisms. Five isolates of *Pseudomonas fluorescens* applied as sett and soil treatment significantly reduced sugarcane red rot disease caused by *Colletotrichum falcatum* (teleomorph *Glomerella tucumanensis*) by inducing systemic resistance (ISR). The *Pseudomonas*-mediated ISR was significantly expressed at a higher level in the susceptible sugarcane cultivars than in the moderately resistant and moderately susceptible cultivars. Among the five *Pseudomonas* strains evaluated for their biocontrol potential against red rot disease at an endemic location, the strains CHA0, EP1, and Pf1 significantly reduced the red rot disease incidence in sugarcane under field conditions (Viswanathan and Samiyappan 2002). Another bacterial biocontrol agent *Pseudomonas chlororaphis* PA23 strain was shown to have multiple mechanisms of biocontrol activity in suppressing the development of canola stem rot disease caused by *Sclerotinia sclerotiorum*. In addition to its ability to suppress the pathogen development by antibiosis, two applications of the strain PA23 induced resistance against *S. sclerotiorum* in canola. Enhanced accumulation of PR proteins and oxidative enzymes including chitinase and β -1,3-glucanase by PA23 strain in canola leaf tissues might account for reduction in infection by *S. sclerotiorum*. The combination of antibiotic production with induction of ISR by *P. chlororaphis* PA23 strain could act synergistically and restrict the pathogen growth and colonization, resulting in significant suppression of disease incidence and intensity (Fernando et al. 2007b). Black leg disease of canola caused by *Leptosphaeria maculans* (anamorph *Phoma lingam*) was suppressed by *Bacillus cereus* DFE4 strain and *B. amyloliquefaciens* DFE 16 strain by producing the lipopeptide antibiotics iturin A, bacillomycin D, and surfactin, and also by inducing systemic resistance. The direct antifungal activity of antibiotics was considered a more dominant mechanism of black leg disease suppression (Ramarathnam et al. 2011).

Paenibacillus illinoisensis strain KJA24 was evaluated for its biocontrol potential for the suppression of *Phytophthora capsici*, infecting pepper (chilli) plants. The growth

response of pepper plants and kinetics of pathogenesis-related (PR) protein production were determined after challenge inoculation with *P. capsici* and combined inoculation with the pathogen and the biocontrol agent (BCA). Root mortality of plants treated with BCA and inoculated with *P. capsici* was significantly reduced. The activities of β -1,3-glucanase, cellulase, and chitinase were significantly increased in plants inoculated with the pathogen and BCA to greater levels compared with plants inoculated with the pathogen alone. The activities of these defense-related enzymes were negatively correlated with root mortality. The fresh weight of plants treated with *P. illinoisensis* strain KJA24 was increased, indicating that the bacterial BCA strain induced resistance to *P. capsici* in addition to promoting the growth of pepper plants treated with BCA (Jung et al. 2005). *Paenibacillus polymyxa* MB02-1007 strain exhibited antagonistic activity against *Ralstonia solanacearum* infecting several crops including potato and tomato. Most of the strains (14 of 16) did not interact with the pathogen directly by producing any inhibitory compounds in dual cultures. The chitinase activity was enhanced by *P. polymyxa* and chitosan (a known inducer of resistance to pathogens in treated plants) in the presence or absence of the pathogen. *P. polymyxa* increased chitinase activity as a soil drench or seed treatment by 68% and 78%, respectively. Changes in β -1,3-glucanase activity induced by the BCA were less marked compared with those of chitinase activity. The growth of plants treated with the bacterial BCA was substantially increased, in addition to suppression of the development of disease symptoms induced by *R. solanacearum* (Algam et al. 2010).

13.1.4.3 Mechanisms of Action of Mild Strains of Viruses

Different mechanisms of action of viral biocontrol agents have been indicated by various investigations. Cross-protection is the major mechanism by which the viruses protect the plants against virulent/severe strains. Different mechanisms of cross-protective activity of mild strains have been suggested to explain the suppression of severe symptoms induced by severe strains of the wild-type virus. The exhaustion of precursors for virus replication was proposed as a mechanism of action of mild strains of viruses such as *Tobacco mosaic virus* (TMV). In preimmunized plants, the mild strain may utilize all available precursors required for the establishment of the challenge virus, resulting in the failure of development of severe strain to reach titers sufficient to produce the characteristic symptoms of severe strain. Evidence for this suggestion was indicated by the finding that a significant correlation was shown between the decrease in the amount of microsomal RNA and the degree of cross-protection between the two strains of TMV (Hageman 1964). However, all viruses and strains have been reported to replicate in susceptible plants, utilizing the same amino acids and nucleotides available in plant hosts. The unavailability of these basic materials for virus replication being a limiting factor does not seem to be a possible mechanism of action of mild strains.

The virus strain used for preimmunization may occupy the specific site(s) required for its replication, resulting in the exclusion of the severe (challenging) strain from the same site(s) and consequent failure of infection by the severe strain. Requiring different sites for replication, the unrelated viruses may be able to infect the plants already infected by the mild strain used for preimmunization (Bawden 1950). The virus-specific RNA polymerase may be a site for which there may be competition between the protecting strain and challenging strain and the latter may have a competitive disadvantage with the strain already established in plant (Barker and Harrison 1978). The specificity

of ribosome-binding may be altered by the virus-induced polypeptide in favor of the protecting viral RNA. The mRNA of the protecting strain may occupy most of the available ribosomes, creating an unfavorable condition for the development of the challenging strain (Ziemiecki and Wood 1976). The phenomenon of cross-protection is generally observed between closely related viruses or strains, implying the existence of virus nucleotide sequence similarity required for competition. The results of investigation of cross-protection between strains of *Grapevine fan leaf virus* (GFLV) support the hypothesis of competition between virus strains as a mechanism of cross-protection. The rate of GFLV infection was markedly lower in plants initially infected with GFLV-GHu strain (19%) than in those initially infected with ArMV-Ta strain (40–55%) (Komar et al. 2008). The GAC mutant of *Zucchini yellow mosaic virus* (ZYMV) involving amino acid changes conferred complete cross-protection against the wild-type (parent) strain of ZYMV. The squash plants were resistant to ZYMV at 5 days after inoculation with GAC mutant. The definitive reaction was triggered as the mild strain initiated the infection process (within 5 days after protective inoculation). After stimulation of defense systems of the host plant, the defective (mutated) HC-Pro proteins of the GAC strain was unable to arrest the plant's defense responses, resulting in sharp reduction in accumulation of mild strain. Low levels of mild strain could be detected at this stage, indicating the ability of mild strain to survive. The low titer of the mild strain continued to stimulate and maintain the plant defense responses, resulting in a high level of protection against the severe strain of ZYMV. In the initiation stage of cross-protection, the protective action may result from competition for replication factors that are already used by the protective strain. At the maintenance stage, cross-protection is virus-dependent and a low titer of the protective virus is needed to stimulate the plants to continuously synthesize host factors to maintain the protection mechanism at the required level (Lin et al. 2007; Chao et al. 2010).

The phenomenon of cross-protection by mild strain against severe strain of the same virus has been suggested to be due to posttranscriptional gene silencing (PTGS). The protecting strain induces RNA silencing against its own RNA and homologous sequences of the closely related strains, and it may function as an elicitor of a natural antiviral response RNA silencing (Goldbach et al. 2003). Preimmunization of citrus with *Citrus tristeza virus* PB61 strain protected sweet orange seedlings more effectively against orange stem-pitting (OSP) isolate PB235 and OSP isolate PB155, possibly because PB235 had close nucleotide sequence homology to PB61 than to PB155. This hypothesis based on RNA-mediated defense was proposed to account for the cross-protection provided by the mild isolates of CTV. Infection with protective strain triggered the host to produce a ds-RNA-specific nuclease which targeted the viral RNA for degradation to low concentrations. This condition may lead to the formation of small nucleotide fragments such as those detected during PTGS. Other transcripts – homologous or those similar to the silenced gene – may also be degraded if they infect the plant after establishment of PTGS initiated by the CTV mild strain (Zhou et al. 2002). Involvement of posttranscriptional gene silencing as a mechanism for cross-protection of plants by mild strain against the severe strain was also indicated in other host–virus combinations. Posttranscriptional gene silencing was considered to be functionally involved in the cross-protection of gladiolus by the attenuated isolate of M11 of *Bean yellow mosaic virus* (BYMV) against the parent strain (Xu et al. 2006). Infection of plants by a mutant strain of *Cucumber mosaic virus* (CMV) resulted in the generation of a strong systemic silencing signal directed against CMV-specific RNA

sequences. It is more likely that protection occurred through either induction of very highly localized RNA silencing or by competition between strains for host cells or resources (Ziebell et al. 2007).

13.1.5 Improvement of Biological Control Potential of Biotic Agents

The efficacy of various biotic agents in suppressing the development of phytopathogens has been assessed by different methods under in vitro, greenhouse, and field conditions. The genetic potential of biological control agents (BCAs) may be expected to be important in its antagonistic activity against the plant pathogens. The genetic diversity of a BCA has to be determined by subjecting all available isolates to analyses by different molecular techniques such as RAPD, DNA fingerprinting, and PCR-based assays. If genes controlling the biosynthesis of antimicrobial compounds or enzymes can be identified, such information may facilitate the development of methods to enhance the biocontrol potential of the microorganism(s) selected as the most efficient for genetic manipulation. Attempts have therefore been made to improve the biocontrol potential of the microorganisms through various biotechnological methods. Some of the approaches examined to enhance the efficacy of biotic agents in suppressing the development of plant pathogen(s) include: genome analysis; identification of genes controlling the production of metabolites with antifungal and antibacterial activity; characterization of gene products; induction of mutation, detection, or transfer of gene(s); increasing the copy number of basic gene(s); and protoplast fusion. Widespread application of biotic biocontrol agents for the management of crop diseases has not been possible because of variable levels of control provided by them and commercial conditions that are to be satisfied, as in the case of chemicals that are recommended for different crop diseases. Enhancement of biocontrol potential of the biotic agents is therefore essential if they are to be acceptable as alternative products to chemicals. Information on the genes controlling the biosynthesis of antimicrobial compounds or enzymes may facilitate the choice of suitable methods to enhance the biocontrol potential of microorganism(s) selected for genetic manipulation. Genetic modification through biotechnological methods has been attempted to improve the performance of the most effective naturally occurring strains of biocontrol agents.

Superoxide dismutases (SODs) are considered as the important line of antioxidant enzyme defense systems by removing the superoxide anion, since the superoxide anion initiates a radical chain reaction that results in other reactive oxygen species (ROS) provoking damage to DNA, proteins, and membrane lipids (Krutika and Subramanian 2007). In order to increase the tolerance levels to temperature and salt concentration, *Trichoderma harzianum* was transformed with the SOD gene via *Agrobacterium tumefaciens* (At) transformation technique with an efficiency of 60–110 transformants per 10^7 spores. The successful transfer of SOD gene consisting of 751 bp sequence of nucleotides in transformants was confirmed by Southern blotting. No adverse effect on the growth of transformants was observed, while the biocontrol activity of the transformants was not altered. The genetically modified *T. harzianum* strain by SOD recombinant technology exhibited greater tolerance to salt and heat, facilitating its survival in adverse environments where it had to act on the pathogen *Sclerotinia sclerotiorum*. Antifungal activities of the transformants were assessed under abiotic stresses. The transformants exposed to 40°C for 3 days and 2 mol L⁻¹ NaCl at 27°C for 5–10 days

were tested for their antifungal activity. The inhibition rates of transformants compared with the wild-type strain were 83.96 and 60.13, respectively, after exposure to 40°C and 2 mol L⁻¹ NaCl, indicating the higher levels of tolerance of the transformants to heat and salt stress (Yang et al. 2010).

Biocontrol agents may be applied alone or in combination with fungicides to enhance the effectiveness of crop disease control. It is therefore essential that BCAs are tolerant to the fungicides to enable them to provide additive effects to reduce disease incidence and/or severity. UV mutagenesis and protoplast fusion techniques were combined for the production of fungicide-resistant strains of the cold-tolerant strains of *Trichoderma harzianum* and *T. atroviride*. The methyl-1-(butylcarbonyl) benzimidazole-2-yl carbamate (MBC-) resistant mutants of both species of *Trichoderma* showed total cross-resistance to benomyl and thiabendazole fungicides, whereas the wild parent strains could not develop in the presence of 20 µg mL⁻¹ of the fungicides. The possible mechanism of development of MBC resistance in UV-induced mutants could be a nucleotide substitution in the *tub2* β-tubulin gene. Interspecific protoplast fusion was carried out between carbendazim- and tebuconazole-resistant mutants of both parental strains. Putative haploid recombinants with stable resistance to both fungicides were produced in the case of *T. atroviride*. The fungicide polyresistant fusants exhibited a satisfactory level of antagonism against *Fusarium culmorum* and *Microdochium nivale*, causal agents of wheat Fusarium head blight disease complex and also other fungal pathogens *Pythium debaryanum* and *Fusarium oxysporum*. The results indicated that the combination of UV mutagenesis and protoplast fusion techniques could be used as tools for improving multiple-fungicide resistance in *Trichoderma* strains with desired levels of biocontrol potential against economically important plant pathogens (Hatvani et al. 2006).

Acidovorax citrulli, causal agent of cucurbit bacterial fruit blotch (BFB), is seedborne and affects the fruit production substantially in many countries. A *A. citrulli* nonpathogenic strain with type III secretion system AACOO-1ΔhrcC was generated. This mutant was nonpathogenic to watermelon, but retained its ability to colonize germinating watermelon seed. Treatment of watermelon seed naturally infested with *A. citrulli* with its mutant reduced the seed-to-seedling transmission of BFB pathogen by 81.8% compared to control seeds. Another *A. citrulli* antagonist *A. avenae* strain AAA99-2 was able to reduce BFB seedling transmission by 74.6% in seed samples from the same lot. In addition, when the female watermelon blossoms were protected with AACOO-1/ΔhrcC and subsequently challenged with AACOO-1, the resulting seed lots displayed only 8% BFB seedling transmission. This reduction was not significantly different from seeds from blossoms protected with AAA99-2 (4%), but significantly less than those from blossoms treated with 0.1 M phosphate-buffered saline (36%). The results suggested that the nonpathogenic *A. citrulli* showing a higher level of biocontrol potential might be a potential candidate for seed treatment in a comprehensive BFB management program (Johnson et al. 2011).

13.1.6 Transformation Crop Plants with Genes of Biological Control Agents

Different *Trichoderma* species have been shown to suppress many plant pathogens effectively by producing antibiotics and enzymes such as chitinases, proteases and glucanases that directly affect the mycelial growth of fungal pathogens. Various strains of *T. harzianum* produced endochitinases CHIT-31, CHIT-33, CHIT-37, CHIT-42, and CHIT-52 which differed in their molecular weights and structure

(Markovich and Konova 2003). Transgenic plants overexpressing endochitinase (CHIT) from *T. harzianum* were found to be tolerant to fungal diseases. The effect of expression of CHIT-36 in carrot plant (*Daucus carota*) tissues was investigated. Transgenic carrot plants constitutively expressing CHIT-36 endochitinase were generated. A plasmid containing *chit36* gene under the control of CaMV 35S promoter and the *nptII* selection gene was introduced into polyethylene glycol (PEG-) treated carrot protoplasts. All transformed plants contained the transgenes, as revealed by PCR assay with *nptII*- and *chit36*-specific fragments, respectively. In vitro assays using detached leaves and petioles of transformed carrot plants showed less severe symptoms of infection by *Alternaria dauci*, *A. radicina*, and *Botrytis cinerea*. The resistance response depended on the transgenic clone. However, all clones had significantly enhanced tolerance to *A. radicina* and *B. cinerea* on an average of 50%. Progress of disease induced by *A. dauci* was slower only in two transgenic lines, whereas other clones showed enhanced level of susceptibility to *A. dauci*. The results indicated that the levels of resistance of carrot to *A. radicina* and *B. cinerea* might be improved by transformation of carrot clones with chitinase gene(s) of biocontrol agent *T. harzianum* (Baranski et al. 2008).

13.2 Evaluation of Abiotic Agents for Biological Control Potential

The abiotic agents of plant and animal origin, which are naturally derived products, have been evaluated for their effectiveness in suppressing the development of diseases caused by microbial plant pathogens infecting various crops cultivated in different agroecosystems. The seeds or propagules may be infected by soilborne and airborne pathogens, in addition to the pathogens carried in and/or on the seeds and propagules. It is essential to understand the complex conditions existing in the soil. The host plants interact with the microbial pathogens which adversely affect plant growth, whereas other microorganisms associated with rhizosphere may be either beneficial for plant growth directly, or antagonistic to soilborne pathogens and indirectly reduce the adverse effects of plant pathogens, providing conditions for better yields. The soils that suppress pathogen development and consequent failure of disease incidence are known as suppressive soils. In contrast, soils that favor disease development are termed conducive soils, resulting in reduction of yield of grains and other products. Soil suppression is a property primarily conferred by the soil microbial community (Narayanasamy 2013).

13.2.1 Effects of Addition of Organic Matter

The application of organic matter to the soil improves crop yield mainly by enhancing soil fertility rather than through provision of nutrients to the plants. In addition, many microorganisms antagonistic to plant pathogens are activated following addition of organic matter to the soil, leading to suppression of disease development. Organic matter incorporated into the soil is called amendment, whereas organic matter placed or spread on the soil surface is known as mulch. Organic amendments added to the soil promote biological destruction of pathogen inoculum through germination-lysis

mechanism and intensification of microbiological activity, resulting in the decay of pathogen propagules. The potential of organic amendments to suppress *Ralstonia solanacearum* race 3 biovar 2, causing potato brown rot disease of tubers, was assessed in Dutch sandy and clay soils as well as in sandy soils of Egypt. Organic amendments significantly increased disease incidence and pathogen survival in Dutch sandy and clay soils, which correlated with high organic carbon in the organic Dutch soils. Cow manure amendment significantly reduced disease incidence in organic Dutch sandy soils, but did not affect the bacterial populations. However, cow manure reduced bacterial population densities in Egyptian sandy soils, most probably due to microbial competition. A clear shift in population was detected by denaturing gradient gel electrophoresis (DGGE) technique in Dutch sandy soils after application of cow manure. Suppressiveness of soil towards *R. solanacearum* might be related to soil characteristics and microbial communities which exhibit variations depending on the soil type and organic matter content. Disease suppression was lost following treatment of the soil with methyl bromide fumigation or heat, indicating that a biological principle could be the main factor in enhancing soil suppressiveness towards soilborne pathogens such as *R. solanacearum* (Messiha et al. 2007a, b).

Effective alternate management practices have to be developed for soilborne potato diseases transmitted through seed tubers, since they remain persistent problems. Compost amendments, along with two biocontrol agents (BCAs) *Bacillus subtilis* and *Rhizoctonia solani* hypovirulent isolate (Rhs1A1), were evaluated alone or in combination for their efficacy in reducing soilborne diseases and increasing the tuber yield over three seasons. Both BCAs reduced multiple soilborne diseases, namely stem and stolon canker by 20–30%, black scurf by 30–58%, and common scab by 10–34%, compared to the untreated controls. The combined treatment of *B. subtilis* and *R. solani* hypovirulent isolate provided nominally better control than individual treatments. However, these treatments did not have any effect on tuber yield. On the other hand, all compost amendments from different sources increased total and marketable tuber yield substantially (11–37% and 17–51%, respectively) relative to the controls. Compost treatments did not reduce incidence of black scurf and common scab diseases, but some reduction in *Rhizoctonia* canker was observed. The combination of compost-BCA treatments did not provide significantly better effect than individual treatments, but combined beneficial effects from both components were indicated in this investigation (Larkin and Tvantzis 2013).

The effects of compost amendment and grafting bell pepper onto resistant or partially resistant rootstocks on the incidence of *Phytophthora* blight disease caused by *Phytophthora capsici* were assessed under artificially or naturally infested soil using five sweet pepper cultivars – Fenice, Sienor Marco, Fiume, Kaiman, and Cuneo – as non-grafted controls. Disease incidence in the control plots ranged over 73–100%. The cultivars Cuneo and Kaiman grafted onto Tecnico F1 rootstock provided complete suppression of the disease development. By grafting all susceptible cultivars onto Robusto, Brutus, Snooker, and Capsifort, disease incidence was reduced by 68–93%, 86–100%, 67–100%, and 55–78%, respectively. Under natural infestation conditions, the addition of compost to the soil and the interaction of compost-amended soil with grafting were significant factors contributing to the reduction of disease incidence. The effect of grafting was significant in reducing the percentage of diseased plants in all trials. The addition of compost to the soil was an effective and low-cost disease management option, particularly when combined with susceptible not-grafted hybrids of bell pepper or when partially resistant rootstocks were cultivated (Gilardi et al. 2013).

Cultivation and incorporation of specific green (manure) crops into the soil as a means to manage specific biologically active resident soil microbial communities has been evaluated for the control of soilborne plant pathogens that are capable of infecting seeds/propagules when planted later. The effects of green manure crops buckwheat and canola and crop sequences on potato scab caused by *Streptomyces scabies* and Verticillium wilt induced by *Verticillium dahliae* were assessed in field trials conducted for 2 years. Densities of indigenous streptomycetes and in vitro pathogen inhibitory activities were also determined. Tubers grown in buckwheat-treated soil had significantly lower Verticillium wilt ratings, while tuber yield was increased significantly. Potatoes grown in soils planted with corn or alfalfa in the previous year had significantly lower Verticillium wilt incidence and potato scab ratings, as well as higher yields than in soil previously planted to potato. *Streptomyces* from soils collected from green manure (GM-) treated plots tended to have greater in vitro pathogen inhibitory activity on *Streptomyces* than those from the soils from fallow-treated plots. In addition, pathogen inhibitory activity of *Streptomyces* was often negatively correlated with plant disease incidence and positively correlated with potato yield. The streptomycete communities in GM-treated soils were consistently in greater proportions of pathogen antagonists than in communities in fallow-treated plots in the second season of experimentation. The GM crops may selectively favor the abundance or activity of antibiotic producers within the soil microbial community (Wiggins and Kinkel 2005).

The relative increase in inhibitory activity of the streptomycetes community was frequently associated with a decrease in disease development and an increase in potato yields. The effect of application of cultivars Jubilee Sweet corn and Jubilee Super sweet corn as green manure was investigated for suppressing the development of Verticillium wilt disease of potato induced by *Verticillium dahliae*. The sweet corn cultivars reduced the disease incidence by 60–70%. These treatments did not reduce the pathogen populations directly, but the colonization of potato feeder roots and in potato tissues of stem pieces by *Verticillium dahliae* was reduced. Feeder-root colonization was positively correlated with Verticillium wilt incidence ($P \leq 0.05$) and negatively correlated with yield. In addition, corn green manures increased populations of several fungi such as *Ulocladium* and *Fusarium equiseti*. When potato was grown consecutively for 2 years, the beneficial effects of sweet corn green manure was entirely lost; however, following 2 consecutive years of potato, a single sweet corn crop was enough to restore the original benefit and enhancement of tuber yield by four-fold. The results clearly showed the increases in potato tuber yields could be sustained by incorporating the sweet corn plant materials as green manures into the soil (Davis et al. 2010).

Disease suppressive potential of composts to suppress the development of onion black mold caused by *Aspergillus niger* was assessed by testing the leachates prepared from alfalfa and sunflower stalks. The onion seeds were treated with the leachates of composts at 10 and 20% concentration, and their ability to induce production of antifungal compounds and to suppress the black mold disease incidence was determined. The leachates reduced the disease incidence in sets, but not disease severity in onion seedlings. The antifungal activities of the fractions of the leachates, obtained using thin-layer chromatography, did not differ. The presence of fluorescent pseudomonads and *Pantoea agglomerans* was detected in the leachates of alfalfa and sunflower stalk composts. The sunflower compost leachate had higher populations of *P. agglomerans* compared to alfalfa compost leachate. Both leachates inhibited mycelial growth of the

pathogen in agar tests, indicating that the bacterial BCAs might have a role in the induction of antifungal compounds effective against onion black mold disease (Özer and Köycii 2006).

Compost amendments incorporated into soil or planting mixes have been reported to provide protection not only to soilborne plant pathogens but also to airborne plant pathogens infecting aerial plant organs, such as foliage and inflorescence/seeds. The efficacy of composted cannery wastes and yard wastes in suppressing bacterial spot disease caused by *Xanthomonas vesicatoria* and *X. axonopodis* pv. *vesicatoria* was determined. The disease incidence was reduced by composted yard wastes when disease pressure was high in the conventional tomato production system. Marketable yield was increased by 33% in compost-amended organic plots, whereas no increase in marketable yield could be realized in compost-amended conventional plots (Abbasi et al. 2002). The effectiveness of tomato or pepper residue composts combined with cattle or chicken manure to suppress bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) was assessed. Application of composts reduced the disease incidence by 70–100% under natural infection of mature plants and artificial inoculated conditions. Populations of *Cmm* in composts declined to undetectable levels within 15–20 days, while those in peat remained high for 35–40 days. Likewise, colonization of compost-grown tomato plant tissues by the pathogens was reduced to 0–20% compared to plants growing on peat or perlite which showed 53–90% colonization. The results indicated that application of compost could be an effective component of integrated management system for bacterial canker disease affecting tomato (Yogev et al. 2009).

13.2.2 Effects of Products from Plant Sources

Plant tissues contain secondary metabolites such as essential oils, and extracts of various plant tissues have been shown to have antifungal, antibacterial, and antiviral properties. Various nonhosts of plant pathogens have therefore been screened for the presence of antimicrobial compounds that may be useful for the management of crop diseases. The leaf extracts of *Arbus precatorius* alone or in combination with *Pseudomonas fluorescens* Md1 were evaluated for their ability to suppress the development of *Colletotrichum falcatum*, incitant of sugarcane red rot disease. The plant extracts and the bacterial BCA were used to treat the sugarcane setts (planting material) planted in pots under greenhouse conditions. Red rot incidence was reduced to 24.2% and 20% in setts treated with plant extract and bacterial BCA, respectively. Treatment with plant extract was not effective in reducing red rot infection under field conditions. However, the combination of plant extract and *P. fluorescens* applied as foliar spray or soil drench reduced the incidence of red rot disease to the minimum level (3%). The results indicated the effectiveness of the combined application of *A. precatorius* extract and *P. fluorescens* Md1 for the suppression of red rot disease in sugarcane (Jayakumar et al. 2007).

The biocontrol potential of dravya, a commercially produced aqueous plant extract, was assessed for suppression of cotton bacterial blight caused by *Xanthomonas campestris* pv. *malvacearum*. Seed soaking in dravya (1:500) followed by foliar spray three times at intervals of 10 days commencing from 20 days after sowing (das) reduced the bacterial blight disease incidence by 66%, 70%, and 74% as determined at 40, 60, and 80 das, respectively. The mechanism of action of dravya appeared to be through

induction of resistance in cotton plants against cotton bacterial blight disease. The results indicated that the plant extract had potential for use as an ecofriendly component of the integrated management system for cotton bacterial blight disease (Raghavendra et al. 2007).

The inhibitory effect of Chinese leek (*Allium tuberosum*) on banana Fusarium wilt pathogen *Fusarium oxysporum* f.sp. *cubense* (*Foc*) was determined. Treatment of soil in pots with Chinese leek extract reduced disease incidence and disease severity index by 58% and 63%, respectively, in the banana cultivar Baxi (AAA) and by 79% and 81%, respectively, in the cultivar Gungfen No. 1 (AAB) under greenhouse conditions. In the plate assays crude extracts of Chinese leek entirely suppressed the mycelial growth of *Foc* race 4, inhibiting the proliferation of spores by 91%, and caused 87% mortality of conidia. Adopting a rotational crop system including Chinese leek reduced the Fusarium wilt disease incidence and severity index by 88–97% and 91–96%, respectively, under field conditions. In addition, inclusion of Chinese leek as a rotational crop improved the crop value by 36–86% in areas heavily infested with *Foc* during 2007–2009. The results revealed the potential of Chinese leek in suppressing the development of banana Fusarium wilt disease and also improving the quality of banana fruits (Huang et al. 2012).

Products extracted from plants have been evaluated for their inhibitory activity against plant pathogens. Volatile antimicrobial compound allicin (diallyl-thiosulfinate) present in garlic juice inhibited the germination of sporangia, encysted zoospores, and subsequent germ tube elongation in *Phytophthora infestans*, causative agent of late blight disease, both in vitro and in vivo on tomato leaf surface. Disease severity in infected seedlings was reduced by spraying garlic juice containing allicin ($55\text{--}110\mu\text{g mL}^{-1}$) with an effectiveness of c. 45–100% mortality (Portz et al. 2008). The inhibitory activity of garlic juice was also assessed against other plant pathogens. Allicin effectively suppressed the development of seedborne pathogens *Alternaria* spp. infecting carrot, *Phytophthora infestans* causing late blight in tomato and tuber blight in potato, and *Magnaporthe grisea* infecting rice. Reduction in diseases was apparently due to the direct action of allicin on these pathogens (Slusarenko et al. 2008). The inhibitory effects of the root exudates of two garlic cultivars Gailiang (G064) and Gangshan (G025) on *Phytophthora capsici*, infecting pepper, were investigated. Different concentrations of root exudates (25–100%) of cultivar G025 showed lower inhibitory effect than that of cultivar G064. At 100% concentration of root exudates, G064 and G025 showed inhibition of mycelial growth by 69.24% and 49.06%, respectively. High-performance liquid-chromatography (HPLC) analysis showed that the root exudates from G064 contained greater amounts of allicin than that present in root exudates of cultivar G025. The pathogen hyphae treated with root exudates of G064 were mishappened, fragmented, and had a smaller diameter as well as empty cytoplasmic contents in the cell wall, as revealed by scanning electron microscope. These adverse effects were not observed in the control and G025 treatments. The results indicated that the root exudates of cultivar G064 had significant fungicidal effect on *P. capsici* (Azam Khan et al. 2011).

Essential oils, a group of plant-derived compounds, contain a mixture of different terpenoid compounds and their oxygenated derivatives. These oils have a wide spectrum of antifungal activity against plant pathogens. The essential oils (EOs) oregano, thyme, dictamnus, marjoram, lavender, rosemary, sage, and pennyroyal were evaluated for their inhibitory activity against *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) using plate assays. The multiplication of *Cmm* was entirely inhibited by oregano, thyme, dictamnus, and marjoram essential oils at relatively low concentrations ($85\text{--}300\mu\text{g mL}^{-1}$).

Thymol was the main component of oregano oil, while thyme, dictamnus, and marjoram oils were rich in carvacrol (Daferera et al. 2003). Five essential oils extracted from *Cymbopogon citratus*, *Monodora myristica*, *Ocimum gratissimum*, *Thymus vulgaris*, and *Zingiber officinale* were evaluated for the comparative inhibitory effect on five seedborne bacteria, namely *Acidovorax avenae* subsp. *avenae*, *Burkholderia glumae*, *B. plantarii*, *Xanthomonas oryzae* pv. *oryzae*, and *X. oryzae* pv. *oryzicola*. The essential oils (EOs) at 1:1 dilutions showed different degrees of inhibition of the bacterial pathogens, as deduced from inhibition zones using agar diffusion method. Among the EOs tested, *O. gratissimum* and *T. vulgaris* oils were more effective with mean inhibition zones of 14 mm and 10 mm in diameter, respectively, against all five bacterial pathogens tested. The EOs from *O. gratissimum* and *T. vulgaris* were evaluated for their ability to reduce the seed transmission of *A. avenae* subsp. *avenae* (Aaa) and seed infection of *X. oryzae* in naturally infected seeds by the cassette holder method and liquid assay, respectively. EO applied as a slurry significantly reduced the seed transmission of Aaa by 60–80% and from seeds with infection by 32% on three rice cultivars. The vigor of rice seedlings growing from treated seeds was significantly enhanced. Treatment with EOs reduced the total cell counts of bacteria (92–97%), while the percentages of germination of treated seeds were increased by 13–23% (Nguefack et al. 2005).

The effectiveness of essential oils, organic acids, extracts of stinging nettle and golden rod, Biosept (grapefruit extract), and Tillecur to suppress the development of seedborne pathogens was assessed. Thyme oil, oregano oil, cinnamon oil, clove oil, and Biosept inhibited the development of *Xanthomonas campestris* pv. *campestris*, *Clavibacter michiganensis* subsp. *michiganensis*, *Alternaria dauci*, and *Botrytis aclada* to the maximum extent. The fungal pathogens were inhibited to a lesser degree by organic acids compared to bacterial pathogens. Treatment of cabbage seeds for 30 min with selected essential oils or Biosept at 0.1–1.0% eliminated >99% of the total bacterial populations associated with cabbage seed. They also reduced the percentage of seeds contaminated with fungi from 70% to less than 10%, as indicated by blotter tests. Cinnamon oil and Biosept at >1% affected seed germination. Organic acids exhibited variable antimicrobial effects, but the organic acids ascorbic acid, propionic acid, acetic acid, and lactic acid at concentrations of 2.5% or higher generally reduced the seed-associated bacteria. Among the organic acids, only propionic acid reduced seed germination at concentrations >1.0%. Thyme oil was the most effective natural compound, showing potential for the suppression of seedborne pathogens tested (van der Wolf et al. 2008). Hydrodistilled essential oils and n-hexane extracts from *Origanum acutidens*, *O. rotundifolium*, and *O. vulgare* were analyzed by GC and GC-MS techniques to determine their chemical composition. Essential oils and hexane, chloroform, acetone, and methanol extracts were evaluated for their antibacterial activity against 25 phytopathogenic bacterial strains. The wide spectrum antibacterial activity of essential oils was due to the phenolic compounds such as carvacrol and thymol. The compounds carvacrol and thymol possessed higher antibacterial activity. The results suggested that the essential oils might be valuable antibacterial agents that could be used as seed disinfectant (Dadasoglu et al. 2011).

The inhibitory effects of clarified neem oil (CNO) in 0.1% Triton on aflatoxigenic *Aspergillus* spp. were determined using submerged cultures containing either 0.5 mL or 0.1 mL of CNO. Fungal growth and aflatoxin B1 production were determined in an atmosphere of volatiles emitted from 0.25–1.0 mL CNO added to the plates. The radial growth of *Aspergillus* sp. was reduced by 7–29% and aflatoxin B1 production by 0–67%. GC/MS assessments of the head space volatiles of the CNO indicated that the reduction

in pathogen growth and aflatoxin B1 was probably due to low-molecular-weight hydrocarbons, aldehydes, alcohols, and sulfur compounds emitted at 30% in dry culture (Zeringue et al. 2001). Plant-derived gum biopolymers containing plant growth-regulating hormones have been evaluated for their effect on the development of plant diseases. The effect of application of xanthan gum on the development of *Bipolaris sorokiniana* infecting barley was assessed. Barley plants of cultivars 127, 128, and 129 were treated with xanthan gum and the plants were challenged with the pathogen at different periods after treatment. The treated plants exhibited an increase in the levels of proteins, as well as in the activity of β -1,3-glucanase when compared with the extract from healthy plants. The most important macromolecules in the defense-related functions were demonstrated to be pathogenesis-related (PR-) proteins which accumulated following accumulation of the resistance inducer (Castro and Bach 2004).

Gum biopolymers containing plant growth-regulating hormones with priming potential without any side effects were evaluated for their inhibitor activity against pearl millet downy mildew pathogen, *Sclerospora graminicola*. The gum exudates of *Acacia arabica*, *Moringa oleifera*, *Carica papaya*, and *Azadirachta indica* were evaluated for their synergistic effect. Seed priming with exuded gum biopolymer combined with metalaxyl (Apron 35 SD) fungicide was performed to determine their effect on pearl millet seed quality, growth parameters, and resistance to *S. graminicola*. Seeds of cultivar 7042S were primed with gum biopolymers and metalaxyl 35SD. Seed germination and vigor were synergistically enhanced using gum biopolymers solution (1:2 w/v) with 3 g kg^{-1} metalaxyl 35SD. *A. arabica* and *A. indica* gum polymers alone or with fungicide resulted in seed germination of >91%. Seed priming with fungicide (6 g kg^{-1}) gave 89% seed germination and was not significantly different from control. A similar trend in vigor was recorded among all treatments. Seed priming with gum biopolymers alone provided different levels of disease protection when compared with control. Treatment with the combination of *A. arabica* or *A. indica* gum and metalaxyl at 3 g kg^{-1} was superior in protecting pear millet plants against the downy mildew disease with 86% reduction in disease incidence and better growth of plants (Sudisha et al. 2009).

Durum wheat bran contained various monomeric forms of phenolic acids of which ferulic acid was found to be the most abundant. The effect of natural phenolic acids from wheat bran on the biosynthesis of the mycotoxin type B trichothecene by *Fusarium culmorum* was assessed in vitro. When the liquid cultures of *F. culmorum* were supplemented with a natural wheat bran extract, the production of trichothecene was completely inhibited. The phenolic acid treatment resulted in a drastic reduction in the expression level of structural trichothecene biosynthetic genes. The inhibitory efficiency of the natural phenolic acids extract was significantly higher than that of a reconstituted mixture containing similar concentrations of monomeric forms. To elucidate the full repression of type B trichothecene production induced by natural phenolic acid from wheat bran, two possibilities were therefore suggested: (i) a synergistic impact of monomeric and dimeric forms of phenolic acids; and (ii) involvement of an unidentified oligomeric form able to efficiently repress toxin yield. The most abundant dimer of ferulic acid, the 8-5'-benzofuran dimer, was synthesized and inhibited the trichothecene biosynthesis to the same extent as the monomer of ferulic acid (Boutigny et al. 2010).

Microdochium majus, incitant of snow mold, seedling blight, and foot rot of small grain crops, causes severe losses in yield and quality. As options for the control of plant diseases in organic cultivation system are limited, aqueous extracts of botanical powders prepared from chamomile, meadow sweet, thyme, and Chinese galls were evaluated for

their inhibitory effects on conidial germination and mycelial growth of *M. majus*. Based on the in vitro results, three botanicals were tested under controlled environmental conditions by applying them as powders with different seed coating adhesives. Seed treatments with warm water, a bacterial product, or a botanical were evaluated under field conditions for 3 consecutive years. The Chinese galls treatment was the most effective in suppressing conidial germination and mycelial growth by 97% and 100%, respectively, and the treatment reduced the disease incidence from infested seeds by 59%. Grain yield was increased by treatment with Chinese galls, the bacterial product, and warm water to the extent of 19, 10, and 10%, respectively, compared to the control plots. The results indicated that Chinese galls might be useful for application to organic crops against infection by *M. majus* (Vogelgsang et al. 2013).

13.2.3 Effects of Products from Animal Sources

Chitosan is the most commonly tested product of animal origin for its efficacy in suppressing the development of pathogens infecting various crops. Chitosan, a deacetylated form of chitin, is a natural biodegradable fiber (polymer) derived from crustacean shells such as crabs and shrimps. Chitosan has antifungal properties, leading to direct suppression of the development of fungal pathogens. In addition, chitosan has been shown to induce resistance to pathogens by stimulating defense mechanisms naturally operating in plants. Defense responses have been shown to be triggered following activation of signaling pathways involving salicylic acid (SA) and jasmonic acid (JA) in most compatible plant-host-parasite interactions. The effects of SA and methyljasmonate (MeJA) applied to *Tilletia laevi*-inoculated wheat (*Triticum aestivum*) seedlings at 1, 2, or 3 weeks following emergence on infection were assessed. Further, transcript levels of defense-related genes were determined. Application of SA or MeJA coordinately activated transcripts of different groups of defense-related proteins and reduced common bunt infection. Three patterns of defense gene expression following SA and MeJA treatments were differentiated. Transcripts of Chi1, Chi3, Chi4, PR-1, PR-1.2, Glu1, and a lipase that were upregulated in response to MeJA treatment, their expression potentiated by infection by *T. laevis*, and the highest transcript levels were associated with the 3 week seedling stage. Transcripts of Glu2 responded almost exclusively to SA were not potentiated by infection, and the highest expression was observed at the 3 week seedling stage. Transcripts of ns-LTP-1, ns-LTP-2, and Glu3 were upregulated in response to both SA and MeJA, not potentiated by infection, and the highest transcript levels were observed at the 1 week seedling stage (Lu et al. 2006).

The antibacterial properties of two kinds of chitosan with different molecular weights and degrees of *N*-acetylation were evaluated for the antibacterial activities against rice bacterial pathogens *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola*, causing bacterial leaf blight and leaf streak diseases. Both kinds of chitosan solutions showed strong antibacterial activity against both rice bacterial pathogens and significantly reduced disease incidence and severity when compared with control under greenhouse conditions. However, the interaction between chitosan and rice pathogens was influenced by the type and concentration of chitosan, the bacterial species, and the contact time between chitosan and bacteria. The direct antibacterial activity of chitosan may be attributed to both membrane lysis and destruction of biofilm. In addition, both chitosan solutions significantly increased the activities of phenylalanine ammonia lyase (PAL), peroxidase, and polyphenol oxidase in rice seedlings following inoculation of

two bacterial pathogens compared with control. Chitosan protected the rice plants against rice bacterial pathogens causing leaf blight and streak diseases through direct antibacterial activity and indirectly by induced resistance (Li et al. 2013).

The ability of chitosan as seed treatment to induce resistance in durum wheat (*Triticum durum*) against seedborne fungal pathogen *Fusarium graminearum*, one of the main causal agents of root and foot rot in wheat, was assessed. The efficacy of seed treatment with chitosan was evaluated by biochemical analyses including the following treatments: seed treated; seed treated and inoculated with pathogen; seed not treated and inoculated; and seeds not treated and not inoculated. The enzymatic activities of some enzymes involved in defense mechanisms were analyzed. Seed treatment induced changes in the activities of guaiacol peroxidase (POD), ascorbate peroxidase (APX), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL) and increased the phenol contents. Disease incidence on plants was significantly reduced by chitosan treatment under greenhouse conditions. Under field conditions, seed treatment with chitosan reduced the disease severity and enhanced quantitative yield parameters, indicating the effectiveness of chitosan seed treatment as effective disease management strategy by improving plant defense responses to the disease caused by *F. graminearum* (Orzali et al. 2014).

Oligochitosan is prepared by hydrolysis or degradation of chitosan. It is water soluble and more effective than chitosan in eliciting multiple plant defense responses, such as H_2O_2 production and stimulation of defense-related enzymes. *Phytophthora capsici* with no chitin in its cell wall was highly sensitive to oligochitosan with EC_{50} and MIC values of 100 and $580 \mu g mL^{-1}$, respectively. Oligochitosan at low concentrations inhibited pathogen development at different stages in the life cycle of *P. capsici*, including zoosporangia production, zoospore release, cystospore germination, and induction of rupture of released zoospores. The addition of ATP did not reduce inhibition of zoospore release and zoospore rupture by oligochitosan. Treatment with oligochitosan induced leakage of electrolytes from the mycelium of *P. capsici*, indicating that oligochitosan might also act on the cell membrane by upsetting osmotic pressure as for chitosan. Transmission electron microscopic observations showed slight distortion of some hyphal cell walls of *P. capsici* in the presence of oligochitosan ($10 \mu g mL^{-1}$). The most pronounced structural alteration of the hyphae treated with oligochitosan ($10 \mu g mL^{-1}$) was found to be disruption of endomembrane system, especially vacuole and secretory vesicles such as plasmalemmasomes in hyphal tips. The polycationic nature of oligochitosan might contribute to the antifungal and multiple modes of action, including induction of disease resistance in treated plants (Xu et al. 2007).

The effectiveness of chitosan in suppressing the development of potato common scab disease caused by *Streptomyces scabies* was assessed when applied alone or in combination with *Streptomyces melanosporofaciens* strain EF76, producing geldanamycin. The combined application of chitosan and strain EF76 was the most effective treatment in reducing common scab incidence under greenhouse conditions, but field application of EF76 strain did not allow the selection of geldanamycin-resistant actinomycetes (GRA) in the bulk soil during the potato-growing season. However, the number of GRA on harvested potato tubers was significantly increased in treatments containing chitosan than in other treatments, suggesting that chitosan might promote the establishment of the antagonistic actinomycetes on progeny tubers. The results showed that combined application of chitosan and EF76 might suppress the development of potato common scab disease by direct effect on the pathogen and indirectly by inducing resistance in potato plants (Prévost et al. 2006).

A naturally occurring spontaneous recovery of plants infected by phytoplasmas is a known phenomenon. Hence, a strategy to facilitate recovery of plants from severe phase by stimulating the plant defense systems was evaluated for potential application for the management of grapevine Bois noir (BN) disease. Five commercial products consisting of chitosan (ChitoPlant, ChitPro), Phosetyl-Al (Aliette), two different mixtures of glutathione (Kenal and Valagro), and oligosaccharines (Olvis) and BTH (Bion) were evaluated for their efficacy by applying aqueous solutions on the canopy on BN-infected grapevine plants. Of the 744 plants treated, 214 were PCR-positive for the presence of BN and showed typical symptoms of BN. In 2007, all treatments increased the percentage of recovered plants in comparison to the control. Bion, Olvis, Kendal, and Aliette yielded the highest percentages of recovered plants which were significantly ($P \leq 0.05$) greater, the recovery ranging from 23.8% (Aliette) to 147.2% (Kendal). None of the plants treated with inducers of resistance showed phytotoxic symptoms. PCR assays failed to detect BN phytoplasma in recovered vines. The results indicated the usefulness of resistance induced to different degrees by different chemicals (Romanazzi et al. 2009).

13.2.4 Effects of Organic Compounds

The ability of various organic compounds to suppress the development of diseases incited by microbial pathogens in different crops has been assessed; most of them have been shown to lack direct inhibitory effect on pathogen development, but have the potential to stimulate the defense mechanisms of host plants. These compounds induce the same spectrum of systemic acquired resistance (SAR) genes to levels comparable with those induced by the biotic inducers of disease resistance (Ward et al. 1991). A wide range of organic compounds has been reported to have the potential for use as inducers of systemic resistance to many economically important crop diseases.

The mechanism of induction of systemic resistance by salicylic acid (SA) has been studied using the model plant *Arabidopsis thaliana*. The SAR regulatory protein non-expressor of PR genes (*NPR1*) is activated by salicylic acid (SA) through redox potential. These changes drive systemic expression of antimicrobial PR proteins and facilitate their secretion by upregulating protein secretory pathway genes (Mou et al. 2003; Wang et al. 2005). Exogenous application of salicylic acid (SA) triggers the translocation of *NPR1* into the plant cell nucleus. The expression of a large number of genes is altered, following SA treatment (Yao et al. 2003). Induction of many genes by SA via the endogenous *NPR1* was confirmed by RNA blot analysis and real-time RT-PCR assay (Wang et al. 2005). The mechanism of treatment of potato tubers with acetyl salicylic acid (aspirin) for protection against soft rot disease caused by *Erwinia carotovora* subsp. *carotovora*-225 (Ecc-225) was assessed. Acetyl salicylic acid was applied at five concentrations from 0.0125 to 0.05% (w/v) at pH 7.0 which did not inhibit the bacterial growth. This compound at concentrations 0.0125, 0.025, and 0.05% in water adjusted to pH 7.0 were able to produce considerable reduction of soft rot. The results indicated that inhibition of development of soft rot disease in potato tubers could be achieved by applying acetyl salicylic acid (López-López et al. 1995).

Exogenous application of salicylic acid (SA) and methyl jasmonate (MeJA, biologically active derivative of jasmonic acid) induced resistance in wheat against common bunt disease caused by *Tilletia laevis*. The responses of the defense-related pathogenesis-related (PR-) genes of wheat seedlings treated with SA and MeJA on infection at 1, 2, or 3 weeks following emergence of seedlings were investigated. Application of SA or

MeJA coordinately activated transcripts of different groups of defense-related proteins, resulting in reduced infections by *T. laevis*. Transcripts of *chi1*, *chi3*, *chi4*, *PR-1*, *PR-2*, *Glu1*, and a lipase were upregulated due to MeJA treatment and their expression was potentiated by infection by *T. laevis* (Lu et al. 2006). Perception of both general and specific pathogen-associated molecules triggers defense responses in plants via signal transduction cascades and transcriptional activation of numerous genes. In chickpea (*Cicer arietinum*), putative genes potentially involved in defense responses including the rapid synthesis of PR-proteins, the presence of an oxidative burst, and synthesis of a putative cell-wall-strengthening proteins and antimicrobial proteins have been identified (Coram and Pang 2006). The responses of three chickpea genotypes treated with defense-signaling compounds SA, MeJA, and aminocyclopropane carboxylic acid (ACC) to *Ascochyta rabiei* causing Ascochyta blight disease were investigated using microarray technology. The observations were validated by quantitative RT-PCR assay. The coregulation of transcripts between treatments for each genotype with varying levels of disease resistance showed that large proportions of transcripts were independently regulated by ACC, MeJA, or SA (Jalali et al. 2006).

The effect of exogenous application of salicylic acid (SA) on host resistance to rice blast pathogen *Magnaporthe grisea* was determined. Rice leaves were treated with SA at 8 mM concentration. Blast lesion production in treated rice leaves was suppressed. Activities of defense-related enzymes peroxidase and polyphenoloxidase were stimulated in SA-treated rice leaves. Analysis of crude leaf extracts of leaves indicated the presence of rice phytoalexins, oryzalexin A, C, and E, and momilactone A following root application of SA. The hydrolysates of conjugated antifungal compounds from SA-treated rice plants caused greater inhibition of conidial germination of *M. grisea* than those from control plants. The results showed that exogenous application of SA to rice plants might result in an increase in the concentrations of compounds involved in the resistance as well as antifungal compounds, which might inhibit the pathogen development directly (Daw et al. 2008).

The potential of salicylic acid (SA), a key phytohormone required for defense against some biotic and abiotic stresses, was assessed for reducing infection by *Plasmodiophora brassicae*, an obligate parasite causing club root disease of broccoli (*Brassica oleraceae* var. *italica*) with a number of survival and dissemination strategies. Experiments were conducted under controlled conditions in growth chambers, glasshouse, and field conditions. Exogenous application of SA to the leaves where it promoted the upregulation of the pathogenesis-related genes *PR-1* and *PR-2* in a systemic acquired resistance (SAR-) like response was observed as early as 24 h post-treatment under controlled conditions. Concentrations of SA >20 mM significantly reduced both shoot and root weights when applied exogenously, but lower concentrations had little measurable effect on plant growth. When SA was applied at >5 mM to plants, there was a significant reduction (25–65%) in gall formation at 6 weeks postinoculation with *P. brassicae*, indicating that the pathogen development could be suppressed by application of SA. A combination of SA and jasmonic acid (JA) also reduced disease severity (25–35%) induced by *P. brassicae*. The results indicated that application of SA could reduce the disease severity appreciably (Lovelock et al. 2013).

The efficacy of organic compounds in inducing resistance against virus diseases has been assessed in some pathosystems. Elimination of viruses from infected plants or propagules has been demonstrated to be an effective virus disease management strategy. Thermotherapy has been successfully applied to eliminate the viruses from

plants and propagules. Enhancement of thermotolerance of plants by treating with chemicals may be a useful approach to increase the chances of obtaining a higher percentage of virus-free plants. The ability of salicylic acid (SA) to increase the thermotolerance of potato microplants infected with *Potato virus X* (PVX) was assessed. Potato microplants were cultured with or without 10^{-5} M SA for 4 weeks, then subcultured without SA and exposed to 42°C for 30 days. Survival of SA-treated microplants was more consistent compared to untreated control plants. SA treatment also improved the post-thermotherapy subculture. All of the surviving microplants treated with SA were virus-free compared to 40–65% of virus-free microplants in the controls. In another group of 30 potato genotypes infected with PVX, 98% of the surviving SA-treated microplants became PVX-free after thermotherapy compared to 75% of untreated control plants. SA-treated plants had lower catalase activity and higher H_2O_2 levels (López-Delgado et al. 2004).

A nontoxic synthetic analogue of salicylic acid (SA), benzo (1, 2, 3)-thiadiazole-7-carbothioic acid-S-methyl ester (BTH) has been demonstrated to be an effective inducer of resistance in many crops against diseases caused by microbial pathogens. Fusarium head blight (FHB) disease caused by *Fusarium graminearum* occurs in most countries cultivating wheat. The possibility of improving the resistance of wheat plant to the FHB pathogen using BTH was explored. Wheat spikes were treated with BTH prior to anthesis, and transcript accumulation and resistance to FHB were determined. BTH-treated wheat spikes were examined for the expression of six defense response genes (*PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, and peroxidase) and five wheat chemically induced (WCI) genes that were induced by BTH wheat leaves. All five WCI genes were induced by BTH in spikes. However, none of the six defense-related genes was induced in the spikes. In contrast, the defense-related genes were induced by *F. graminearum* infection, whereas the WCI genes were not induced. The results indicated that the pathway for induction of the defense response genes by *F. graminearum* was distinct from the BTH-induced pathway. Disease assessment showed that BTH did not provide significant Type I or Type II resistance to wheat spikes spray- or point-inoculated with *F. graminearum*, respectively, and that the application of BTH for FHB management was an ineffective approach (Yu and Muehlbauer 2001).

The responses of two rice cultivars Tetep (resistant to *Magnaporthe grisea* (Mg), rice blast pathogen, and *Cochliobolus miyabeanus* (Cm) rice brown spot pathogen) and Nakdong (susceptible to both pathogens) to BTH treatment and conidial germination fluid (CGF) of *Cm* were assessed. Treatment with CGF induced rapid cell death in both cultivars, possibly due to phytotoxins produced by *Cm*. Pretreatment with CGF induced significant resistance to *Mg* in cultivar Nakdong, but not to *Cm*. Likewise, BTH significantly enhanced resistance to only *Mg*, but it failed to protect cultivar Nakdong against *Cm*. Enhancement of resistance in cultivar Nakdong to *Mg* by BTH or CGF treatment was correlated with more rapid induction of three PR genes. The results suggested that rice defense mechanisms against rice brown spot disease were distinct from those against blast disease and their expression patterns in response to *Cm* infection were nearly identical in both susceptible and resistant cultivars. It appeared that rice might employ distinctly different mechanisms for its defense against these two fungal pathogens (Ahn et al. 2005).

Elicitors of systemic acquired resistance (SAR) have been shown to reduce disease incidence and/or severity in different crops. Benzo (1, 2, 3) thiadiazole-7-carbothioic acid-S-methyl ester (BTH), acibenzolar-S-methyl, and the rhizobacteria *Pseudomonas*

aeruginosa KMPCH and *P. fluorescens* WCS417 were evaluated for their potential to induce resistance in susceptible and moderately resistant bean (*Phaseolus vulgaris*) to anthracnose pathogen *Colletotrichum lindemuthianum*. BTH induced local and systemic resistance when bean leaves were immersed in BTH solutions at (10^{-3} to 10^{-7} M BTH) concentrations 3 days before the challenge inoculation. BTH at high concentration (10^{-3} M) induced resistance to anthracnose disease, although it induced phytotoxic symptoms at this concentration. Soil and seed treatment with 1 mg kg^{-1} BTH protected beans against anthracnose. BTH-mediated resistance was effective in both susceptible and moderately resistant plants. *P. aeruginosa* KMPCH strain could only induce resistance to *C. lindemuthianum* in a moderately resistant interaction. On the other hand, KMPCH-567, a salicylic acid (SA) mutant of KMPCH, failed to induce resistance, indicating that SA was required for induction of resistance in bean against the anthracnose pathogen. In contrast, *P. fluorescens* WCS417 could induce resistance to *C. lindemuthianum* in both susceptible and moderately resistant interactions in bean-anthracnose pathosystem (Bigirimana and Höfte 2002).

The effect of BTH, an elicitor of SAR in chickpea against blight disease caused by *Didymella rabiei*, was assessed under field conditions. Multiple foliar applications of BTH were effective under economic threshold levels. However, multiple applications reduced the yields. The reduction in yield could therefore be compensated for by applying a combination of BTH and the contact fungicide mancozeb. One spray of BTH (50 ppm) followed by one spray of mancozeb (0.2%) significantly increased the grain yield to 1.241 t ha^{-1} , compared to 0.922 t ha^{-1} obtained with three sprays of BTH alone (Sharma et al. 2001).

Acibenzolar-*S*-methyl (ASM, a derivative of BTH) has been reported to be effective in inducing resistance in several crops against different diseases caused by microbial pathogens. The plant resistance activator ASM, the signaling molecules salicylic acid (SA), and methyl jasmonate (MeJA) were evaluated by seed treatment for their ability to protect melon seedlings against gummy stem blight and white mold diseases caused by *Didymella bryoniae* and *Sclerotinia sclerotiorum*, respectively. Infection of melon seedlings by *D. bryoniae* was entirely suppressed by MeJA application. Necrotic lesions similar to hypersensitive response (HR) appeared on all inoculated seedlings and prevented pathogen invasion into healthy tissues. ASM (BTH) seed treatment also effectively restricted *D. bryoniae* infection, although the percentage of necrotic lesions in comparison with water-soaked lesions was significantly lower than that present in MeJA-treated seedlings. ASM (BTH) protected melon seedlings against *S. sclerotiorum* by producing a high percentage of necrotic lesions. A lower level of resistance was also induced by MeJA seed treatment. The enhanced level of resistance of tissues following seed treatment with ASM (BTH) and MeJA was associated with a rapid increase in the activity of the pathogenesis-related (PR) proteins, chitinase and peroxidase. MeJA also induced a rapid and transient accumulation of lipoyxygenase. Furthermore, ASM (BTH) and MeJA treatments resulted in differential induction of de novo synthesis of particular isoenzymes of these proteins. The results indicated that ASM (BTH) and MeJA applied to melon seeds might activate metabolic pathways in seedlings, leading to induction of resistance against different pathogens (Buzi et al. 2004).

The effectiveness of BTH in comparison with an avirulent strain of *Pseudomonas syringae* pv. *maulicola* (*Psm*) in inducing resistance in canola (*Brassica napus*) against *Leptosphaeria maculans* and virulent strains of *Psm* was assessed. Application of BTH increased systemic resistance (SAR) against *L. maculans* and virulent strains of *Psm*

to a greater extent than localized preinoculation of plants with avirulent strain. Accumulation of PR genes including *BnPR-1* and *BnPR-2* was induced by BTH and avirulent strain of *Psm*. The levels of transcripts of PR genes induced were greater in BTH-treated plants than in plants preinoculated with avirulent strain. Development of SAR in *B. napus* plants expressing a bacterial salicylate hydroxylase transgene (*NahG*) that metabolized salicylic acid (SA) to catechol was appreciably compromised. These plants accumulated only reduced levels of PR gene transcripts compared with nontransformed control. The results indicated that SAR development in *B. napus* had many hallmarks of classical SAR, including long-lasting and broad host range resistance associated with PR gene activation and requirement of salicylic acid (Potlakayala et al. 2007).

The potential of acibenzolar-*S*-methyl (ASM) for its ability to protect pepper (chilli) fruits against infection by *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) was assessed following spray application of ASM on pepper plants. Protection offered by ASM could be observed when the inducer was completely degraded. It appeared that the protection was due to the activation of systemic acquired resistance (SAR) (Buonaurio et al. 2002). Pepper plants carrying one or more major genes for resistance (*R*) to *Xav* were sprayed with ASM, followed by challenge inoculation with incompatible strains of *Xav*. At 3 weeks after inoculation, the ASM-treated plants showed significantly fewer lesions induced by both incompatible (hypersensitive) and compatible (susceptible) responses than the number of lesions formed on untreated plants. The results indicated the usefulness of SAR inducer to increase the durability of genotype-specific resistance conferred by *R* genes (Romero and Ritchie 2004). Seed and/or seedling treatment with ASM or the bacterial biocontrol agent (BCA) *Pseudomonas fluorescens* (*Pf*) improved the seed germination and seedling vigor relative to the untreated controls. Under field conditions, a combination of ASM and *Pf* significantly reduced the severity of bacterial spot disease and increased seedling biomass and tomato yield compared to untreated controls (Abo-Elyousr and El-Handawy 2008).

The protective potential of acibenzolar-*S*-methyl (ASM) against tomato bacterial canker disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) was assessed. Pretreatment of tomato plants with ASM reduced the severity of the canker disease and the growth of *Cmm* was reduced in planta. Development of resistance in ASM-treated plants required an interval of 1–7 days between inducer application and challenge inoculation with *Cmm*. The level of protection by ASM reached its maximum when challenge inoculation was performed at 3 days after ASM application. Reduction in disease severity (up to 75%) was correlated with the suppression of growth of *Cmm* during the time course of infection. ASM-treated tomato plants showed higher activities of peroxidase, chitinase, superoxide dismutase, and glutathione-*S*-transferase compared with untreated control plants. ASM appeared to have dual action during interaction with the bacterial pathogen by direct inhibitory effect on the bacterial population and also by enhancement of defense-related enzymes in treated tomato plants, resulting in a higher level of resistance to bacterial canker disease in tomato (Baysal et al. 2003). The effect of ASM and the bacterial BCA *Bacillus cereus* isolate L2-1, applied either alone or in combination, on the suppression of cotton bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) was determined. Treatment with ASM reduced the disease severity to the maximum extent (57.8%), while L2-1 reduced the severity to 25.48% of the control. The combined application of ASM + L2-1 was less effective compared to ASM treatment alone. Application of ASM

alone induced the maximum increase in the activities of phenylalanine ammonia lyase (PAL), peroxidase, and β -1,3-glucanase as well as lignin deposition, especially at 14 days after treatment (Ishida et al. 2008).

Various kinds of organic compounds have been evaluated for their protective potential against crop diseases. Imidacloprid, a neonicotinoid insecticide, breaks down in plant into 6-chloronicotinic acid which is closely related to the SAR-inducer isonicotinic acid (INA). Soil application of imidacloprid was able to effectively suppress the development of citrus canker disease caused by *Xanthomonas citri* subsp. *citri* (*Xcc*). Potted Swingle citrumelo seedlings (*Citrus paradisi* x *Poncirus trifoliata*) were treated with imidacloprid, isonicotinic acid (INA), and acibenzolar-*S*-methyl (ASM) (known SAR inducers) as soil drenches or with ASM as foliar spray at 1 week prior to inoculation of immature leaves with *Xcc*. Seedlings were reinoculated four times over a 24 week period. Soil application of SAR inducers reduced canker lesions up to 70% compared with untreated inoculated plants. Lesion size was significantly reduced in treated plants, compared with untreated control treatment. Populations of *Xcc* per leaf were reduced following soil treatment with imidacloprid and INA and ASM. SAR induction in treated plants was confirmed by expression of the PR-2 gene (β -1,3-glucanase) (Francis et al. 2009).

The efficacy of inducers of systemic acquired resistance (SAR) acibenzolar-*S*-methyl (ASM), imidacloprid (IMID), and thiamethoxam (latter two are insecticides) in suppressing the development of citrus canker disease caused by *Xanthomonas citri* pv. *citri* (*Xcc*) was assessed by treating the soil. Imidacloprid (IMID), a neonicotinoid, was used to reduce canker disease incidence through control of the leaf miner (*Phyllocnistis citrella*) which could assist the bacterial pathogen to gain entry into the citrus leaf tissue. Thiamethoxam, another soil-applied neonicotinoid used for insect control, was included for comparison. The effects of 1, 2, or 4 soil applications of these compounds were compared with 11 foliar sprays of copper hydroxide and streptomycin applied at 21 day intervals. Canker disease incidence was assessed as the percentage of the total leaves with lesions. All treatments significantly reduced foliar incidence of citrus canker on the combined spring-summer-fall flushes. Sprays of copper hydroxide and streptomycin were effective for reducing canker incidence on shoot flushes produced throughout the season compared to untreated controls, whereas soil-applied SAR inducers reduced foliar disease depending on the rate, frequency, and timing of application. Except for the treatment with four applications of ASM at 0.2 g ai per tree or two applications of imidacloprid, SAR inducers were ineffective in reducing foliar infection by *Xcc* on the flushes that were present during the tropical storm. In 2009, treatments significantly reduced the incidence of foliar canker on the combined spring-summer-fall flushes, but not all treatments of spring-summer-flushes with SAR inducers were effective compared with untreated control. As the occurrence of copper-resistant strains of *Xcc* was observed, soil application of SAR inducers could be an alternative option for the management of citrus canker disease caused by copper-resistant strains (Graham and Myers 2011).

The protective activity of acibenzolar-*S*-methyl (ASM, Bion 50 WG) against *Xanthomonas campestris* pv. *vitians* (*Xcv*), causing bacterial leaf spot in lettuce, was assessed. Aqueous solution of ASM was sprayed on lettuce seedlings (c. 200 μ L of 0.2 mg mL⁻¹ for each seedling). Application of ASM reduced the disease index remarkably. The resistance induced by ASM treatment was discernible up to 11 days after treatment (dat). Plants in control

treatment showed rapid development of disease symptoms. Disease severity was reduced by 75% in ASM-treated seedlings at 7 dat and reduced disease development was observed until 11 dat. ASM treatment resulted in marked reduction in the growth of *Xcv* in plant tissues. The time between initial treatment with ASM and subsequent inoculation with *Xcv* significantly affected the induction of ISR, the optimum interval being 48 h after ASM application. In ASM-treated seedlings, a significantly lower *Xcv* population was recorded up to 96 h postinoculation compared to untreated plants (Yigit 2011).

The nonprotein amino acid, β -aminobutyric acid (BABA), was reported to induce resistance in plants against many diseases caused by plant pathogens. The effect of application of BABA on the development of potato late blight disease caused by *Phytophthora infestans* was investigated using two potato cultivars, Bintje and Pampeana, with different levels of horizontal resistance. Pretreatment of potato foliage with BABA at 30 days after emergence provided 60% protection in cultivar Pampeana against *P. infestans*. The expression of defense molecules such as glucanases, chitinases, and phenolic compounds was stimulated following BABA treatment (Altamiranda et al. 2008). The responses of potato cultivars with different levels of resistance to BABA-induced resistance against *P. infestans* were assessed. The effect of BABA on development of another pathogen, *Fusarium solani*, infecting potato tubers was also determined. Potato plants receiving four applications of BABA over the entire crop cycle, produced potato tubers with greater resistance to both *P. infestans* and *F. solani* than those from nontreated potato plants. Tuber slices from treated plants inoculated with *P. infestans* showed an increase in phenolic and phytoalexin contents. In the potato–*F. solani* interaction, infected tubers from BABA-treated plants exhibited less proteolytic activity than the infected and nontreated tubers. Treatment with BABA improved the growth of potato plants, in addition to providing protection against fungal pathogens. The degree of enhancement of resistance of potatoes depended on the natural (genetic) resistance of potato cultivars concerned (Olivieri et al. 2009).

The potential of β -aminobutyric acid (BABA) to protect potato plants against late blight pathogen *Phytophthora infestans* was influenced by different factors. Under greenhouse conditions, the durability of resistance induced by BABA, the dose–response relationships in susceptible (Bintje) and partially resistant (Ovatio and Superb) cultivars, and the effects of combined application of BABA and fungicides were studied. The lesion size was reduced by treatment with BABA by an average of 40–50% compared with untreated controls. The effect of induced resistance by BABA was progressively reduced at 4–5 days after application. Combined application of BABA and fungicides appeared to have an additive effect in suppressing disease development both under greenhouse and field conditions. With an increase in the concentrations of BABA, the protective effect became stronger proportionately. The partially resistant potato cultivars Ovatio and Superb reacted to lower concentrations of BABA. On the other hand, no significant effect of BABA could be seen under field conditions. However, BABA application compensated for a 20–25% reduction in the dose of fungicide Shirlan, as the combination was as effective as the full dose of Shirlan alone. The results indicated that BABA could be applied as a component of a disease management system against potato late blight disease (Liljeroth et al. 2010).

BABA induced resistance (IR) alone may not be sufficient to suppress the late blight disease development to the required level. Defense responses, such as hypersensitive response (HR), secretion of defense-related proteins, and production of catalase,

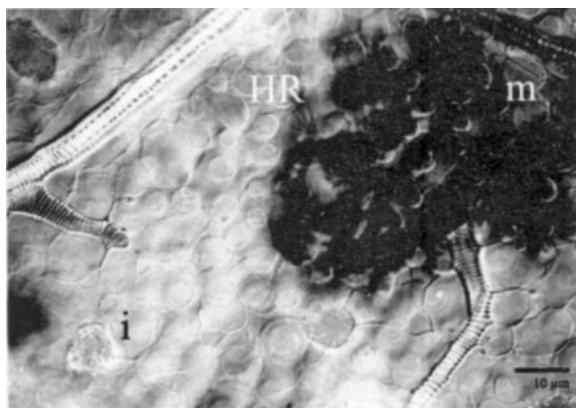


Figure 13.2 Effect of BABA application on the development of defense responses in potato cultivars challenged with *Phytophthora infestans*. Note hypersensitive response (HR-) like lesions formation in leaves treated with BABA, irrespective of infection at all intervals after inoculation; i: idioblast; m: mesophyll cells; HR: HR-like lesions in BABA-treated potato cultivar Ovatio at 48 h postinoculation. Source: Bengtsson et al. (2014). Reproduced with permission of John Wiley & Sons.

phenols, and the SA-marker PR-1, were studied in the potato-*Phytophthora infesta* pathosystem using potato cultivars Bintje and Ovatio with different levels of resistance to late blight pathogen. Leaves of cultivars Bintje and Ovatio were analyzed after foliar treatment with BABA. Visibly recognizable HR-like lesions were formed at 2 days post-treatment with BABA, irrespective of levels of infection by *P. infestans*. After staining with trypanblue, observations under the microscope revealed the induction of a small hypersensitive response by callose as well as production of H_2O_2 . When examined under the microscope, these HR-like lesions showed the presence of trypan blue-stained clusters of five or more dead mesophyll cells, both inside and outside the inoculation site, as well as in mock inoculated potato leaves (Fig. 13.2). The HR-like lesions at the site of penetration were enlarged in proportion to the progress of pathogen invasion. Rapid activation of various defense responses and a significant reduction in *P. infestans* growth were observed in leaves treated with BABA. In the more resistant cultivar Ovatio, the activation was both faster and stronger than in Bintje. Molecular and chemical analyses revealed the presence of soluble phenols, such as arbutin and chlorogenic acid, and the activation of PR-1. The role of secreted proteins in the development of resistance to late blight pathogen was investigated. SDS-PAGE analysis of protein secreted in the apoplast of cultivars Bintje and Ovatio showed accumulation of defense marker protein PR-1 (15kDa) at basal level at 2 days post-treatment with BABA at lower concentration (0.1 or 1.0 mM) or water. However, with an increase in concentration of BABA (10 mM), the apoplastic accumulation of PR-1 was appreciably enhanced in both cultivars, the increase in PR-1 level being greater in cultivar Ovatio than in Bintje. The results showed that BABA modulated the defense responses in potato in such a way that did not require release of elicitors from *P. infestans*. The efficiency of BABA-IR might differ based on cultivar differences (Bengtsson et al. 2014).

The comparative efficiency of protecting sunflower plants by BABA, BTH, and 2,6-dichloroisonicotinic acid (INA) against the downy mildew disease caused by *Plasmopara halstedii* was assessed using susceptible (S), partially resistant (MR), and highly resistant (HR) cultivars. By employing *P. halstedii*-specific primers, no detectable pathogen

marker transcript accumulation was recorded in the hypocotyls of S and HR cultivars. However, pretreatments with any one of three resistance inducers reduced the transcript accumulation in both the infected S and MR lines. Pretreatment with BTH before inoculation considerably enhanced defense-related enzyme activities in the infected S and HR genotypes, but not in MR plants. The results indicated that the resistance-inducing compounds might improve resistance in all of the sunflower genotypes to downy mildew disease and increase the activities of peroxidase and polyphenol oxidase, as well as accumulation of mRNAs of glutathione-S-transferase, defensin, and catalase. However, the activation of these enzymes did not correlate with the degree of resistance, but rather with the amount of necrotic tissues (Körösi et al. 2011).

The level of protection provided by BABA to *Brassica napus* (canola) against *Leptosphaeria maculans* was determined. BABA exerted direct inhibitory activity against *L. maculans* in the in vitro assays. The EC₅₀ concentration of BABA was similar to that of the fungicide tebuconazole. Both spore germination and hyphal growth of *L. maculans* were inhibited by BABA. Suppression of disease progression in plants and antifungal activity in vitro were weaker. In contrast to BTH, the effect of BABA on disease development was nearly independent of timing of treatment, indicating possible antifungal activity in plant. Quantification of multiple hormones and an expression analysis indicated that treatment with BABA induced synthesis of salicylic acid (SA) and expression of SA marker gene PR-1. However, no evidence for priming of SA responses to *L. maculans* was obtained. The antifungal activity of BABA against *L. maculans* could be another possible mechanism of action by which BABA might provide protection to *B. napus* (Sašek et al. 2012).

The efficacy of DL-β-aminobutyric acid (BABA) in suppressing the development of tomato bacterial spot disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) was assessed. Tomato plants (3 weeks old) were treated with different doses of BABA (250–1000 μg mL⁻¹), followed by inoculation with the suspension of *Cmm* (10⁸ CFU mL⁻¹). No antibacterial activity of BABA was indicated by in vitro assays. Foliage sprays of 500 μg mL⁻¹ of BABA suppressed disease development by up to 54% at 14 days after inoculation. Populations of *Cmm* were reduced by 84% in planta by BABA, compared with control treatment at 4 days after inoculation. In BABA-treated tomato plants, higher activities of phenylalanine ammonia lyase (PAL) and peroxidase (PO) and higher levels of H₂O₂ were observed at 1 day after application relative to those in control plants. The results suggested that application of BABA might result in induction of defense-related enzymes contributing to the enhancement of resistance in tomato to bacterial canker disease (Baysal et al. 2003).

Maize Fusarium ear rot disease caused by *Fusarium verticillioides* negatively impacts grain yield and quality because of contamination with mycotoxins (fumonisin) which are harmful to humans and animals. The disease management strategy of using chemical elicitors of disease resistance to reduce infection by *F. verticillioides* and mycotoxin contamination was evaluated. Five resistance elicitors, namely BABA, BTH, harpin protein, 2,6-dichloronicotinic acid (INA), and methyl jasmonate (MeJA), and a fungicide containing difenconazole (triazole) and azoxystrobin (strobilurin) as active ingredients were tested for their effectiveness under field conditions in multi-site field trials. After inoculation of maize plants with *F. verticillioides* isolate MRC 826 (high fumonisin producer), disease severity was determined by visual rating and the contents of fumonisins in grains were quantified by HPLC technique. Neither the resistance elicitors nor the fungicide consistently reduced the Fusarium ear rot and/or fumonisin contamination.

Treatment of maize with BTH resulted in significant reduction in ear filling and yield in field trials. Treatment effects on fumonisin content were influenced by maize genotype and trial location. The results showed that the resistance elicitors tested may not be an acceptable option for the management of maize ear rot disease (Small et al. 2012).

The ability of 2,6-dimethoxy-1,4-benzoquinone (DMBQ) to induce resistance in rice against blast pathogen *Magnaporthe grisea* was assessed. DMBQ inhibited spore germination and appressorium formation at concentrations of $>50\text{ }\mu\text{g mL}^{-1}$. When rice leaves were pretreated with DMBQ at $10\text{ }\mu\text{g mL}^{-1}$, lesion formation was inhibited compared with controls and there was no antifungal activity at this concentration of DMBQ. Induction of resistance was at a greater level in the leaf sheaths pretreated with DMBQ. Generation of H_2O_2 and expression of defense-related enzymes PAL, POX, and PR-10 were observed in rice leaves pretreated with DMBQ, indicating that DMBQ might protect rice plants against rice blast disease (Ueno and Yoshikiyo 2014).

Glycerol, an ecofriendly, nontoxic, edible, and biodegradable sugar alcohol, was evaluated for its effectiveness in suppressing development of pod rot disease of cocoa caused by *Phytophthora capsici*, using leaf disc bioassay. Exogenously applied glycerol is absorbed by plant cells and further phosphorylated to glycerol-3-phosphate (G3P) by glycerol kinase in the cytosol. An increase in G3P and decrease in oleic acid levels are considered to be associated with induction of the defense pathway in plants. The effect of exogenous application of glycerol on the endogenous level of G3P within the treated leaves was evaluated using ultrahigh-performance liquid-chromatography mass spectrometry (UHPLC-MS). The cocoa leaves of cultivar SCA6 were treated with glycerol (100, 500, and 1000 mM) or water for 3 days consecutively and inoculated with *P. capsici* after the last treatment with glycerol. All glycerol treatments drastically reduced the average lesion size relative to the control. There was no significant difference in the average sizes of lesions formed on leaves treated with 100, 500, and 1000 mM concentrations of glycerol, indicating that glycerol at 100 mM concentration was sufficient to suppress disease development by inducing resistance to *P. capsici*. Glycerol treatment significantly upregulated the expression genes governing the biosynthesis of a set of pathogenesis-related (PR-) protein in treated plants. Expression of *TcChi1* gene was significantly induced due to application of 100 and 500 mM glycerol application, whereas PR-3Q and PR-5 were not significantly induced. Expression of *TiChi4* was upregulated by a factor of 5 and 30 by 100 and 500 mM glycerol treatments, respectively, compared with water-treated controls. The results indicated that a low-cost and effective strategy of applying glycerol may be useful for managing cocoa pod rot disease caused by *P. capsici* (Zhang et al. 2015).

13.2.5 Effects of Inorganic Compounds

The induction of resistance in crops to diseases caused by microbial pathogens using inorganic compounds has been demonstrated. Phosphites (Phi) are alkali metal salts of phosphorous acid and have the potential to protect plants against infection by pathogens. Seed tubers and foliage of potato cultivars Shepody and Kennebec were treated with Phi to determine their ability to induce resistance against *Phytophthora infestans*, *Fusarium solani*, and *Rhizoctonia solani*. A high level of resistance to *P. infestans* was induced by Phi, whereas the levels of resistance to *F. solani* and *R. solani* were intermediate and low, respectively. Furthermore, seed potato tubers treated with calcium or potassium phosphites (CaPhi and KPhi) as 1% commercial product emerged earlier

than the untreated tubers. Spray application of Phi on the foliage two to four times at different doses provided high levels of protection against *P. infestans* in both potato cultivars. A higher level of protection was observed in cultivar Kennebec when CaPhi was applied, whereas the protective effect of KPhi was greater in cultivar Shepody. Expression of β -1,3-glucanases was induced at different times after treatment, but no correlation between β -1,3-glucanase expression and level of foliar protection could be established. On the other hand, Phi positive protection effects did not produce negative effects on plant growth. Leaves in treated plants were darker green in color than those in untreated plants. Increase in Rubio protein and a delay in setting of leaf senescence in Phi-treated leaves were also seen (Lobato et al. 2008).

The comparative efficacy of potassium phosphite (foliar fertilizer), DL-3-aminobutyric acid (BABA, Bion), and benzothiadiazole (BTH) in suppressing the development of Fusarium head blight (FHB) disease of winter wheat, caused by *Microdochium majus* (= *M. nivale* var. *majus*), was assessed using detached leaves for preliminary assessment. Aqueous solutions of the test chemicals were sprayed on greenhouse-grown winter wheat plants at 7 days prior to inoculation of wheat heads with the pathogen. The number of bleached spikelets per inoculated spike reflected the disease development and severity. Potassium phosphite (KPhi) applied as pretreatment foliar spray reduced the FHB disease development. Spraying plants with foliar fertilizer reduced the disease severity due to *F. culmorum* by up to 40%. Reduced development disease induced by *M. majus* was observed in detached leaves pretreated with the foliar fertilizer. The foliar fertilizer inhibited the mycelial growth of *M. majus* and *F. culmorum*, indicating its fungistatic activity accounting for its inhibitory effect on disease development. No significant reduction in disease development was observed in wheat pretreated with BABA or Bion, although these compounds tended to reduce disease development, especially when applied in combination with other potential resistance inducers. The results showed that an aqueous solution of potassium phosphite was effective in suppressing FHB disease in winter wheat (Hofgaard et al. 2010).

Application of silicon (Si) has been reported to enhance resistance of rice plants to blast disease caused by *Magnaporthe grisea*. The enhanced resistance of rice plants treated with Si appeared to be strongly associated with the production of phytoalexins momilactones A and B. Leaf extracts from plants treated with Si^+ , followed by inoculation with *M. grisea*, contained higher concentrations of momilactone phytoalexins compared with those from untreated rice plants (Si^-). Stimulation of terpenoid pathway occurred more efficiently in Si^+ plants. The lower blast disease severity on leaves of Si^+ plants at 96 h after inoculation with *M. grisea* might be due to stimulation of terpenoid pathway in Si^+ plants (Rodrigues et al. 2005). Silicon induced a rapid, but transient burst of H_2O_2 at 24 h after inoculation. Application of Si to rice plants significantly altered the activity of catalase and lipoxygenase and the concentration of malondialdehyde (indicative of lipid peroxidation) in rice plants. The results suggested that rice plants might respond to Si by increased H_2O_2 accumulation and lipid peroxidation, which in turn may be linked to host defense mechanisms such as lignin production, oxidative cross-linking in the cell wall, phytoalexins production, and hypersensitive reaction (Sun et al. 2010).

The effect of the application of calcium silicate (Si^+) or calcium carbonate (Si^-) on the development of Phytophthora blight disease of bell pepper caused by *Phytophthora capsici* was assessed. Calcium silicate or calcium carbonate was incorporated in No. 2 peat mix and seedlings were planted after 6 weeks. The soil was infested with two

isolates of *P. capsici* (Cp30 and Cp32). The roots and stem samples were examined for the presence of lesions on crown and stems and wilting of plants. The area under disease progress curve (AUDPC) and area under wilting progress curve (AUWPC) were calculated based on the data gathered. Relative lesion extension (RLE) was deduced as the ratio of vertical lesion expansion to stem length at 9 days after planting. An increase of 40% in Si concentration in the roots was observed, but not in the stems of bell pepper plants in the Si⁺ treatment, compared to Si⁻ treatment. The AUDPC was reduced by 15.4% and 37.5%, while AUWPC was reduced by 29% and 33.3% in two experiments, compared with control treatments. RLE values also showed a reduction in Si⁺ treatment. The dry root weights and stem weight also registered increases as compared with control treatment, indicating growth promotion effect due to Si application (French-Monar et al. 2010).

Fusarium (Panama) wilt disease of banana caused by *Fusarium oxysporum* f.sp. *cubense* (Foc) is spread primarily through infected suckers (propagules) which may not exhibit symptoms of infection. It is difficult to manage the disease once the pathogen has been introduced via suckers into the field, where it becomes soilborne. The protective ability of silicon (Si) amendment against the incidence of Fusarium wilt disease was investigated. The banana cultivars Grand Nain (resistant) and Maca (susceptible) were grown in plastic pots with soil amended with Si at 0 or 0.3 g kg⁻¹ of soil (Si⁻ and Si⁺ respectively) and inoculated with Foc race 1. Relative lesion length (RLL) and asymptomatic fungal colonization in tissue (AFCT) were determined at 40 days after inoculation. Si content of roots was significantly increased by 35.3% in Si⁺ treatment compared with Si⁻ treatment, the resistant cultivar showing a greater increase than the susceptible cultivar. Grand Nain and Maca plants in Si⁺ treatment showed significant reductions of 40% and 57.2% for RLL, respectively, compared with plants in Si⁻ treatment. A similar beneficial effect of reduction in resistant and susceptible cultivars due to Si amendment was observed for AFCT compared with unamended control treatments. Si amendment increased the levels of activities of defense-related enzymes in plants, indicating that the mechanism of action of Si might be by inducing resistance in banana plants against Fusarium wilt disease (Fortunato et al. 2012).

The comparative protective potential of sodium nitrite, chitosan, and Tillekur (known elicitors of disease resistance) to suppress seedborne infection by *Didymella lycopersici* in tomato seeds was assessed. Sodium nitrite was highly effective when applied at a concentration of 300 mM L⁻¹ for 20 min. Tillekur showed a similar level of protection as sodium nitrite, while chitosan was less effective in reducing seedborne fungal infection. However, phytotoxicity of Tillekur limited its use for treatment of tomato seeds. The results indicated the effectiveness of sodium nitrite in reducing seedborne infection of tomato seeds by *D. lycopersici*. In addition, the low cost of acidified sodium nitrite could be an additional factor for considering it as an effective candidate for replacing the fungicide applied against seedborne fungal pathogens (Kasselaki et al. 2008).

The effect of calcium nitrate on the development of potato soft rot disease caused by *Pectobacterium carotovorum* subsp. *brasiliense* was investigated under field conditions. Sprouted potato tubers of cultivar BP1 were planted in plots treated with different fertilizer combinations. The harvested progeny tubers were inoculated with *P. carotovorum* subsp. *brasiliense*. Calcium amendment increased the activities of defense-related enzymes, phenylalanine ammonia lyase (PAL), polyphenoloxidase (PPO), and peroxidase (PO) involved in the metabolism of phenolics. Calcium amendments significantly reduced the maceration activity of the bacterial pathogen in tuber tissues. Chlorogenic

acid, caffeic acid, and calcium contents, but not ferulic acid, were significantly higher ($P < 0.05$) in tuber peels or potato plants grown in calcium-treated plots. The reduced maceration could be due to the presence in the tubers of increased levels of caffeic acid and chlorogenic acid which possessed antimicrobial properties (Ngadze et al. 2014).

13.3 Methods of Application of Formulated Products of Biological Control Agents

The effectiveness of different kinds of biotic biological control agents in suppressing disease development has been assessed under greenhouse conditions and some of them under field conditions. Very few of them have been advanced to formulation stage however, because of the many strict requirements to be satisfied and the huge costs involved in obtaining toxicological and environmental data. Biological control agents (BCAs) that have been selected based on their potential for large-scale application for the management of crop diseases are formulated by adopting various procedures. The formulated products are applied to treat the seeds, propagules, or plants as either protective and/or curative treatments, depending on the mechanisms of action of the BCAs. In addition, the BCA formulations may be applied as soil treatment or foliar spray. The efficacy of the formulated products in reducing the disease incidence and/or severity is compared with the unformulated fresh fungal or bacterial species to determine the extent of loss of biocontrol activity, if any, due to the process of formulation and storage for various durations. Commercially available products have to be evaluated by state-owned research institutions and are then recommended for large-scale application by farmers (Narayanasamy 2013).

13.3.1 Treatment of Seeds

Biocontrol agents (BCAs) are applied as liquid, powder, or granular formulations containing single strains or a mixture of strains of fungal or bacterial BCAs. The treatment of seeds and propagules with BCA products has been the more frequently employed method of application since seed/propagule treatment is easier and more economical due to requirement of lesser quantities of BCA product. Soil/foliar applications require large quantities of BCA products to protect seedlings/grown plants against soilborne and airborne pathogens that can infect seeds and propagules later. Seed treatment with BCAs is adopted to suppress soilborne pathogens that can infect young seedlings and become systemic, infecting the inflorescence/seeds at heading stage.

Pseudomonas fluorescens as suspension or talc-based powder was used to coat rice seeds to protect plants against the rice sheath blight disease caused by *Rhizoctonia solani* and blast disease caused by *Magnaporthe grisea*. The bacterial BCA was able to spread to roots, stems, and leaves of plants growing from the treated seeds, offering protection against these important diseases of rice (Mew and Rosales 1986; Vidhyasekaran et al. 1997). In a later investigation, two strains of *Pseudomonas fluorescens* (PF1 and PF2) were applied as talc-based formulation to treat seeds, root, and foliage of rice plants, and to the soil. The talc-based formulations significantly reduced sheath blight disease. The effectiveness of the strain mixture was greater than the individual strains in suppressing the development of sheath blight disease under greenhouse and field conditions. The BCA formulations were equally effective as the fungicide carbendazim.

In the field trials, the maximum disease reduction achieved was 62.1%. Application of talc-based formulations of *P. fluorescens* was also effective against the important rice leaf folder (*Cnaphalocrocis medinalis*) by altering the feeding behavior of the insect and adversely affecting the development of the insect (Radjacommare et al. 2002).

Treatment of barley seeds with *Pseudomonas chlororaphis* MA342 was effective against net blotch caused by *Dreschlera teres*. Soon after seed treatment, the bacterial BCA could be detected under seed glume when the seeds were sown. An irregularly distributed area of bacterial aggregation could be observed, indicating the epiphytic colonization of glume cells. The bacteria were not seen within the embryo. Bacterial aggregates were regularly present in the groove of each seed formed by the base of the coleoptiles and scutellum, suggesting that the BCA could localize the seed tissues with the pathogen which facilitates the action of antifungal compounds produced by *P. chlororaphis* strain MA342 (Tombolini et al. 1999).

The formulated product of *Bacillus subtilis* was used to treat the peanut (groundnut) seeds cultivar Shulamit for the control of the seedborne pathogen *Sclerotium (Athelia) rolfsii*, causing southern blight disease. The adverse effects of *S. rolfsii* on the nodulation, leghemoglobin, and nitrogenase activity of peanut were counteracted by the treatment with *B. subtilis*. In addition, the changes in crop vigor index, total nitrogen content, and survivability of both *Rhizobium* spp. and *B. subtilis* was found to be related to compatibility between them. They showed occasional synergism between them (Abd-Allah and El-Didamony 2007).

The comparative efficacy of three fungal biological control agents (BCAs), *Trichoderma viride*, *T. harzianum*, and *Gliocladium virens*, and five neem products in suppressing the development of anthracnose disease of bean caused by *Colletotrichum lindemuthianum* was assessed. *T. viride* and the neem product Wanis ($1000\mu\text{L mL}^{-1}$) were the most effective in suppressing the mycelial growth of the pathogen. These treatments alone or in combination with carbendazim were evaluated after seed treatment under field conditions, using two bean cultivars Baspa (with high field resistance) and two highly susceptible cultivars grown by farmers. Naturally infected seeds were treated with *T. viride* and Wanis, and these two treatments were effective in reducing seed-borne infection. However, seed treatment alone with biocontrol agent, neem product, and carbendazim were not effective. Disease incidence and severity ranged from 21.22–25.63 and 14.14–17.47, respectively, which were statistically on par under field conditions. Integration of BCA with carbendazim provided maximum protection against bean anthracnose disease (Padder et al. 2010).

The beneficial effect of *Rhizobium* spp. as fixers of atmospheric nitrogen in the root nodules formed in leguminous plants is well known. The capacity of *Rhizobium leguminosarum* biovar *phaseoli* to trigger resistance in common bean (*Phaseolus vulgaris*) against common bacterial blight (CBB) disease was assessed. *R. leguminosarum* bv. *phaseoli* was applied as seed treatment on two tolerant lines Ks 51103 and BF13607 and the susceptible Khomein and line Ks21479 and tested under field conditions. *R. leguminosarum* bv. *phaseoli* reduced the CBB disease severity and improved plant growth in the field trials. The disease severity decreased to the maximum extent at 15 days after inoculation with *Rhizobium*, but under field conditions maximum reduction of CBB disease occurred at 35 dai. Treatment with *Rhizobium* improved the 100 seed weight to the maximum extent in cultivar Khomein (Osdaghi et al. 2011).

Cotton bacterial blight disease caused by *Xanthomonas campestris* pv. *malvacearum* reduces the yield substantially both quantitatively and qualitatively. The bacterial

biocontrol agents (BCAs) *Pseudomonas aeruginosa* strain 7NSK2 and *P. fluorescens* strain CHA0 were evaluated for their ability to reduce disease severity. Cotton seeds were treated with BCA formulations after acid-delinting and neutralization of seeds. Both BCAs effectively reduced the disease severity by inducing resistance to the pathogen and increasing the plant biomass (fresh weight) (Fallahzadeh-Mamaghani et al. 2009). The biocontrol potential of talc-based formulations of *Pseudomonas fluorescens* containing Pf32 and Pf93 strains and *Bacillus subtilis* containing B49 strain in suppressing the development of the cotton bacterial blight disease under greenhouse and field conditions was assessed. The BCA strains survived well as talc-based formulation. Application of mixture of Pf32, Pf93, and B49 to seed, soil, and foliage significantly reduced the bacterial blight incidence, and the plant growth and numbers of bolls were increased by the BCA application under field conditions. The yield levels of bolls from BCA-treated plots were increased due to application of selected strains of *P. fluorescens* and *B. subtilis* (Salaheddin et al. 2010).

Carrot seed infection by *Alternaria dauci* and *A. radicina* results in pre- and post-emergence mortality of seedlings. Isolates of *Clonostachys rosea* were found to be as effective as the fungicide iprodione in protecting the seedlings. Isolate IK26 of *C. rosea* was employed to bioprime a carrot seed lot with high level of seed infection by *A. radicina* (29%) and *A. dauci* (11%), and a seed lot with a low level of infection (4% and 7% respectively). The seeds were primed with water alone (hydropriming) or with the addition of *C. rosea* IK726 (biopriming). During the 14 days of hydropriming, the disease incidence of *A. radicina* and *A. dauci* was dramatically increased to two- and five-fold respectively, regardless of initial level of seed infection. In contrast, biopriming reduced the incidence of *A. radicina* to 2.3% and that of *A. dauci* to 4.8%, while the level of infection by both pathogens was reduced to 0.5% on bioprime seeds with lower level of infection (Jensen et al. 2004).

The integration of good agricultural practices (GAPs) with biological control strategies was considered as a sustainable way of achieving reliable suppression of colonization of maize grains by *Fusarium verticillioides* and mycotoxin (fumonisins) contamination of grains. Field trials were conducted in two locations to assess the effect of seed treatment with *Trichoderma harzianum* strain T22 on *F. verticillioides* kernel colonization and on fumonisin accumulation under various natural conditions. An average reduction of 58% in fungal infestation and 53% in mycotoxin contamination was observed in the 3 year experiments. The results suggested that seed biopriming with *T. harzianum* T22 strain could provide a promising and ecofriendly strategy for the management of *F. verticillioides* affecting maize (Ferrigo et al. 2014).

Clonostachys rosea strain ACM941 parasitized *Gibberella zeae* (anamorph *Fusarium graminearum*), resulting in the reduction in severity of Fusarium head blight (FHB) disease of wheat caused by this pathogen. The efficacy of CLO-1, a formulated product of strain ACM941, in reducing perithecial production on various crop residues was assessed in comparison to the registered fungicide Folicur (tebuconazole) under field conditions. When applied on *G. zeae*-inoculated corn, soybean, and wheat residues in the spring of 2009 and 2010, the formulation CLO-1 significantly inhibited perithecial production on all crop residue types, reducing daily perithecial production (DPP) by 93.1% on corn residue, 94.4% on soybean residue, and 84% on wheat residue compared to untreated control. When applied on naturally infected wheat residues in the autumn of 2009 and 2010, CLO-1 significantly reduced DPP in the following growing season by 72.3% on peduncles, 51.0% on spikelets, and 57.2% on stems. The effects were numerically better but not significantly different from those obtained by the fungicide

used as a positive control. The results suggested that CLO-1 could be an effective bio-fungicide in suppressing the development of FHB disease caused by *G. zeae* by reducing the initial inoculum of the pathogen required for floral infection (Xue et al. 2014).

The biocontrol potential of the fungal biocontrol agent (BCA) *Muscador albus* (capable of producing volatile compounds) applied as seed treatment or an in-furrow soil treatment to suppress the development of wheat common bunt (CB) disease caused by *Tilletia caries* was assessed. For seed treatments, dry rye grain culture of *M. albus* was ground into powder and applied at a rate of 125 mg kg⁻¹ of seed to wheat seed infested with *T. caries* teliospores. The BCA culture was cracked into small particles and applied in the furrows at the rate of 4 g m⁻¹ of row length, along with teliospores-infested seed during sowing. Treatments were evaluated during two growing seasons and two planting dates beginning in early spring, when soil temperatures were optimal for disease development (5–10°C) and then at c. 3 weeks later. In the first year, treatments in the first seeding date reduced the common bunt disease from 44% diseased spikes in untreated controls to 12% and 9% in seed and in-furrow treatments, respectively, and from 6% in control to 0% in both treatments in the second seeding date. In the second year, the disease incidence was reduced from 8% in the controls to 0.5% and 0.25% for seed and in-furrow treatments, respectively, in the first seeding date and from 0.75% in controls to 0% in both seed and in-furrow treatments in the second seeding date. The results indicated the potential of *M. albus* for inclusion as a component of a disease management system in organic crop production where options for CB management are limited (Goates and Mercier 2011).

Formulations containing the plant growth-promoting rhizobacteria (PGPR) *Bacillus megaterium* A6 were evaluated for their biocontrol potential against *Sclerotinia sclerotiorum*, causing serious yield losses in oilseed rape and many other crops worldwide under field conditions. Treatments containing strain A6 in pellet and wrap formulations resulted in the reduction in disease incidence in oilseed rape and seed yields that were similar to those from the chemical-treated plots and significantly greater than the untreated control at two locations. Strain A6 applied to oilseed rape seed in two formulations promoted growth in the greenhouse pot experiments using autoclaved soils. The mean shoot dry weight per pot and mean total N% were increased per plant relative to their respective controls containing formulated oilseed rape seed without A6 strain and the untreated control. Both formulations (pellet and wrap) provided stable *B. megaterium* A6 ($\geq 10^6$ CFU) and seed germination (>85%) over a 6 month period at room temperature. The results indicated that the formulations of *B. megaterium* had the potential for advancement to commercial exploitation (Hu et al. 2013a).

13.3.2 Treatment of Propagules/Transplants

Microbial plant pathogens may be carried by propagules or transplants that may or may not exhibit symptoms of infection. The propagules such as tubers, rhizomes, corms, bulbs, cuttings, or setts have to be treated with formulated products of biocontrol agents (BCAs) to reduce the incidence of diseases in the fields. Sugarcane red rot disease caused by *Colletotrichum falcatum* is primarily transmitted via infected setts. Propagation of disease-free planting materials coupled with different kinds of sett treatments (hot air/hot water) prior to planting is advocated for the management of the red rot disease. Treatment of setts with a suspension of talc formulation of *Pseudomonas fluorescens* for 1 h followed by incubation for 18 h prior to planting was reported to reduce the incidence of red rot disease and also enhance the plant growth (Viswanathan and Samiyappan 2002).

Potato silver scurf disease caused by *Helminthosporium solani* spreads through seed tubers during storage. The minitubers of cultivar Red Norland potato were immersed in a suspension of the fungal BCA *Acremonium strictum* for 3 min and air-dried. The treated and untreated tubers were inoculated with *H. solani* by spraying conidial suspension of the pathogen. *A. strictum* reduced the sporulation and spore germination of *H. solani*. The BCA was effective when applied as a protective treatment on infected tubers (Rivera-Varas et al. 2007). *Phytophthora infestans* causes tuber blight in addition to late blight disease on potato plants in the field. The efficacy of a formulated product of the fungal BCA *Chaetomium globosum* in suppressing the development of disease in potato plants was assessed. The BCA was applied as tuber, soil, and foliar treatment at 1 mL kg⁻¹ of tubers, 1 mL kg⁻¹ of farm yard manure (FYM) as soil application, and 0.7% as foliar spray application. Disease incidence was reduced by 28% under field conditions. Application of *C. globosum* increased the tuber yields in two field trials, indicating the usefulness of the formulated product of *C. globosum* (Shanthiya et al. 2013).

The endophytic actinomycete *Streptomyces griseorubiginosus* strain S96 was evaluated for its potential in suppressing the development of banana Fusarium (Panama) wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*) race 4. The roots of plantlets were immersed in a suspension of strain S96 (10⁶ CFU mL⁻¹) for 1 h before planting, followed by inoculation with *Foc* after 3 weeks. The wilt disease severity was reduced significantly, while the mean fresh weight of the plant was increased (Cao et al. 2005). Tumorigenic strains of *Agrobacterium vitis* infect grapevine, rose, and tomato, causing crown gall diseases. Nonpathogenic *A. vitis* strain VAR03-1 suspension was used to soak the roots of grapevine seedlings for 1 h before planting in pathogen-infested soil. The incidence of tumor formation was significantly reduced by strain VAR03-1 (Kawaguchi et al. 2007). In a later investigation, treatment with VAR03-1 significantly reduced the number of plants infected and disease severity induced by the pathogen in rose and tomato as in the case of grapevines. The strain VAR03-1 was more inhibitory to pathogen development than the nonpathogenic strain K84 of *A. rhizogenes*. Under field conditions, strain VAR03-1 was found to be more efficient in controlling grapevine crown gall caused by *A. vitis*. The BCA strain VAR03-1 could multiply and establish a population averaging 10⁶ CFU g⁻¹ of roots in the rhizosphere of grapevine and persisted on the roots of grapevine for 2 years (Kawaguchi et al. 2008).

The biocontrol potential and plant growth-promoting activity of talc-based formulations of two rhizobacterial strains and their mixtures were assessed for suppressing the development of vascular wilt and corm rot of gladiolus caused by *Fusarium oxysporum* f.sp. *gladioli*. The BCA strains *Bacillus atrophaeus* S2BC-2 and *Burkholderia cepacia* TEPF-Sungal were applied for corm dressing and soil treatment. Treatment with strain mixture resulted in the highest spike and corm production of 100% and 150%, respectively, with less vascular wilt and corm rot incidences by 73.6% and 58.4% over pathogen-inoculated control under greenhouse conditions. In the field experiments the strain mixture also suppressed vascular wilt and corm rot incidence (by 48.6% and 46.1%) over nonbacterized control. The effectiveness of the strain mixture in disease suppression was comparable to the fungicide carbendazim, in addition to enhancement of spike and corm yield with an average of 58.3% and 27.4%, respectively, compared to the control (Shanmugam et al. 2011).

The effectiveness of rhizobacterial strain and a fungal BCA antagonist in suppressing the development of ginger rhizome rot caused by *Fusarium oxysporum* f.sp. *zingiberi*

and soft rot and yellows due to *Pythium splendens* was determined. *Burkholderia cepaica* TEPF-Sungal, *Bacillus subtilis* S2BC-1, and *Trichoderma harzianum* S17TH were evaluated for their efficacy in disease suppression. The rhizomes were treated by soaking them in aqueous solutions of talc-based formulation (20 g L^{-1}) of individual strains and their mixtures. After air-drying, they were planted in endemic fields during 2009–2010. The strain mixtures reduced yellows and rhizome rot by 45.9% and 49.3%, respectively, compared to control. The strain mixture treatment was more effective than the fungicides (carbendazim + mancozeb). Among treatments, the strain mixture S17TH + TEPF-Sungal was more effective than individual BCA strains. This strain mixture also increased rhizome yield (Shanmugam et al. 2013).

13.3.3 Treatment of Soils

The soil environments include several microorganisms interacting with each other and the plants. Organic matter and other nutrients are applied for improving plant growth which is likely to be reflected in the enhancement of crop yields with acceptable levels of quality of the produce. Several fungal/bacterial biocontrol agents (BCAs) have been reported to possess the required level of antagonistic activity in in vitro assays, but very few of them exhibited effectiveness and the potential for utilization in suppressing pathogen development consistently under field conditions. The fungal BCAs *Trichoderma viride*, *T. virens*, and *T. harzianum* have been widely employed for the control of several soilborne pathogens which may become seedborne or propagule-borne accounting for both quantitative and qualitative yield losses. The plant growth-promoting rhizobacteria (PGPRs), such as strains of *Pseudomonas* spp. and *Bacillus* spp., have the capacity to induce systemic resistance in treated plants, providing protection to the plants against diseases caused by microbial pathogens.

Nontoxicogenic *Aspergillus* spp. have been employed to competitively exclude toxigenic fungal pathogens from the soil. Aflatoxin contamination of corn grains could be reduced by introducing competitive non-aflatoxin-producing strains of *A. flavus*. Aflatoxin contamination in corn was reduced by field application of wheat grains preinoculated with nonaflatoxigenic *Aspergillus flavus* strain NRRL 30799. The reliability and efficiency of replacing wheat grains with bioplastic formulation Mater-Bi®, applied to serve as carrier matrix to this formulated BCA, were assessed. Mater-Bi® granules were inoculated with a conidial suspension of NRL30797 strain to achieve a final cell density of approximately 10^7 conidia/granule. Incubation of 20 g soil samples with Mater-Bi® granules for 60 days resulted in 10^{42} and 10^{53} propagules of *A. flavus* per gram of soil in microbiologically active (nonsterile) and sterilized soils, respectively. The degree of soil colonization by the BCA was not enhanced by increasing the number of granules applied to the soil. The bioplastic formulation was highly stable under soil environment. Mater-Bi® granules had the potential for substituting wheat grains as a carrier of application of the BCA strain for reducing aflatoxin contamination in corn (Accinelli et al. 2009). In a further investigation, field application of inoculated bioplastic granules induced a rapid shift in the competition of *A. flavus* with a significant decrease in the relative abundance of indigenous aflatoxigenic isolates. Application of bioplastic granules at 30 kg ha^{-1} was more efficient in replacing aflatoxigenic isolates than a 15 kg ha^{-1} dosage. The aflatoxin contamination was reduced by 86–92% at 30 kg ha^{-1} dosage of bioplastic granules in 2009 and 2010 (Accinelli et al. 2012).

13.3.4 Treatment of Foliage of Plants

Seed infection may be the resultant of interaction between airborne inoculum of fungal or bacterial pathogens infecting aerial plant parts such as leaves, stem, and inflorescence. Biocontrol agents (BCAs) have been used to protect the aerial plant parts to prevent seed infection through local or systemic infections. Seedborne pathogen *Alternaria radicina* causes black rot disease of carrots. *Ulocladium atrum*, the fungal antagonist, was applied to plants at flowering stage in seed production fields. Application of *U. atrum* resulted in reduction in seed infestation by *A. radicina* and no negative effects of the antagonists on seed quality were observed. *U. atrum* acted as a strong competitor during substrate colonization of senescing tissues of carrot plants and the BCA could enter necrotic flower parts before establishment of *A. radicina* on seeds (Kohl et al. 2004). The biocontrol potential of *Pseudomonas chlororaphis* PA-23, *Pseudomonas* spp. DF41, and *Bacillus amyloliquefaciens* BS6 and E16 strains to suppress the development of *Sclerotinia sclerotiorum*, incitant of stem rot disease of canola (*Brassica napus*; infection generally commencing from petals) was assessed. The suspensions of the BCA strains were sprayed at 30 and 50% bloom stages, followed by inoculation with ascospore suspension of *S. sclerotiorum*. *P. chlororaphis* PA-23 and *B. amyloliquefaciens* strain BS6 significantly reduced the disease incidence. The inhibition of ascospore germination and stimulation of host plant defense-related enzymes were observed following application of strain PA-23. The strain PA-23 and BS6 provided protection to canola plants to the same level that could be obtained by applying the fungicide Rovral Flo® (iprodione). Double-spray application of the BCA strains was not more effective in reducing disease incidence than the single spray of the BCA strains tested (Fernando et al. 2007b).

The ability of *Pseudomonas fluorescens* isolated from maize rhizosphere and its formulation was assessed to reduce the incidence of maize ear rot caused by *Fusarium verticillioides* and fumonisin accumulation in maize kernels. *P. fluorescens* was formulated using corn starch, wheat bran, and talc powder, and applied as seed treatment and foliar spray. Pure culture of *P. fluorescens* and the formulations increased plant growth and vigor as reflected by seed germination, seedling vigor, plant height, 1000-seed weight, and yield compared to controls. The pure culture of *P. fluorescens* used as seed- and spray-treatments enhanced the growth parameters and reduced the incidence of maize ear rot disease and the levels of fumonisins to the maximum extent compared with formulated products. The pure culture and formulations of *P. fluorescens* showed potential for reducing fumonisin contamination of maize kernels intended for human and animal consumption (Nayaka et al. 2009).

13.4 Integration of Biological Control with Other Management Practices

13.4.1 Integration with Fertilizer Application

Among the crop management practices, application of fertilizers is of vital importance to sustain better plant growth and consequent higher yields. Combined application of the mycoparasitic biocontrol agent *Coniothyrium minitans*, effective against *Sclerotinia sclerotiorum* and causal agent of stem rot disease of oilseed rape, and the compound

fertilizer (N-P-K) at various concentrations significantly reduced the number of apothecia produced from the sclerotia of the pathogen under field conditions. The compound fertilizer did not adversely affect the ability of *C. minitans* to infect the sclerotia of the pathogen or to suppress the carpogenic germination of sclerotia of the pathogen. *C. minitans* was compatible with the fertilizer when applied at planting of oilseed rape. The combined application of *C. minitans* and the fertilizer could achieve the twin objectives of suppressing development of stem rot disease and increasing production efficiency of oilseed rape, in addition to saving labor costs (Yang et al. 2011).

13.4.2 Integration with Fungicide Application

The strategy of integration of biological control agents with fungicides has been evaluated to reduce the quantity of fungicides applied against fungal pathogens affecting different crops. Microorganisms with biological control potential were found to be ubiquitous in rice ecosystems. The bacterial BCAs *Bacillus* sp. and *Burkholderia cepacia* applied as a mixture reduced the sheath blight disease (*Rhizoctonia solani*) severity by the maximum extent at harvest, compared with individual strains. These strains seemed to exhibit synergism in acting on the pathogen. However, the synergistic effect was not observed consistently in all tests under field conditions. The control efficiency ranged over 50–90% with a high variance. To improve the effectiveness of disease control, a half-dose of the commonly used fungicide/antibiotic such as Jingangmycin in China and validamycin in Vietnam was used as a mix with BCAs. The combination of BCAs and fungicides was found to be effective in suppressing the development of rice sheath blight disease (Mew et al. 2004).

An integrated management system for rice sheath blight disease caused by *Rhizoctonia solani* was evaluated for its effectiveness. *Streptomyces philanthi* RM-1-138, commercial formulation of *Bacillus subtilis* (Larminar®), *B. subtilis* strain NSRS89-24 + MK007 (Biobest), fungicides, carbendazim, validamycin, propiconazole, and mancozeb were applied alone or in combination with *S. philanthi* RM-1-138. All treatments inhibited the mycelial growth and sclerotial production of *R. solani* to different extents under in vitro conditions. The four fungicides did not have any detrimental effect on the BCA, even at high concentrations (up to 100 µg mL⁻¹). All treatments, namely *S. philanthi* RM-1-138, the commercial formulation, or fungicides alone or in combination, provided protection to different extents (47–60%). Combined application of the BCA and carbendazim offered the best protection to rice plants with the maximum reduction of sheath blight disease incidence up to 74% (Boukaew et al. 2013).

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14

Crop Disease Management

Chemical Application

Various disease management strategies such as use of disease-free seeds and propagules, cultural practices, and enhancement of host plant resistance have been applied to prevent the introduction of pathogens and/or buildup of pathogen populations, resulting in reduction in disease incidence and further spread. These methods are indirect and their effects are not readily perceptible, since they act on the host plants and facilitate their vigorous development. On the other hand, pathogen management methods involving the use of biological control agents (BCAs) and chemicals have direct adverse effects on the development of pathogens. The effects of chemical application for disease management either as preventive or curative measure are highly visible and more effective against some pathogens compared with biocontrol agents. However, there are limitations for the adoption of chemical application for disease management, necessitating the need for more economically feasible or ecofriendly methods. Use of chemicals is primarily determined by the increase in the net profit that the farmers can realize. The chemicals may have antifungal, antibacterial, antiviral, insecticidal, and nematocidal properties. High-value crops such as fruit and vegetable crops are protected by frequent application of chemicals, whereas cereals receive only minimum protection by a few applications of chemicals. The blemish-free market demands and economic value per unit of crops such as apples, citrus, grapes, and potatoes may lead to more frequent application of chemicals, resulting in the emergence of strains of the pathogens with tolerance/resistance to certain chemicals. In contrast, enhancement of host resistance and adoption of appropriate cultural practices are the tactics generally applicable to agronomic crops, keeping the chemical application to a bare minimum because of the low unit value of produce from cereals (Narayanasamy 2002).

The application of chemicals may become unavoidable in some situations. If susceptible, high-yielding cultivars have to be grown in environments conducive for disease development, protection with chemicals has to be offered to save these cultivars. During a period of rapid development of epidemics, large-scale application of chemicals has to be resorted to in order to restrict the buildup of pathogen inoculum and secondary spread of the disease. Cultivation of crops under glasshouse conditions leads to disease spread at a faster rate once the pathogen has infected a few plants, which form potential foci of infection. Intensive application of chemicals may be the primary option for keeping the disease at bay under such conditions. Seed crops and seed stocks have to be protected by an effective schedule of chemical application to keep the infection below

tolerance levels prescribed for the disease(s) concerned. Fungicides with specific activity were found to be essential for maximizing yields of crops including staple food crops such as cereals, wheat, rice, and maize (Anderson 1995). Substantial benefits of maintaining food quality may be realized by application of fungicides for the control of fungi-producing mycotoxins such as aflatoxins, ergot toxins, *Fusarium* toxin, patulins, and tenuazonic acid (Pohland 1993; Schneider and Dickert 1994). Cultivars with vertical resistance may need larger amounts of chemicals than the cultivars with horizontal resistance to a pathogen, as in the case of potato late blight pathosystem. Likewise, protection against more virulent (aggressive) strains/races may demand greater frequency of chemical application to reduce the disease severity. Cultural practices such as fertilization, planting density, and irrigation practices markedly influence the microenvironment in the field, which in turn affects the disease development. When the microenvironment is conducive for disease development, the chemical at a particular dosage may become less effective in reducing the disease intensity to the required level. Use of fungicides may be optimized if their application is based on an effective prediction model system developed for the disease concerned, as in the case of potato late blight disease. It may be possible to save on costs of chemicals and labor, since fewer applications may suffice if fungicides are applied based on prediction rather than calendar-based recommendations (Chapter 9).

Among the chemicals used against microbial plant pathogens, fungicides are more numerous than bactericides or viricides. These chemicals may be grouped based on their mode of action, target site of activity, and nature of active ingredients (ai). Based on their activity, they may be categorized as fumigants, protectants, and eradicants (therapeutants). The chemicals may be grouped as nonsystemic (contact) and systemic based on their ability or inability to move from the site of application and act on the pathogen present in plant tissues far away.

14.1 Application of Fungicides

Fungicides may be applied against fungal pathogens as seed treatment, soil treatment, or foliar spray to protect the seeds, reduce the soilborne inoculums, and protect the plants against airborne pathogens that may infect floral parts directly when they are formed.

14.1.1 Seed/Propagule Treatment

Organomercuric compounds were used for treating seeds against seedborne fungal pathogens and for drenching soils against soilborne pathogens. These organomercurials were banned in most countries because of their high mammalian toxicity. Elemental sulfur used previously for seed treatment was replaced by other chemicals because of low levels of effectiveness and their nonsystemic nature. Control of internally seedborne fungal pathogens was a difficult problem, as none of the nonsystemic protectants could offer satisfactory control of these pathogens. When used for seed treatment, benomyl, chloroneb, and thiabendazole were absorbed by soybean seeds. Benomyl (methyl-1-(butylcarbamoyl) benzimidazole-2-yl carbamate (MBC)) has been applied as seed treatment to protect the emerging seedlings against several fungal pathogens. Benomyl was found to localize in the cotyledons (Gray and Sinclair 1971). The presence

of MBC in the seed coat and cotyledons of soybean seeds could be detected when soybean plants were sprayed with benomyl. MBC could move through the stems and pod walls to reach the seeds of soybean plants treated with benomyl (Ellis and Sinclair 1975). Use of organic solvents as carriers of fungicides offered more effective protection to seeds and eliminated pathogens from infected seeds (Papavizas et al. 1978). Infusion of chemicals into seeds is effective especially for elimination of pathogens from small seeds of crops such as tomato, pepper, cabbage, and mustard (*Brassica* spp.). Carrot seed infection by *Alternaria dauci* was effectively reduced by treating the seeds with thiram (0.2%) and iprodione (0.25%) for 24 h (Strandberg 1984). Seedborne fungal pathogen *Phytophthora infestans* in tomato seeds was suppressed substantially by soaking the seeds in 0.05% O-hydroxy diphenyl for 15 min. In the case of crops with small-sized seeds with irregular surface, pelleting may be more efficient in protecting the seeds. It may be possible to provide larger loads of fungicides around the seeds and to vary the spatial orientation of active materials within the pellet. Gum arabic, starch, carboxymethyl cellulose (CMC), gelatin, methyl cellulose, polyvinyl alcohol, and polyoxyethylene glycol-based waxes may be used as binders for pelleting (Agarwal and Sinclair 1996). Paraffin or vegetable oils may be employed as adhesives and water-dispersible fungicides are mixed in water to form a slurry which is coated on seeds (Taylor and Harman 1990; Kitamura et al. 1991).

A survey of the loose smut frequencies in certified barley seed lots in Norway during 1997–2002 revealed the prevalence and occurrence of loose smut disease in greater frequencies than previously presumed. High loose smut infection frequencies were also recorded in fields sown with carboxin-treated seeds, suggesting a reduced effectiveness of the fungicide in eliminating the pathogen *Ustilago nuda* from the seeds. Greenhouse and field experiments were conducted to assess the efficacy of combinations of fungicides for treating two barley cultivars. Seeds of cultivars Otira and Fager with 14.4% and 6.9% infection, respectively, were treated with Fungazil (carboxin + imazalil) and Premis Robust (triticonazole + imazalil). Fungazil (at normal rates) reduced loose smut frequencies by approximately 50% or less. At twice the normal rate, loose smut symptoms in crops growing from seeds treated with fungicides were reduced by 60–80%. Premis Robust was more effective in eliminating the pathogen from barley seed than Fungazil C (Brodal 2006). Seed treatment of barley seed with 2% carboxin effectively eliminated *Ustilago hordei*, causing loose smut disease (van Schmeling and Kulka 1966). Carboxin at 55 g of active ingredient per 100 kg or 110 g ai/100 kg seeds of wheat and barley was applied for the control of loose smut of wheat and barley caused by *Ustilago tritici* and *U. nuda*, respectively. Three isolates of *U. tritici* and two isolates of *U. nuda* were not affected by seed treatment with carboxin at recommended dose (55 g ai) in Canada. However, the incidence of these two diseases was significantly reduced at doubled dosage of carboxin, indicating the development of tolerance of the pathogen isolates to carboxin (Menzies 2008). The effectiveness of preventive activity of the fungicide thiram was compared with biocontrol agent *Trichoderma harzianum* and *Gliocladium roseum* and two products Signum and Natural II to reduce the seed infection by *Verticillium* spp. in spinach seed production crops. Seed treatment with thiram and Signum reduced the seed infection in harvested seeds when applied in the naturally infested and uninfested soil. The soil type had a significant influence on the subsequent disease pressure, and a nonsignificant effect of seed treatment in suppressing the development of *Verticillium* spp. was observed in both years (2010 and 2011) of experimentation (Olesen et al. 2014).

The cereal fungicide Charisma containing fusilazole and famoxate (famoxadone) reduced the infection of wheat grains by *Fusarium culmorum* by 67.0 to 80.3%, whereas tebuconazole, azoxystrobin, and carbendazim could reduce infection by 34 to 67%, 9.6 to 35.8%, and 37 to 44% respectively. Charisma treatment also improved the percentage of seed germination to a level greater than that which could be achieved by treatment with other fungicides. In addition, application of Charisma at early and mid-anthesis stages of crop development resulted in the lowest level of deoxynivalenol (DON, vomitoxin) in wheat grains (Greenfield and Rosall 2001).

In another investigation, metconazole, azoxystrobin, and tebuconazole were evaluated for the management of *Fusarium* head blight (FHB) pathogens infecting winter wheat. The effectiveness of the fungicides was assessed by competitive polymerase chain reaction (PCR) assay based on primers derived from the biosynthetic gene for trichodiene synthase (*Tri5*). No correlation between FHB disease severity and deoxynivalenol (DON) concentrations in harvested grains could be observed. However, the population of trichothecene-producing *Fusarium* strains was positively correlated with DON in grains. Metconazole and tebuconazole reduced *Fusarium* population in grains significantly, while azoxystrobin was ineffective. It was suggested that the fungicides, by altering the proportion of trichothecene-producing *Fusarium* spp., may affect the FHB severity and DON content of grains (Edwards et al. 2001).

Seedborne *Microdochium majus* and *M. nivale* are the primary sources of inoculum for the incidence of *Fusarium* seedling blight disease in wheat. The efficacy of seed treatment with the fungicides fludioxonil and carboxin + thiram and fludioxonil + difenconazole at 4, 8, 12, and 16°C was assessed for the extent of protection provided by the fungicides applied as seed treatment. The combination of fludioxonil + difenconazole showed improved performance compared with fludioxonil alone. Generally seedling emergence was improved and the severity of stem base disease symptoms on emerged seedlings was reduced for both fungal pathogen species by treating the seeds with the abovementioned fungicides (Glynn et al. 2008).

Losses due to *Fusarium* head blight (FHB) disease caused by *Fusarium graminearum* epidemics in spring wheat were common in the Red River Valley of Minnesota and North Dakota, USA. In order to reduce the disease incidence, the efficacy of fungicides applied on spring wheat cultivars with different agronomic and economic characters and resistance levels was assessed. Propiconazole (63 g ai ha⁻¹), tebuconazole (126 g ai ha⁻¹) or propiconazole followed by tebuconazole were applied at Feeks growth stages. The net returns from FHB moderately susceptible (MS) cultivars in low-disease environs was 8% greater than the moderately resistant (MR) cultivars, but differences were not significant when disease was moderate. Deoxynivalenol (DON) accumulation in grain of MS and MR cultivars was not affected by fungicide treatment. The results indicated that MS cultivars were economically more advantageous to grow than MR cultivars in both disease environments. However, MR cultivars consistently produced grains with higher quality compared with MS cultivars, since kernel quality was largely independent of fungicide treatment and was linked to quality characteristics of cultivars. Cultivar selection, rather than fungicide treatment using tebuconazole, was therefore considered to be the most effective tool to be used for producing high-quality grains in disease-prone environments (Hollingsworth et al. 2008).

Strobilurin fungicides and an azole compound were evaluated for their efficacy in suppressing the development of *Fusarium culmorum*, causal agent of *Fusarium* head blight (FHB) disease of wheat. Visual assessment, enzyme-linked immunosorbent assay

(ELISA) for detection of mycotoxin, 1000-grain weight and yield were the parameters used for evaluation. As these parameters were interrelated significantly, they could be employed to assess the efficacy of the fungicides (Chala et al. 2003). Inoculum of a marked *nit*-mutant of *Fusarium moniliforme* inoculum was utilized to evaluate the quantitative significance of seedborne disease transmission. Greenhouse and field experiments showed that seedborne isolates were responsible for up to 50% of ear rot disease caused by *F. moniliforme*. Seed treatment with the fungicide prochloraz reduced seedborne transmission and protected corn plants against seedling blight disease. In addition, elimination of seedborne inoculum reduced incidence of ear (kernel) rot and avoided buildup of soil inoculum associated with the planting of infected corn seeds (Galperin et al. 2003). The Fungicide Technology Initiative created a mechanism for developing, funding, and implementing multistate “Uniform Fungicide Trials” (UFTs) in the USA (McMullen 1998). Data from the UFTs suggested that demethylation inhibitor (DMI) fungicides prothioconazole and tebuconazole may provide superior control of *Fusarium* head blight (FHB) disease and reduce deoxynivalenol (DON) accumulation compared to tebuconazole (Bradley and McMullen 2008).

A meta-analysis, defined as the quantitative synthesis of the results from multiple individual investigations, is regarded as an objective approach for integrating and interpreting results and drawing conclusions. The quantitative research synthesis was performed using data collected from UFTs conducted at multiple locations across the US wheat-growing regions. A meta-analysis of the effect of tebuconazole (Folicur 3.6 F) on FHB disease and DON content of wheat grain was performed using data collected from UFTs. Response ratios (mean disease and DON levels from tebuconazole-treated plots, divided by mean disease and DON levels from untreated check plots) were calculated for each of 139 investigations for tebuconazole effect on FHB index (IND) and field or plot-level disease severity (i.e., mean proportion of diseased spikelets/spike), and 101 studies for tebuconazole effect on DON contamination of harvested grain. Tebuconazole was more effective at limiting IND than DON contamination. Tebuconazole showed greater impact in spring wheat than in winter wheat ($P < 0.01$). There was considerable variability (unexplained) in percentage of disease reduction by tebuconazole (Paul et al. 2007). In a later study, the combination of prothioconazole and tebuconazole was found to be the most effective treatment in suppressing the development of FHB disease (52% over control), followed by metconazole (50%), prothioconazole (48%), tebuconazole (40%), and propiconazole (32%). Metconazole was the most effective in reducing DON contamination (45%), followed by prothioconazole alone (43%) or when combined with tebuconazole (42%), tebuconazole (23%), and propiconazole (12%). The efficiency of fungicides was greater in spring wheat compared to winter wheat in suppressing both FHB and DON contamination. The results suggested that reduced efficacy of fungicides in winter wheat might be related to longer anthesis periods in wheat compared to spring wheat (Paul et al. 2008).

The stage at or near anthesis, or at full head emergence, was generally considered to be the crucial time for fungicide applications, regardless of wheat cultivars for reducing the incidence of *Fusarium* head blight (FHB) disease and mycotoxin accumulation in grains. But the most crucial time for *Fusarium graminearum* infection and mycotoxin accumulation in barley differed between open- (chasmogamous) and closed- (cleistogamous) flowering types. The chasmogamous cultivars were highly susceptible at anthesis, whereas cleistogamous cultivars were considerably resistant to FHB at anthesis but became susceptible shortly after the extrusion of spent anthers, which provided the

initial base for colonization by *F. graminearum*. The optimal timing for chemical application for the management of FHB in barley may vary depending on the flowering type of the cultivar. Thiophanate-methyl was applied at different developmental stages commencing from prior to anthesis to 30 days after anthesis (daa) under artificial inoculation conditions in the field, in which inoculum of spores was provided throughout the experimental asduration. The optimal timing of chemical control of FHB and mycotoxin accumulation was found to be the time around the beginning of spent anther extrusion rather than at anthesis. Application as late as 30 daa was also able to reduce mycotoxin accumulation in grains, but not FHB incidence (Yoshida et al. 2008).

In a later investigation, the impact of the time of spray application of fungicides on the incidence of FHB disease and mycotoxin accumulation in grains of winter wheat at harvest time was investigated under field conditions. In addition, changes in *Fusarium* population composition were also determined in three topoclimatological zones of Luxembourg. FHB incidence and severity were significantly different between locations, the central zone (Christnach) having highest incidence. The wheat grains were contaminated by deoxynivalenol (DON) in all samples tested with a range of 261–1588 $\mu\text{g kg}^{-1}$ seeds. Treatment with prothioconazole, spiroxamine, chlorothalonil, epoxiconazole, fenpropimorph, cyprodinil, and propiconazole and time of spray application had a significant effect on the level of DON contamination. Unexpectedly, a single application of chemicals showed lower DON concentrations, whereas plots treated with three sprays showed greatest DON levels. The *Fusarium* species varied strongly from location to location in their distribution, each one being predominant in different locations (Giraud et al. 2011).

Isolates of *Fusarium asiaticum* and *F. graminearum*, the primary incitants of *Fusarium* head blight (FHB) disease of wheat, showed high resistance to carbendazim (a benzimidazole fungicide, MBC) which was extensively applied for the management of FHB disease in China. In the attempt to overcome this problem, the baseline sensitivities of *F. asiaticum* and *F. graminearum* isolates to pyroclostrobin (quinine outside inhibitor, QoI) and epoxiconazole (a sterol demethylation inhibitor) were determined. The EC_{50} values of pyroclostrobin in inhibiting mycelial growth of 126 *F. asiaticum* isolates and 64 *F. graminearum* isolates were 0.12–0.135 and 0.010–0.105 $\mu\text{g mL}^{-1}$, and in inhibiting conidial germination were 0.047–0.291 and 0.042–0.255 $\mu\text{g mL}^{-1}$, respectively. The corresponding EC_{50} values for epoxiconazole were 0.12–0.95 and 0.16–0.93 $\mu\text{g mL}^{-1}$ for inhibition of mycelial growth and conidial germination, respectively. The results suggested that there was cross-resistance between MBC and pyroclostrobin or epoxiconazole. In the protective and curative applications, pyroclostrobin and epoxiconazole applied at 200 and 300 $\mu\text{g mL}^{-1}$ provided over 75 protective and curative control efficacy in all treatments. Under field conditions, both pyroclostrobin and epoxiconazole applied at 225 g ai ha^{-1} provided over 80% protection in 2010 and 2011 at two sites where MBC resistant isolates were recovered, indicating that effective protection offered by these fungicides could be a pointer for their use as alternatives to MBC compounds (Chen et al. 2012b).

Application of fungicides is one of the most practical methods of managing *Fusarium* head blight (FHB) disease of wheat. However, the effect of fungicide has not been found to be sufficiently high or consistent. Fungicide efficacy varied between years and among locations, even when standard protocols were followed. Further, the fungicide application was less effective in reducing deoxynivalenol (DON) accumulation in grains compared to FHB incidence. Fungicide thiophanate-methyl applied at anthesis (0 daa)

Table 14.1 Effect of timings of fungicide application at anthesis on infection parameters and mycotoxin accumulation in wheat cultivar Norin 61 in 2006 (Yoshida et al. 2012).

Infection parameters	Treatments ^a				P (ANOVA)
	I-1	II-1	II-2	II-3	
Days after anthesis	0	0 + 10	0 + 20	0 + 30	–
FHBI (%)	79	71	75	77	0.660 (NS)
FHBS (0–100)	9	7	9	8	0.773 (NS)
FDK (%)	6.7	5.8	3.3	5.6	0.168 (NS)
DON + NIV (µg g ^{−1})	2.7a	2.0ab	1.1b	1.8ab	0.028 (S)
Control efficacy for FHB S	36	49	38	41	–
Control efficacy for DON + NIV	−15	17	52	25	–

^a Values for each treatment are the mean of three or four replications per treatment; NS: not significant; S: significant.

had a crucial effect on reducing FHB in 2005 and 2006, although the efficacy varied between years. In terms of reducing mycotoxin (DON and NIV) accumulation however, fungicide application was effective in 2005 (control efficacy 50) but had no effect in 2006. When thiophanate-methyl was applied again at 20 days after anthesis, the control efficacy was improved from 50 to 85 in 2005 and from −15 to 52 in 2006 (Table 14.1). The results showed that the fungicide application at 20 daa reduced mycotoxin concentration in mature grain without reducing FHB severity, whereas application at anthesis (0 daa) was crucial for reducing FHB incidence (Yoshida et al. 2012).

The impact of seedborne *Microdochium nivale* var. *nivale* and var. *majus* inoculum and seed treatment with a carboxin + thiram mixture on the development of wheat seedling blight disease and on subsequent stem colonization by the pathogens and growth of winter wheat cultivar Cadenza was investigated at temperatures favorable (3°C) and unfavorable (22°C) to the pathogen development under greenhouse conditions. Seedlings grown at 3°C showed severe blight symptoms and greater colonization of stems of blighted seedling in untreated control when compared with the seedlings grown from fungicide-treated seed. Symptoms did not develop in seedlings grown at 22°C. The vigor of seedlings from fungicide-treated seeds was better than that of untreated control plants. The vigor of seedlings grown at 22°C was not perceptibly affected by the pathogen. Carboxin + thiram seed treatment decreased the extent and severity of stem colonization on plants from seedlings grown at 22°C (Haigh et al. 2009).

Gaseous chlorine dioxide (ClO₂) applied to rice seeds at a concentration of 20 ppm strongly suppressed mycelial growth of *Fusarium fujikuroi*, *F. verticillioides*, and *F. proliferatum*. The incidence of *Fusarium* spp. in dry seed with 87% seed moisture content (SMC) tended to decrease as the concentration of ClO₂ increased from 20 to 40 ppm. In the soil, stem rot was reduced from 56.9 to 15.4%, the number of normal seedlings increased from 0.4 to 25.5% with water-soaked seeds (33.1% SMC) holding moisture in the endosperm and embryo, and the effectiveness of disinfection using ClO₂ increased, even with only 20 ppm for 4 h, suggesting the requirement of moisture for the activity of ClO₂. Removal of palea and lemma from seeds significantly decreased the incidence of *Fusarium* spp. to 3.0%. The results showed that gaseous ClO₂ could be

used to disinfect rice seeds infected with *Fusarium* spp. Effectively, and the presence of optimum moisture level around the pathogens in the seed was an important factor for the disinfestation of seeds by ClO_2 (Jeon et al. 2014).

Winter wheat is seriously affected by dwarf bunt disease caused by *Tilletia controversa* in Canada. The efficacy of seed treatments with fungicides was assessed under inoculated field conditions at two locations in Central Ontario for 3 years, using eight wheat cultivars representing various market requirements. Three rates of two combinations of fungicides, namely difenconazole + metalaxyl-M (Dividend XLTRA) and carbathiin + thiram (Vitaflo 280) and a biological control agent (BCA) *Clonostachys rosea* strain ACM 941 were applied as seed treatments. Dwarf bunt disease incidence was significantly reduced ($P < 0.05$) by all seed treatments compared with untreated controls. Different rates of Dividend XLTRA were highly effective, providing 98–99% control of the disease. Vitaflo 289 and the BCA were less effective, reducing the disease incidence by 50% and 35%, respectively. The chemical seed treatments significantly increased emergence of seedling by 5–8% and yield by 2–4% compared to the control. As there was no significant interaction between wheat cultivars and Dividend XLTRA treatment for all measured parameters, this fungicide was concluded to be an effective tool for the management of dwarf bunt disease in winter wheat in Canada (Xue et al. 2007).

Seedborne infection by barley net blotch pathogen *Stagonospora nodorum* reduced the grain yield substantially. Seed treatment with carboxin + thiram (Anchor) reduced net blotch disease incidence by nearly 100% compared to 81% by imazalil (Fungazil A25), 60% by ipconazole + imazalil (Rancona i-MIX), and 54% by triconazole + prochloraz + pyrimethanil (Rubin TT). In addition, Anchor increased the yield significantly (3–7%), 1000-kernel weight (3–13%), grain volume weight (3–9%), and protein content (1–7%) in the first year of the trial, but not in the second year. The correlation between percent infected kernels in the conventional osmotic method and results of real-time PCR assay was high, showing a potential for complementarity between traditional plate test methods and molecular methods. The fungicide treatment could be used as a preventive measure for reducing the incidence of net blotch disease on the seedlings developing from infected barley seeds (Hysing and Wiik 2013).

The efficacy of fungicides in inhibiting the spore germination and mycelial growth of *Ascochyta rabiei*, causal agent of chickpea blight, was determined in vitro. Of the 13 fungicides tested, treatment of seeds with benomyl + thiram, carbendazim, and carbendazim + chlorothalonil reduced the infection by *A. rabiei* by more than 85% on vacuum infiltration of naturally infected seeds. Coating the seeds with polymers did not increase the effectiveness of protection offered by the fungicides. Azoxystrobin, chlorothalonil, and mancozeb were the most effective under field conditions. With increased disease pressure, the efficacy of the fungicides declined (Demirci et al. 2003).

The effectiveness of cyazofamid, applied as seed treatment, was assessed for the suppression of pearl millet downy mildew caused by *Sclerospora graminicola*. Sporangial production, zoospore release and motility were inhibited by cyazofamid at 0.3 mg mL^{-1} concentration. No phytotoxic symptoms due to treatment with the fungicide concentrations up to 2.0 mg mL^{-1} as seed treatment or $1\text{--}10 \text{ mg mL}^{-1}$ as foliar spray were visually observable. Seed treatment followed by a single foliar application provided effective control of downy mildew disease. Cyazofamid did not have any systemic activity and exhibited strong curative activity, but only moderate translaminar activity with marginal reduction in disease incidence (34.8%). Loss of fungicidal activity over time was very low, indicating stable residual and rainfastness activity. The results indicated

the potential of cyazofamid for use as an effective treatment against pearl millet downy mildew disease (Jogaiah et al. 2007).

Fungicides were applied as seed treatment and foliar spray to reduce the incidence of anthracnose disease caused by *Colletotrichum lindemuthianum* in dry bean (*Phaseolus vulgaris*). Pyraclostrobin and azoxystrobin fungicides were applied under low and high disease pressure conditions at two locations and at four stages of plant growth. For seed treatments, a combination of diazinon + captan + thiophanate-methyl provided superior control of the anthracnose disease on leaf and pod tissue under low and high disease pressure conditions. The combined effect of seed treatments and foliar spray timings improved plant health, extended crop maturity, and increased yield over seed treatment alone. Sequential fungicide treatments were more effective than single applications of fungicides in most environments. Under moderate to severe disease pressure, two fungicide applications and a seed treatment might be needed to maximize economic returns to growers (Gillard et al. 2012). In a further study, seed treatment with metalaxyl-M + fludioxonil + azoxystrobin was found to be equally effective as treatment with diazinon + captan + thiophanate-methyl. The addition of a surfactant to azoxystrobin increased seed yield and net return under high disease pressure. Spray application of pyraclostrobin outperformed azoxystrobin in reducing disease incidence and increased the yield levels under high disease pressure and net return under low and high disease pressure conditions (Gillard and Ranatunga 2013).

The most effective suppression of bacterial fruit blight (BFB) disease of watermelon caused by *Acidovorax avenae* subsp. *citrulli* (*Aac*) is by exclusion of the pathogen from seeds and other sources. Treatment of cucurbit seeds with hydrochloric acid (HCl) was effective in eliminating the pathogen from seed (Hopkins et al. 1996). Peroxyacetic acid (PA) and HCl were evaluated for their comparative efficacy in eliminating *Aac* from watermelon seeds. Peroxyacetic acid at low concentrations eliminated *Aac*, *Didymella bryoniae*, causal agent of gummy stem blight (GSB) disease, and *Fusarium oxysporum* from microbial suspensions. Treatment of seed contaminated with *Aac* and *D. bryoniae* with PA at $1600\mu\text{g mL}^{-1}$ and higher concentrations for 30 min were effective in preventing seed transmission of BFB and GSB pathogens. Exposure of seeds to PA at 3200 and $6400\mu\text{g mL}^{-1}$ for 15 min did not reduce seed germination and the vigor of triploid watermelon seed, even when seeds were not rinsed after treatment. PA at $1600\mu\text{g mL}^{-1}$ was as effective against BFB as the HCl at $10,000\mu\text{g mL}^{-1}$ (Table 14.2). PA was as effective or more effective than HCl in preventing seed transmission of BFB to cantaloupe seedlings as in watermelon. An effective wet seed treatment procedure included a 30 min treatment with PA at $1600\mu\text{g mL}^{-1}$ followed by seed drying at low humidity in a 40°C drying oven for 48 h. Approximately 10 to 100-fold higher concentrations of HCl were required to achieve the efficacy levels in equivalence to PA for the elimination of BFB pathogen from watermelon seeds (Hopkins et al. 2003).

Fusarium graminearum, a cereal pathogen, causes pod blight, seed and root rot, and pre-emergence damping-off of soybean. Seed infection by *F. graminearum* and *F. sporotrichoides* and accumulation of mycotoxins deoxynivalenol (DON) and HT-2 mycotoxins produced by them were detected (Clear et al. 1989). The efficacy of captan, fludioxonil, mefenoxam + fludioxonil, azoxystrobin, trifloxystrobin, and pyraclostrobin applied as seed treatment was assessed using the rolled-towel assay and 2.5×10^4 and 2.5×10^5 macroconidia mL^{-1} of *F. graminearum* for inoculation. All fungicides were able to reduce disease incidence significantly compared to the untreated controls ($P < 0.05$). The effectiveness of treatment was observed in varying levels depending on the fungicide.

Table 14.2 Effect of treatment with PA in combination with different seed drying temperatures on seed transmission of BFB in watermelon (Hopkins et al. 2003).

Treatment ^b	Temperature (°C) ^c	BFB (%) ^a	
		Test I	Test II
Untreated	GH	47b	68b
320 (µg mL ⁻¹)	GH	2c	1d
1600 (µg mL ⁻¹)	GH	0c	1d
Untreated	40	85a	86a
320 (µg mL ⁻¹)	40	0.5c	4c
1600 (µg mL ⁻¹)	40	0c	0d
Untreated	30	48b	66d
320 (µg mL ⁻¹)	30	2c	3c
1600 (µg mL ⁻¹)	30	0.3c	0d
Untreated	25	80a	90a
320 (µg mL ⁻¹)	25	2c	4c
1600 (µg mL ⁻¹)	25	0.8c	0.3d

^a Means of values of four replications followed by the same letters are not significantly different as per DMRT test ($P = 0.05$); ^b BFB-contaminated seeds treated with PA solutions for 30 min; ^c Seed drying temperatures

Seed treated with captan or fludioxonil produced plants with significantly reduced disease severity across all isolates of pathogens and concentrations, while azoxystrobin-treated seeds showed highest disease severity in seedlings. There was no significant difference ($P < 0.05$) between high and low fungicide rates for fludioxonil or captan for disease severity. No significant effect of strobilurin seed treatment on disease severity was observed. Trifloxystrobin and pyraclostrobin treatments had significantly lower disease severity than the untreated seed (Ellis et al. 2011). The efficiency of seed treatments with fungicides for reducing damping-off disease of pea caused by *Rhizoctonia solani* was assessed. Seeds of pea cultivar Mikado were treated with carbendazim, carbendazim + thiram, captan, iprodione, iprodione + thiram, metalaxyl-M + fludioxonil, pencycuron, procymidone, and tolyfluamid. Treated seeds were sown in soil inoculated or uninoculated with *R. solani*. Seedling emergence was reduced in inoculated soil and most effective treatments for the control of *R. solani* were carbendazim, pencycuron, iprodione, and carbendazim + thiram (da Silva et al. 2013).

Planting materials (propagules) have to be protected by applying chemicals prior to planting, although they might have been obtained from disease-free mother plants. Potato tubers may carry fungal, bacterial, and viral pathogens. Preplanting treatments of potato seed tubers with sodium hypochlorite solution (as NaOCl, 500 ppm for 8 min), thiophanate-methyl (TPM, as EsoutTM, 50 g ai/100 kg seed tubers) or a combination treatment with NaOCl followed by TPM were evaluated for their efficacy to reduce the incidence of black scurf disease caused by *Rhizoctonia solani* and common scab caused by *Streptomyces scabies* on potato tubers cultivar Russet Burbank. The treatment with

combination of NaOCl and TPM reduced *R. solani* on progeny tubers at harvest and after storage. However, the incidence of scab on tubers even in untreated checks was low, making the assessment of effectiveness of the chemicals against *S. scabies* unreliable. The combination treatment might have killed the majority of *R. solani* sclerotia on the tuber surface, and the combination treatment with TPM following NaOCl disinfection further suppressed the mycelial growth of *R. solani*. No phytotoxicity symptoms due to combination treatment were observed (Errampalli and Johnston 2001).

Tuber treatment with fungicides has been shown to be effective for reducing the tuber blight of potato caused by *Phytophthora infestans*. Seed tuber treatment with thiophanate-methyl + mancozeb increased plant emergence and uniform stand of potato crop. The foliar fungicides dimethomorph + mancozeb, cymoxanil + mancozeb, or propamocarb + chlorothalonil were also found to be useful for tuber treatment. However, additional foliar spray with fungicides might be necessary to achieve effective suppression of late blight disease development on potato plants at later stages. Sprout treatment with oxadixyl or Ridomil was more effective than treatment of whole tubers with deep incisions or cut tubers (Singh and Pundhir 2004).

The efficacy of fungicides applied alone or in combination as preplanting seed tuber treatment to reduce the incidence of silver scurf caused by *Helminthosporium solani* on daughter tubers was assessed in three field experiments. The most effective fungicides fludioxonil and prochloraz-Zn, applied as low-volume spray, showed control efficiencies of 88% and 82%, respectively. Propineb and mancozeb applied as a dust treatment had control efficiencies of 78% and 79%, respectively. Treatment with azoxystrobin and imazalil were less effective with control efficiencies of 68% and 43%, respectively. None of the fungicides had any effect on crop yields at all three locations (Tsrer and Pertz-Alon 2004). The effectiveness of chemicals thiabendazole (TBZ), fludioxonil, mancozeb, thiophanate-methyl (TPM), azoxystrobin, sorbic acid, and sodium carbonate was assessed to reduce the incidence of silver scurf disease in progeny tubers at four locations in Washington and Oregon in 1998–1999. Following treatment of seed tubers with the chemicals, significant reduction in disease incidence on progeny tubers was observed to a different extent. Seed tubers treated with fludioxonil, fludioxonil + quintozene, azoxystrobin, or thiophanate-methyl + mancozeb showed significantly less silver scurf disease incidence regardless of locations, compared to untreated control treatments at harvest in the spring in both years. Treatments with TPM, sorbic acid, mancozeb, and sodium carbonate were not effective in reducing the disease incidence, which was not significantly different from untreated control at any location or sampling time ($P < 0.05$). Isolates resistant to thiabendazole and thiophanate-methyl were identified for the first time (Geary et al. 2007).

14.1.2 Soil Treatment

Soilborne pathogens may infect the plants at different stages of plant growth from seedling stage and become systemic, reaching floral parts at the time of heading. Nonsystemic and systemic chemicals may be applied on the seeds or in the soil to interact with the pathogens present in the soil. The seeds function as vehicles to carry the fungicides into the soil where they interact with pathogens in the rhizosphere. Systemic fungicides may act from the seed surface against the soilborne pathogens. In addition, these fungicides may also penetrate the tissues of germinating seeds and emerging seedlings and interact with the pathogen structures, if infection has already been established (Maude 1996).

Dosages of chemicals applied as seed treatments and soil application may indicate that relatively small quantities applied to seed may not be able to exert required pressure on the soil microflora/pathogens. However, seed treatments using high rates of chemicals followed by stem base sprays may significantly reduce the disease incidence as in the case of onion white rot disease caused by *Sclerotium cepivorum* (Entwistle 1986). Metalaxyl applied in furrow provided longer protection to soybean plants against root and stem rot disease caused by *Phytophthora sojae* than when metalaxyl was applied as seed treatment. Soybean cultivars with partial resistance, when planted in the fields with environmental conditions conducive for *Phytophthora* root rot development, could benefit from fungicide seed treatment (Dorrance and McClure 2001).

Soil drenches with fungicides have been suggested to protect the seedlings in the main field known to be infected with soilborne pathogens. The practice of using plastic mulch and furrow irrigation favored the incidence of crown and root rot of capsicum caused by *Phytophthora capsici*. Metalaxyl was drenched to restrict the outbreak of the disease in glasshouse crops in southern Italy. The level of sensitivity to metalaxyl was determined using single-hypha isolates of *P. capsici*. More than 80% of the isolates of *P. capsici* from commercial plastic-house crops showed moderate levels of resistance, indicating the loss of effectiveness of metalaxyl application as soil drenches (Agosteo et al. 2000). The optimal timing of application of simeconazole for reducing the incidence of rice kernel smut was determined under field conditions in Miyagi Prefecture, Japan. The fungicide was applied in the form of granules (1.5%) for soil treatment. The submerged application of simeconazole granules (450–600 g ai ha⁻¹) at 1–5 weeks before heading was highly effective against rice kernel smut, with treatments at 1–2 weeks before heading being the most effective. Submerged application of the fungicide at 2–5 weeks before heading was also highly effective in reducing the false smut disease, with treatment at 3 weeks before heading being the most effective. These periods overlap the timing for optimal application of simeconazole to control rice sheath blight and ear blight. It was concluded that treatment with simeconazole at 2–3 weeks before heading could be followed for the control of these diseases of rice (Tsuda et al. 2006).

14.1.3 Treatment of Aerial Plant Parts

Various fungicides and other chemicals with antimicrobial activity have been evaluated for their capacity to reduce disease incidence severity and/or production of mycotoxins/toxic metabolites by the microbial pathogens. The effects of fungicides on the development of Fusarium head blight (FHB) complex due to *Fusarium culmorum*, *F. avenaceum*, and *Microdochium nivale* were investigated under natural and artificially inoculated conditions in the field. The pathogen populations were estimated using quantitative PCR assay and DON contents were also estimated. Tebuconazole selectively controlled *F. avenaceum* and *F. culmorum* and also reduced DON levels, but it was ineffective against *M. nivale*. In contrast, azoxystrobin selectively arrested the development of disease due to *M. nivale* and allowed greater colonization by toxigenic *Fusarium* species, resulting in higher accumulation of DON in grains. No-break azoxystrobin application 2 days postinoculation increased the production of DON per unit of pathogen in artificially inoculated field trial. The results indicated the potential risk of enhanced DON contamination of wheat grains following treatment with azoxystrobin aimed at control of head blight in susceptible wheat cultivars (Simpson et al. 2001).

The systemic fungicide tebuconazole (Folicur®) was applied a few days before and/or after inoculation of wheat earheads with *Fusarium graminearum* under field conditions. Plants in check plots were not inoculated and protected with the test fungicide. Inoculation with *F. graminearum* reduced the yield significantly by 24.2–45.0%. Preinfectional spray of tebuconazole reduced deoxynivalenol (DON) synthesis in the field to an appreciable level and also decreased FHB, glume blotch, and percentage of *Fusarium*-damaged (infected) kernel (FDK). The levels of *Fusarium* kernel infection after harvest clearly reflected the DON content of wheat grain (Homdork et al. 2000).

Structural and cytological changes induced by tebuconazole in the FHB pathogen *Fusarium culmorum* were studied. Mycelial growth of *F. culmorum* was strongly inhibited by the fungicide placed in filter paper strips soaked in $20\mu\text{g mL}^{-1}$ around the point of inoculum in petridishes. Scanning electron microscopic (SEM) observations showed that the fungicide induced irregular swelling and excessive branching of hyphae, thickening of cell walls, excessive septation, formation of incomplete septa, extensive vacuolization, accumulation of lipid bodies, and progressive necrosis or degeneration of hyphal cytoplasm. Nonmembrane inclusion bodies were often detected in hyphal cytoplasm. The formation of new hyphae (daughter hyphae) inside collapsed hyphal cells was commonly seen after fungicide application. Cytochemical procedures showed that the labeling intensities of chitin and β -1,3-glucan in the cell walls of tebuconazole-treated hyphae were more pronounced than in those of untreated control hyphae. In addition, immunogold labeling with antiserum against deoxynivalenol (DON) revealed that DON was localized in the cell walls, cytoplasm, mitochondria, and vacuoles of the hyphae from the control and the fungicide treatment, but labeling density in the fungicide-treated hyphae decreased dramatically compared with control hyphae, indicating that tebuconazole reduced *Fusarium* toxin production by the pathogen (Kang et al. 2001).

In a later investigation, the effect of fungicides on the incidence of *Fusarium* head blight (FHB) disease in durum wheat, infected kernels, and DON concentration in grain was assessed under field conditions. Single application of prochloraz, tebuconazole, epoxiconazole, or bromuconazole at recommended doses was made to wheat cultivars at the beginning of anthesis stage. The fungicides effectively reduced the disease incidence when the disease pressure was low to medium and when the primary pathogens of FHB were *F. graminearum* and *F. culmorum*. Kresoxim-methyl showed low level of effectiveness in suppressing FHB disease development. Tebuconazole, prochloraz, and bromuconazole were effective in controlling *F. graminearum* and *F. culmorum*. DON concentration in grains of plants inoculated with *F. graminearum* and *F. culmorum* was high, averaging 4.2 mg kg^{-1} in untreated control. Application of tebuconazole reduced DON concentration by 43%, while epoxiconazole was ineffective. DON concentration in naturally infected grains was 1.95 mg kg^{-1} grains, which exceeded the tolerance limit of 1 mg kg^{-1} grains (maximum level) allowed in the USA. Prochloraz, bromuconazole, and tebuconazole application under natural infection conditions reduced the DON concentration from 73 to 96%, while epoxiconazole provided the lowest level of protection. A positive correlation was observed between *Fusarium*-infected grains and level of DON contamination of grains (Menniti et al. 2003).

The efficacy of fungicide application at anthesis was assessed for reducing the *Fusarium* head blight (FHB) disease and production of trichothecene mycotoxins by *Fusarium graminearum*, *F. avenaceum*, *F. poae*, *F. culmorum*, *F. tricinctum*, and *Microdochium nivale* under field conditions in soft wheat, durum wheat, and barley. Deoxynivalenol (DON) and nivalenol (NIV) were detected most frequently.

Azoxystrobin significantly decreased *M. nivale* infection levels, whereas treatment with this fungicide was ineffective against *F. graminearum* and *F. culmorum*. On the other hand, metconazole or tebuconazole significantly decreased *F. graminearum* or *F. culmorum* infection levels, but the effect was variable across the years and the type of fungicide applied. DON concentration in wheat treated with tebuconazole and metconazole was reduced by 46% and 48% respectively, while NIV concentration was not reduced in 2002. However, a significant reduction in *F. graminearum* or *F. culmorum* infection levels was not always associated with significant decrease in DON and NIV mycotoxins in 2000 and 2001. Overall, the fungicide application did not reduce the mycotoxins produced in wheat grains (Ioos et al. 2005).

As wheat is highly susceptible to FHB disease around anthesis stage, the effects of timing of fungicide application on the FHB disease incidence and mycotoxin (DON and NIV) accumulation in wheat grain were determined. The late period of grain development (beyond 20 daa) was considered to be important to determine the final toxin contamination level in wheat. Thiophanate-methyl (TPM) was tested under artificial inoculation conditions in which moisture and inoculum of spores were provided throughout the testing period. Eight treatments representing different application timing (0, 10, 20, and 30 daa) and the number of applications (0–2) were tested for 2 years. The results showed that fungicide application timing could affect FHB development and mycotoxin accumulation. Fungicide application at 20 daa reduced mycotoxin concentration in matured grain without reducing FHB severity. On the other hand, application at anthesis was the critical factor for reducing FHB severity (Yoshida et al. 2012).

The comparative efficacy of a strobilurin and an azole compound was determined for the control of *Fusarium culmorum*, causing Fusarium head blight (FHB), by applying strobilurin twice and azole compound once. Visual assessment, enzyme-linked immunosorbent assay (ELISA), a mycotoxin test, 1000-grain weight, and yield assessment indicated the differing levels of the efficacies of the fungicides in arresting disease development and mycotoxin accumulation in grains. Correlation coefficients calculated for the different parameters were significantly higher, indicating their usefulness for determining the effectiveness of the selected fungicides against *F. culmorum* infecting wheat (Chala et al. 2003). In a 2 year investigation, four fungicides were compared for their effect on FHB disease incidence using two wheat cultivars with different levels of resistance to FHB inoculated with 3-ADON and 15-ADON chemotypes of *Fusarium graminearum*. All fungicides tested, namely prothioconazole, tebuconazole, metconazole, and prothioconazole+tebuconazole, reduced FHB index, percent Fusarium-damaged kernels (FDK%) and deoxynivalenol (DON) levels, and increased yield compared with the nonsprayed control in the moderately resistant cultivar Glenn. In the highly susceptible cultivar Robin also, all fungicides reduced FHB index and increased grain yield relative to the control but inconsistent results for FDK and DON contamination were evident. Differences were seen between the two chemotypes of *F. graminearum* for all parameters in both years except for FDK and yield in 2010. The results indicated the need for protection to reduce FHB incidence and DON contamination for obtaining higher level of quality grains (Amarasinghe et al. 2013).

The effectiveness of postanthesis application of prothioconazole + tebuconazole and metconazole was assessed by conducting seven field experiments in Ohio and Illinois during 2011–2013 to reduce Fusarium head blight (FHB) disease and DON contamination in soft red winter wheat. Fungicides were applied at early anthesis (A), 2 (A + 2), 4 (A + 4), 5 (A + 5), or 6 (A + 6) days after anthesis. Additional inoculation of

Fusarium graminearum was provided for six experiments, and the natural incidence of FHB was taken into account in the other experiment. *Fusarium*-damage kernels (FDK) and DON concentration in grain were quantified. Relative to the control, A + 2 resulted in the highest reduction in disease (FHB index, IND) and DON contamination by 69% and 54%, respectively, followed by A + 4 (62% and 52%), A + 6 (62% and 48%), and A (56% and 50%). Treatments A + 2 and A + 6 significantly reduced IND by 30% and 14%, respectively, relative to the anthesis application. DON accumulation was not significantly reduced by postanthesis application of fungicides. The results suggested that application of fungicides made up to 6 daa might provide better protection against FHB and DON contamination (D'Angelo et al. 2014).

The influence of wet rainy conditions during anthesis on the effectiveness of fungicides for reducing *Fusarium* head blight (FHB) disease and deoxynivalenol (DON) contamination of wheat grains was investigated. Tebuconazole + prothioconazole were applied to wheat spikes at anthesis in combination with the nonionic surfactant Induce. Simulated rainfall of a fixed intensity and duration was applied to separate plots at five different times after the fungicide treatment (0, 60, 105, 150, or 195 min). Spike samples were collected at 4 day intervals after fungicide application and assayed for tebuconazole residue. A similar set of greenhouse experiments was conducted using six post-fungicide application rainfall timing treatments (0, 15, 30, 120, or 180 min). All plants in these trials were inoculated at anthesis with conidia of *Fusarium graminearum* and FHB index (IND) and DON were quantified. In four of five trials, all fungicide-treated experimental units (EUs) had significantly lower mean IND and DON than the untreated check, regardless of rainfall treatment. Among all rainfall treatments, EUs that received the earliest rains after fungicide applications tended to have the highest numerical mean IND and DON, but were generally not significantly different from EUs that received later rains or fungicide without rain. In both years, fungicide residue on wheat spike decreased rapidly with time after application of fungicide, but the rate of reduction varied somewhat between years with a half life of 6–9 days. Rainfall did not have a significant effect on the rate of residue reduction or the level of residue at a fixed sampling time after fungicide application. The results showed that tebuconazole + prothioconazole mixed with the nonionic surfactant was fairly rainfast for a fixed set of rainfall characteristics, and tebuconazole residue did not persist for a long time after application to wheat spikes (Anderson et al. 2014). In China, application of tebuconazole at 125 g ai ha⁻¹ decreased FHB severity by 81.80% and 70.54% in 2012 and 2013 and reduced DON contamination by 78.85% and 38.43%, respectively. The yield losses in fungicide-applied plots were much lower in both years. The results indicated that the fungicide tebuconazole could be used for effective reduction of FHB disease and DON contamination levels in wheat grains in China (Sun et al. 2014).

The effects of azoxystrobin (strobilurin fungicide) on infection of wheat by *Mycosphaerella graminicola* (anamorph *Septoria tritici*), causing leaf blotch disease, were assessed using green fluorescent protein (GFP-) expressing transformants. Azoxystrobin at 125 or 250 g ai ha⁻¹ was applied at various stages of infection process under controlled conditions. GFP transformants showed conserved in vitro sensitivity to azoxystrobin and pathogenicity. Azoxystrobin reduced *M. graminicola* infections by over 90% when applied before or during penetration of the pathogen (15% of incubation phase). Azoxystrobin impaired the growth of intercellular hyphae of *M. graminicola* at post-penetration infection stages when applied at up to 50% of incubation phase. Incubating treated leaves showed infections were viable, but the pathogen growth was

impaired. No necrosis was induced in treated leaves under controlled conditions. The efficiency of azoxystrobin at half dosage was not altered in suppressing the development of wheat leaf blotch pathogen (Rohel et al. 2001).

The effects of curative and preventive applications of azoxystrobin in wheat fields against *M. graminicola* were assessed using quantitative PCR assay and visual monitoring. A nonsystemic protectant chlorothalonil and a systemic curative fungicide epoxiconazole were used as references. Asymptomatic infection by *M. graminicola* could be detected by PCR assay at 3 weeks before symptom expression, facilitating the differentiation of the effects of preventive/curative action of fungicides. When applied 1 week after the beginning of infection, curative activity of azoxystrobin was intermediate between chlorothalonil (low effect) and epoxiconazole. When applied preventively, none of the fungicides completely prevented leaf infection. It appeared that azoxystrobin, as preventive treatment, might delay fungal DNA increase more effectively than epoxiconazole at the beginning of leaf infection. Both curative and preventive treatments increased the time lapse between the earliest PCR detection and the measurement of a 10% necrotic leaf area. Azoxystrobin only slightly decreased the speed of necrotic area increase compared with epoxiconazole. The results showed that azoxystrobin activity towards *M. graminicola* was mainly in lengthening the time lapse between the earliest detection by PCR and the measurement of a 10% necrotic leaf area. Based on the results obtained, it would be possible to have optimal positioning of azoxystrobin treatments against wheat leaf blotch disease (Rohel et al. 2002).

Field isolates of *Mycosphaerella graminicola* were classified into four main phenotypes: wild-type strains (TriS); DMI-resistant phenotypes with low (TriLR); moderate (TriMR); and high (TriHR) levels of resistance to representative triazole fungicides (Leroux and Walker 2010). In the absence of highly resistant wheat cultivar to Septoria leaf blotch disease caused by *M. graminicola*, DMIs were the key fungicides applied for the management of this disease. The quantitative (q) PCR assay was employed to evaluate the preventive and curative efficiency of DMI fungicides to control the more frequently occurring genotypes of *M. graminicola*. Prothioconazole treatments significantly reduced the amount of *M. graminicola* DNA by 80% in preventive treatment and were still 70% efficient when applied as curative treatment at 3 days postinoculation (dpi). Electron microscopic observations revealed that after preventive application of prothioconazole, the spores failed to germinate. Applications of prothioconazole at 7–10 dpi resulted in a high loss of efficiency (>50%) of disease control. Preventive treatment was very effective on the susceptible cultivar Alixan with a protection level of 83.6%; this efficiency declined sharply when inoculated at 7–15 days after treatment (dat) with protection level of 42.4% and 27.2%, respectively. No protective effect of prothioconazole was observed after 15 dat. The protocol of qPCR could be used for effectively determining the optimum time of application of DMI fungicides for the management of Septoria leaf blotch of wheat (Selim et al. 2014).

Rice sheath blight caused by *Rhizoctonia solani* is widespread, and it is difficult to obtain an economically sustainable yield. Fluxapyroxad (3-(1-(difluoromethyl)-1-methyl-N-(3'4'5'-trifluorobiphenyl-2-yl)-pyrazole-4-carboxamide) was reported to interfere with succinate ubiquinone reductases in the electron transport chain of fungi. Isolates (128) of *R. solani* collected during 2008–2010 were characterized for baseline sensitivity to fluxapyroxad. The EC_{50} values of boscalid for inhibition of mycelial growth of *R. solani* isolates ranged over 1.89–2.68 $\mu\text{g mL}^{-1}$ and the average EC_{50} value was $2.212 \pm 0.228 \mu\text{g mL}^{-1}$, indicating that isolates of *R. solani* were less sensitive to boscalid

than fluxapyroxad. The fungicide fluxapyroxad exhibited excellent protective and curative activity against *R. solani* by providing 82.6–94.2% protective or curative control efficacy. In the field trials, fluxapyroxad (at 100 g ai ha⁻¹) was applied twice at 15 days and 30 days. After second application of the fungicide, the control efficacy was 83.4–88%. The control efficacy of boscalid at a dosage of 600 g ai ha⁻¹ was at a lower level (51.7–57.0%). The control efficacy of fluxapyroxad was at higher level compared to boscalid or jinggangmycin and has the potential for providing effective protection against rice sheath blight disease (Chen et al. 2014).

Rice false smut caused by *Ustilaginoidea virens* infects several high-yielding and good-quality varieties in most rice-growing ecological regions in China. Chemical control of the disease using fungicides is an important disease management strategy. The ergosterol biosynthesis inhibitor (EBI) fungicides such as prochloraz, difenconazole, propiconazole, and tebuconazole have been extensively used in China for the management of rice blast and sheath blight diseases. *U. virens* isolates (102) were tested for their sensitivity to the four EBI fungicides during mycelial growth stage. The EC₅₀ ranges of values for prochloraz, difenconazole, propiconazole, and tebuconazole inhibiting mycelial growth of 102 isolates of *U. virens* were 0.04–0.75, 0.04–1.08, 0.04–0.38, and 0.03–0.57 µg mL⁻¹ L⁻¹, with the average EC₅₀ values of 0.32 ± 0.08, 0.45 ± 0.08, 0.19 ± 0.03, and 0.21 ± 0.06 µg mL⁻¹ L⁻¹, respectively. The EC₅₀ values suggested that all pathogen isolates were sensitive to all EBI fungicides tested. Results of field trials showed that two sprays of these fungicides were more effective than a single spray for suppressing false smut disease development. Two sprays of 50% propiconazole EC at 300 g ai ha⁻¹ provided best protection against false smut at both sites during the 2 years (2010–2011), with control efficacy ranging over 71.5–74.3%. The sensitivity of the fungal pathogens to fungicide has to be monitored continuously (Chen et al. 2013).

Ascochyta blight disease of chickpea caused by *Ascochyta rabiei* was a major limiting constraint for chickpea production in Canada. Rates and timings of application of chlorothalonil and azoxystrobin separately and in combination were determined under low and high disease pressure conditions. Fungicide application under dry weather, when disease pressure was low, had no effect on blight disease severity in 1998. Under high disease pressure due to wet conditions in 1999 and 2000, a single fungicide application reduced disease severity but had no effect on yield. Two applications (early + midflowering stages) of chlorothalonil (Bravo R 500) at 1 kg ai ha⁻¹, two applications of azoxystrobin at 125 g ai ha⁻¹ or chlorothalonil + azoxystrobin reduced Ascochyta blight and increased yield. Another formulation of chlorothalonil (Bravo Ultrex®) was less effective, but more effective than mancozeb. Fungicide application had a substantial impact on seed yield. A strong relationship between disease severity and seed yield was indicated by regression analysis. Applying azoxystrobin at early + midflowering stages reduced seed infection to 7–9% from 30–48% in control. Fungicide application did not have any effect on other seedborne fungi such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Fusarium* spp. (Chongo et al. 2003).

The effect of fungicide application frequency, timing, and rotation of different fungicides on the suppression of Ascochyta blight and on yield and seed quality of three chickpea cultivars was assessed during 2002–2004. Five fungicides – chlorothalonil, azoxystrobin, pyraclostrobin, mancozeb, and boscalid – were compared for their efficiency. The disease severity was significantly reduced by fungicide applications to levels below 25% compared to disease severity in untreated controls (21–99%). Increasing the number of fungicide applications was beneficial under high disease pressure conditions, but was not always related to effectiveness of disease suppression.

Application of fungicide at the pre-flowering stage reduced the *Ascochyta* blight severity significantly, increasing the seed yield or 1000-seed weight. No consistent positive impact on seed yield or quality was discernible due to an increase in the number of fungicide applications. Different fungicides showed less improvement in the effectiveness of disease management than the timing and number of fungicide treatments. Inclusion of strobilurin in rotation was beneficial under high disease pressure conditions (Banniza et al. 2011).

The antifungal activity against *Colletotrichum capsici*, incitant of anthracnose and fruit rot of pepper (*Capsicum* spp.), was recorded in in vitro assessments. Greenhouse and field assessments of the efficacy of fungicides showed that foliar spray of propiconazole at 0.1% significantly reduced anthracnose/fruit rot disease of pepper by 70%, followed by difenconazole (0.05%) and carbendazim (0.1%), with a reduction in disease incidence of 58% and 44%, respectively. The fungicides propiconazole, difenconazole, and carbendazim increased fruit yields by 86%, 63%, and 60% respectively (Gopinath et al. 2006). Soybean production is highly hampered because of yield-limiting diseases such as charcoal rot and *Phomopsis* seed decay. Lactofen or azoxystrobin was applied on soybean cultivars and genotypes with different resistance levels to these diseases. Application of lactofen at growth stage R1 and azoxystrobin either at planting, R3 or R6, had no significant effect on severity of charcoal rot, percentage of harvested seed infected by *Phomopsis* spp., or severity of pod blight on genotype Asgrow 4403. PI567562A showed high levels of resistance to charcoal rot and none of the four genotypes was resistant to infection by *Phomopsis* spp. The results indicated that the identification of sources of resistance and the search for new fungicides capable of suppressing the development of diseases seriously affecting soybean production have to be intensified (Cross et al. 2012).

The effectiveness of cyazofamid (4-chloro-2-cyano-N,N-dimethyl-5-p-tolylimidazole-1-sulfonamide) was shown to have specific inhibitory activity against diseases caused by *Phytophthora infestans*. The minimum inhibitory concentration (MIC) was over 60 times lower than that of mancozeb and at least 16 times lower than that of metalaxyl. Cyazofamid at $1.6\text{--}25\text{ mg L}^{-1}$ exhibited not only preventive activity, but also stable residual activity and rainfastness. Cyazofamid at 6.3 mg L^{-1} concentration reduced zoosporangia formation by *Phytophthora infestans* on susceptible host plants by 100%. Cyazofamid also exhibited translaminar and curative activity. Cyazofamid did not show cross-resistance with other commonly used fungicides and, under field conditions, the fungicide provided a high level of control (Mitani et al. 2002). Phosphorous acid (phosphonate or phosphite) is the ionic metabolite of the systemic fungicide aluminum tris-O-ethyl phosphonate (fosetyl-Al). Both phosphorous acid and fosetyl-Al were inhibitory to oomycetes and are systemic in nature (Cohen and Coffey 1986; Ouimette and Coffey 1989).

Phosphorous acid was applied to assess its efficacy in reducing the incidence of potato tuber rots caused by *Phytophthora infestans*, *P. erythroseptica*, and *Pythium ultimum* when applied as foliar spray on potato cultivars at different application timings and rates under different growing conditions in four locations in the Pacific Northwest, USA. Late blight incidence (*P. infestans*) infects tubers after harvest. Foliage of four late-blight-susceptible cultivars and one moderately tuber-blight-resistant potato cultivar was treated with phosphorous acid at 7.49 and $9.37\text{ kg ai ha}^{-1}$. Three applications were not consistently more effective in reducing tuber infection than two applications at the dosage of $9.37\text{ kg ai ha}^{-1}$ when evaluated at three widely separated and diverse locations. Applications at tuber bulking stage beginning were more effective than applications made 4 weeks after initial tuber bulking at one of two locations. Potato

cultivars had an influence on the amount of phosphorous acid required for managing tuber blight, the tuber-blight-resistant cultivar Umatilla Russet needing two applications compared to three required for susceptible cultivar Ranger Russet to achieve the same level of protection. Suppression of pink rot disease (*P. erythroseptica*) by phosphorous acid was not as effective as in the case of late blight disease during the 3 years of investigation. The percentage of reduction in disease incidence was relatively small and three sprays were required for disease suppression in two locations. Control of infection by *Pythium* spp. by phosphorous acid was not up to a recognizable level. The effect of phosphorous acid application on tuber yield was not significant. It was suggested that sound cultural practices such as avoiding excess irrigation of fields, in addition to phosphorous acid application, might be useful for more effective management of tuber rot diseases and a consequent increase in marketable tuber yield (Johnson et al. 2004).

Production of the phytotoxin thaxtomin A is positively correlated with the ability of isolates of *Streptomyces* spp. to induce common scab disease of potato tubers. A strong correlation between the ability of the chemical to suppress common scab symptom development and the ability of the chemical to inhibit thaxtomin A toxicity was indicated. Two isolates of *Streptomyces scabiei* G32 and G20 were used to inoculate the potato tubers. The isolate G32 induced greater severity of common scab disease. The chemicals 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenoxyacetic acid (amide form, 2,4-Dam), and 2,5-dibromobenzoic acid (2,5-DBB) showed consistent and significant ability to reduce the disease (tuber surface cover) across both cultivars, Russet Burbank (moderately resistant) and Desiree (susceptible), when compared with water control. Another chemical 3,5-dichlorophenoxyacetic acid (3,5-D) was able to suppress the disease severity significantly in five of six experiments. The results showed a clear positive relationship between disease development and thaxtomin A-induced necrosis in tuber tissues, and the extent of disease mitigation using foliar chemicals was strongly correlated with their ability to inhibit thaxtomin A toxicity (Tegg et al. 2012).

Watermelon seedlings are susceptible to gummy stem blight pathogen *Didymella bryoniae* which infects seedlings used as transplants in the greenhouse. Seedlings of bottle gourd (*Lagenaria siceraria*) and interspecific hybrid squash (*Cucurbita moschata* x *C. maxima*) were used as rootstocks for grafting watermelon, and the graft wounds provided the entry points for *D. bryoniae*. Fungicides were necessary to protect the grafted plants grown under misted or high relative humidity (RH) conditions provided for rapid graft establishment. Phytotoxicity symptoms on plants treated with fluopyram + tebuconazole or cyprodinil + difenconazole were observed. Four of the five fungicides that were not phytotoxic reduced the incidence and severity of gummy stem blight on seedless watermelon grafted onto bottle gourd cultivar Emphasis and hybrid squash Strong Tosa. Difenconazole and cyprodinil were more effective than mancozeb or water control ($P=0.01$). Although difenconazole and cyprodinil remained unregistered for use on cucurbits, it was suggested that mancozeb or cyprodinil + fludioxonil might be applied against gummy stem blight disease on watermelon and rootstock seedlings during greenhouse production (Keinath 2013).

14.1.4 Development of Resistance in Fungal Pathogens to Fungicides

Fungicides have to be applied for effective management of crop diseases under certain conditions such as unavailability of dependable sources of resistance to the target disease(s) and existence of conducive environmental conditions for disease development.

However, some undesirable effects due to fungicide use have also been observed, necessitating preclusion of indiscriminate and excessive use of the chemicals. Hazards to human health and environment effects on nontarget microbes, in addition to development of tolerance/resistance to fungicides which has been a great concern for the growers and agencies involved in the development of agrochemicals, resulted in the withdrawal or replacement of fungicides to which tolerant/resistant isolates of the pathogen have been identified.

The application of fungicides at frequent intervals for longer periods has been found to be necessary to protect high-value crops and highly susceptible cultivars. Under such conditions, continued use of the same fungicide for several seasons exerts selection pressure on the pathogens to produce new strains or races that may be insensitive/resistant to the fungicide concerned, leading to the withdrawal of the fungicide from further use against the pathogen(s). Resistance or tolerance to fungicide may be defined as reduction in response of a plant pathogen to a fungicide or chemical as a result of application for the suppression of the pathogen concerned. Development of resistance in pathogen occurs at a faster rate against certain fungicides, whereas no single pathogen is known to develop resistance to different groups of fungicides. Development of strains resistant to nonsystemic fungicides used prior to 1970s was rarely seen, but with an increase in the use of systemic fungicides with narrow-spectrum of activity the incidence of pathogen strains with less sensitivity has been increasing rapidly in number. Fungicide resistance may be of two types: (i) field resistance characterized by the development of resistant strains identified by their reduced sensitivity to the fungicide when used as per label recommendations; and (ii) laboratory resistance recognized under in vitro conditions (Staub 1991). Nonsystemic protectant fungicides have multisite and broad-spectrum action and the chances of fungal pathogens developing tolerance to them are less. In contrast, in the case of systemic fungicides with narrow-spectrum action, the development of resistance by substitution of a single amino acid by suitable changes in pathogen DNA sequence may be possible as in *Rhynchosporium secalis*, causal agent of barley leaf blotch disease. Mutations affecting amino acids at positions 198 and 200 appeared to result in resistance to methyl-1-(butylcarbamoyl) benzimidazole-2-yl-carbamate (MBC) (Hollomon and Butters 1994). As the systemic fungicides offer certain advantages such as requirements of small quantities and fungicidal action inside plant tissues, the use of systemic fungicides has increased despite the possibility of development of resistance in the fungal pathogens.

Carbendazim (MBC) failed to be effective against wheat scab disease caused by *F. graminearum* after about 30 years of application in the eastern coast of China. Presence of MBC resistant isolates was detected frequently in this region. The EC_{50} values and minimum inhibitory concentration (MIC) values of MBC inhibiting the mycelial growth of wild-type isolates were less than 0.9 and $1.4 \mu\text{g mL}^{-1}$, respectively. Both field-resistant and sensitive MBC strains shared similar thermosensitivity, fitness, and virulence on wheat ears. Field-resistant strains and UV-induced mutants showed positive cross-resistance to other benzimidazole derivatives and were mainly at intermediate MBC resistance level. No mutation in β -tubulin was detected in *F. graminearum*, whereas mutation in tubulin has led to MBC resistance in other fungal pathogens (Chen et al. 2007).

The emergence of isolates of *Fusarium graminearum* with lower levels of sensitivity to carbendazim, the major fungicide applied for control of Fusarium head blight (FHB) disease, posed a major obstacle for the continued application of this fungicide. Five isolates representing three phenotypes of different carbendazim sensitivity levels

(S, MR, and HR) were randomly selected to study the inheritance of carbendazim resistance by three genetic crosses under field conditions. Each parent in all crosses was marked with a different class of nitrate nonutilizing (*nit*) mutation. The presence of sexual recombinants indicated that outcrossing had occurred in the crosses. Over 100 putative self-crossing or outcrossing perithecia for each cross were randomly sampled on the surface of the haulms of dead rice for each pair of parents. Results indicated that 5.7–20.9% outcrossing frequency occurred in three crosses and confirmed sexual recombination under field conditions. Most of the sexual recombinants possessed fitness levels comparable with those of their parents. Outcrossing between carbendazim-sensitive and -resistant isolates did occur under field conditions and sexual recombination must play a role in the development of carbendazim resistance in the field (Chen and Zhou 2009).

Resistance to carbendazim has been shown to be conferred by mutations in a single chromosomal β -tubulin gene in *Botrytis cinerea* and most other fungal pathogens, due to several allelic mutations. In contrast, carbendazim resistance in *Fusarium graminearum* was not associated with mutation in the corresponding β -tubulin gene. The β -tubulin gene conferring carbendazim resistance in *B. cinerea* was cloned and connected with two homologous arms of the β -tubulin gene of *F. graminearum* by using a double cross-over at the site where β -tubulin gene of *F. graminearum* is normally located (the β -tubulin gene of *F. graminearum* had been earlier deleted). The β -tubulin gene conferring carbendazim resistance in *B. cinerea* did not express this resistance in *F. graminearum*, since the transformants remained very sensitive to carbendazim (Liu et al. 2010).

In a later investigation, isolates of *F. graminearum* (7261) collected in China during 2010–2012 were evaluated for their sensitivity to carbendazim (MBC). The relationship between trichothecene chemotypes of the FHB pathogen and their MBC-sensitivity was studied. MBC-sensitive isolates were more numerous in nivalenol (NIV) chemotype. 15-acetyldeoxynivalenol (15-ADON) chemotype had a limited number of MBC-sensitive isolates. Most MBC-resistant isolates secreted 3-deoxynivalenol (3-ADON). Trichothecene analyses indicated that MBC resistance increased 3-ADON yield and percentage, especially in site-directed mutants at codon 167 in the β 2-tubulin gene (Zhang et al. 2013a). In order to validate the mechanism of development of resistance in *Fusarium graminearum* (*Fg*), the resistance of field isolates to carbendazim through association with mutations in β -tubulin (*tub*) gene was investigated. Site-directed mutagenesis procedure was adopted, followed by gene replacement to change the β 2-tubulin gene of a carbendazim sensitive field strain of *Fg* at residues 50, 167, 198, or 200. The transformants stably retained the transgressed gene and were tested for their sensitivity to carbendazim. The level of resistance differed depending on the mutation. Biological characteristics of the mutants did not show variations from the parent strain. Mutations at codon 50, 167, 198, or 200 of *F. graminearum* of β 2*tub* could lead to resistance to carbendazim and these codons might form a binding pocket (Qiu et al. 2011). *Fusarium graminearum* (*Fg*) has two β -tubulins (*Fgtub1* and *Fgtub2*), whereas *Botrytis cinerea* and other fungal pathogens have only one tubulin. Even after replacing *Fgtub1* with that of *B. cinerea* (*BctubE198A*), the *F. graminearum* transformants were sensitive to carbendazim. The *Fgtub2* of the mutant 20C1 (*Fgtub1* was replaced with *BctubE198A*) and *Fgtub1* of mutant 20D10 (*Fgtub2* was replaced with *BctubE198A*) were deleted. These mutants were also sensitive to carbendazim. The results indicated that *B. cinerea* β -tubulin could complement the function of the two *Fg* β -tubulins, even when both were deleted and expression of the β -tubulin conferring carbendazim resistance differed between fungal pathogens (Liu et al. 2013).

Sunflower downy mildew induced by *Plasmopara halstedii* was managed by seed treatment with metalaxyl-M, a phenylamide compound. Isolates of *P. halstedii* exhibiting resistance to metalaxyl-M were isolated in Spain during 2000, 2004, and 2006. Populations (60) of the pathogen from the field were characterized by virulence (race) and by reaction to metalaxyl-M applied as seed dressing. Race 310 was found predominantly in 3 years in southern Spain (68% of the populations) and races 100 and 330 were also present. Thirty-one percent of the populations were not controlled by metalaxyl-M when applied at the recommended dose of 2.0 g ai kg^{-1} seed and subpopulations collected on plants growing from treated seed were the same race as those from the initial populations. Doses of metalaxyl and metalaxyl-M varying over $0\text{--}8.0 \text{ g ai kg}^{-1}$ seed were applied to the pathogen population present on seed and the incidence of downy mildew depended significantly on the fungicide dose. A dose response-regression analysis showed ED_{50} values of the populations to be between 2.4 and 4.4. The extended and frequent use of the fungicide was considered as the possible reason for the emergence of populations with resistance to metalaxyl-M in Spain. The presence of virulent populations of *P. halstedii* resistant to the fungicide has to be considered in genetic and chemical strategies for the management of sunflower downy mildew disease (Molinero-Ruiz et al. 2008).

Field isolates of *Gibberella pulicaris* (anamorph *Fusarium sambucinum*), incitant of potato dry rot disease, produce trichothecene mycotoxins and were found to be resistant to thiabendazole, a benzimidazole fungicide. In order to characterize the resistance to this fungicide, homokaryons were derived from single ascospores that were produced in vitro from crosses of thiabendazole-resistant *G. pulicaris* field isolates with sensitive isolates of an opposite mating type. The β -tubulin gene was amplified from these homokaryons using degenerate oligonucleotides synthesized to conserved sequences of the β -tubulin gene derived from related fungi. An open-reading frame containing three putative introns and a deduced amino acid sequence of 446 amino acids exhibited a high level of homology to the β -tubulin gene of other fungi. Although nucleotide and amino acid differences were observed, none of them was linked to the thiabendazole resistance. Linkage analysis confirmed that the isolated β -tubulin gene was a single-copy gene and was not linked to thiabendazole resistance of *G. pulicaris* (Kawachuk et al. 2002).

Seedborne *Didymella bryoniae*, causing gummy stem blight disease, is a very destructive disease of watermelon. The infected seeds form the primary sources of inoculum capable of introducing the strains from different locations. *D. bryoniae* has a history of becoming insensitive to systemic fungicides used for the management of crop diseases. The populations repeatedly exposed to high-risk fungicides present in a location were tested for their sensitivity/resistance to fungicide(s) applied repeatedly at the location concerned. However, the unexpected occurrence of resistant populations in fields where fungicides have never been used suggested the introduction of resistant isolates through seeds/external source. Watermelon seed as a potential source of fungicide-resistant inoculum was therefore investigated. Using direct-plating, sweat box, and blotter assays, 5800 seeds from commercial watermelon seed lots were tested for the presence of isolates insensitive to fungicides boscalid, tebuconazole, azoxystrobin, and thiophanate methyl. Some isolates showed resistance to thiophanate methyl. The results showed that watermelon seed naturally infested with *D. bryoniae* might harbor fungicide-resistant isolates which could be introduced into locations where the fungicide had not been applied earlier (Thomas et al. 2014).

Tridemorph was applied to reduce the incidence of corn smut disease caused by *Ustilago maydis*. Mutants of *U. maydis* with low resistance to tridemorph were isolated in a mutation frequency of 7×10^6 after UV-irradiation and selection on media containing $25 \mu\text{g mL}^{-1}$ tridemorph. Genetic analysis with nine mutant isolates resulted in the identification of two unlinked chromosomal loci, *U/tdm-1* and *Utd-2*. The *U/tdm* mutations were responsible for low resistance levels to tridemorph, based on effective concentration causing 50% reduction in the growth rate (EC_{50}) or minimal inhibitory concentration (MIC). Haploid strains carrying both *U/tdm* mutations showed higher levels of resistance to the fungicide, indicating interallelic interaction between nonallelic genes. Cross-resistance studies with the inhibitors of C-14 demethylase showed that the *U/tdm* mutations were responsible for increased sensitivity to the triazoles, triadimefon, triadimenol, propiconazole, and flusilazole and the pyridine pyrifenoxy. Study of the gene effect on the fitness of *U. maydis* showed that *U/tdm* mutations appeared to be pleiotropic, having more or less adverse effects on pathogen growth rate in liquid culture and pathogenicity on young corn plants (Markoglou and Ziogas 2000).

Carboxin was used to treat wheat and barley seeds to protect the seedlings against loose smut pathogens *Ustilago tritici* and *U. nuda*, respectively. Isolates of *U. tritici* (3) and *U. nuda* (2) isolated in 1997 and 1999 in Canada were found to be insensitive to carboxin, based on teliospore germination assay using carboxin-amended medium. Seeds with or without treatment with carboxin at recommended rate of 55 g ai/100 kg or at 110 g ai/100 kg seed followed by inoculation with the pathogen were sown. The growing plants were assessed at heading stage for infection which was expressed as percentage of smutted spikes. Three isolates of *U. nuda* were not affected by treatment with carboxin at 55 g ai/100 kg seed. However, the incidence of loose smut on their respective hosts by three of these isolates was significantly reduced when seeds were treated at 110 g ai carboxin/100 kg seed. The results suggested that these isolates of *U. tritici* and *U. nuda* were tolerant to carboxin rather than resistant. However, the tolerant isolates might subsequently develop resistance to the higher dose of carboxin sooner or later (Menzies 2008).

Fusarium sporotrichoides, a component of Fusarium head blight (FHB) complex, was investigated for the ability to develop resistance to fungicides. Carbendazim-resistant (RS) and control (CS) strains of *F. sporotrichoides* were exposed to graded concentrations (0, 1, 10, and 100 mg L^{-1}) of azoxystrobin in broth media. Significant strain-fungicide interactions were recorded in trichothecene production following exposure to azoxystrobin. At 4 and 8 days of incubation, addition of 10 mg L^{-1} of azoxystrobin stimulated T-2 toxin synthesis ($P < 0.05$) only in RS cultures. In contrast, T-2 toxin enhancement in the CS cultures occurred only on day 8, but at a lower concentration (1 mg L^{-1}). The stimulation of T-2 toxin synthesis depended on strain and azoxystrobin level. Another toxin diacetoxyscirpenol (DAS) production was affected by a more complex set of interactions. As in the case of T2-toxin, DAS production in CS was stimulated ($P < 0.05$ or better) only at low exposure levels of azoxystrobin. In the case of neosolaniol (NEO), the main effect of strain was significant with CS producing consistently more of the mycotoxin than RS. The results indicated that development of resistance to the fungicide might affect trichothecene production by *F. sporotrichoides* on exposure to a second fungicide (D'Mello et al. 2001).

The primary inoculum for *Fusarium graminearum* (teleomorph *Gibberella zeae*), causal agent of wheat Fusarium head blight disease, comprised ascospores available during anthesis. The application of fungicides containing triazoles at the time of anthesis

was effective in the initial year of introduction. Prothioconazole, metconazole, and tebuconazole inhibited ascospore germination, whereas epoxiconazole had only a minor effect within the range of concentrations tested. The inhibitory effect of epoxiconazole on mycelial growth was strongest in strains isolated in 1987 and decreased with time at an average rate of 0.22% per year. The initial efficacy of triazoles against *G. zeae* ascospores at their market introduction increased by a factor of 2.950 within 10 years (epoxiconazole excluded). During the first 10 years following their introduction, the EC_{50} values for metconazole and tebuconazole increased by factors of 1.391 and 1.393, respectively. The results suggested that the decline in *G. zeae* sensitivity against triazoles was slower in the past decades than the increase in sensitivity achieved by development of new and more efficient triazole analogues (Klix et al. 2007).

Quinine outside-inhibiting (QoI) fungicides have been shown to be effective against some seedborne pathogens. They inhibit mitochondrial respiration of the sensitive fungus by binding to outer quinine-oxidizing pocket of the cytochrome bc1 (*cytb*) enzyme complex. Mutants of *Ustilago maydis* with high resistance to azoxystrobin (based on EC_{50} values) were isolated by guanidine mutagenesis and selection on media containing $1 \mu\text{g mL}^{-1}$ of azoxystrobin, in addition to use of 0.5 mM salicylhydroxamate (SHAM), a specific inhibitor of cyanide-resistant (alternative) respiration. Oxygen uptake in whole cells was strongly inhibited in the wild-type strains (sensitive) by azoxystrobin ($1.5 \mu\text{g mL}^{-1}$) in addition to SHAM (1 mM), but not in the mutant isolates. The criterion of cytoplasmic resistance (of intracellular selection) was fulfilled by experiments on the stability of resistance phenotypes. Cross-resistance studies with other fungicides which also inhibit electron transport through complex III of respiratory chain showed that mutations for resistance to azoxystrobin were also responsible for reduced sensitivity to kresoxim-methyl and to antimycin A which act at the Q_0 and Q_1 sites of the cytochrome bc1 complex, respectively (Ziogas et al. 2002).

Sensitivities of fungal pathogens to quinine-outside-inhibiting (QoI) fungicides may be influenced by the induction of alternative respiration or mutational changes of the cytochrome b target site. The expression and impact of the resistance mechanisms during the conidial germination were investigated. In the absence of a host, alternative respiration provided a >500-fold rescue from azoxystrobin during the germination of conidia derived from four wild-type isolates of *Magnaporthe grisea*, causal agent of rice blast disease. The rescue potential during conidial germination was substantially more pronounced than for mycelial growth. In a comparison of wild-type strain and an alternative respiration-deficient mutation, azoxystrobin efficacies in suppressing symptom development differed by a factor of two, with full disease control achieved for both genotypes at $1 \mu\text{g mL}^{-1}$ azoxystrobin. In contrast, conidia derived from two QoI-resistant target site mutants were highly resistant to azoxystrobin and trifloxystrobin and were fully capable of infecting leaf surfaces protected with $10 \mu\text{g mL}^{-1}$ of azoxystrobin. Both target-site mutants emerged spontaneously in the presence of high azoxystrobin doses when residual mycelial growth was supported by alternative respiration. The effective silencing of alternative respiration in protective applications of QoI-inhibiting fungicides might constitute a strategy of slowing down of the emergence of highly resistant target site mutants (Avila-Adame and Köller 2003).

The emergence of strains of *Magnaporthe oryzae*, causal agent of rice blast disease, showing resistance to QoI fungicides necessitated the development of techniques for rapid, reliable, and sensitive monitoring of the resistant/insensitive strains. A single nucleotide polymorphism (SNP) was detected in a QoI-resistant mutant and a wild-type

strain of *M. oryzae*. Two types of PCR-based DNA markers that facilitate sensitive detection of resistant strains were developed, using DNA templates prepared from filter permeated with the pathogen. This simple, rapid, cost-effective, and reliable procedure required only a few hours to yield results without the requirement of any special equipment (Hayashi et al. 2015).

Microdochium nivale and *M. majus* are involved in the Fusarium head blight (FHB) disease affecting cereals in France. The efficacy of strobilurins was found to be altered in providing protection to the expected level in some field experiments. The sensitivity of French isolates of FHB pathogens to strobilurins and the possible mechanisms of resistance were investigated. Half of the strains collected in 2007 were resistant to strobilurins. In addition, most of the isolates displaying strong resistance were found to be mostly *M. majus* isolates. Positive cross-resistance was observed between all strobilurins tested, but not with the phenylpyrrole derivative fludioxonil and the various classes of sterol biosynthesis inhibitors (SBIs). In most strains, resistance was correlated with the G143A substitution in cytochrome b, the molecular target of strobilurins. Two other mechanisms were also detected at lower frequencies. Strobilurin resistance in *Microdochium* was detected for the first time (Walker et al. 2009).

Isolates of *Fusarium graminearum* (teleomorph *Gibberella zeae*) isolated between 1960 and 2009 in Belgium, Canada, Germany, Italy, Luxembourg, or the USA belonged to the three known chemotypes (3-ADON, 15-ADON, and nivalenol (NIV)) and they were screened for their sensitivity to trifloxystrobin using a liquid culture assay. None of the isolates was completely inhibited by concentrations up to 3 mM of trifloxystrobin. The inhibitory capacity was compared with prothioconazole which inhibited the pathogen growth of a standard isolate of *F. graminearum* at concentrations as low as 0.007 mM. The highest level of inhibition obtained using trifloxystrobin ranged over 14–65% among the strains tested. The sensitivity to trifloxystrobin was not affected by the country of origin or by the chemotype of *F. graminearum*. Significant differences in resistance levels between the countries of origin and chemotypes were detected. In addition, strains isolated before the market introduction of strobilurins in 1996 also showed resistance to strobilurins, indicating that this was largely due to natural resistance of *F. graminearum* isolates and not primarily related to intensive use of strobilurin in agriculture (Dubos et al. 2011).

Azoxystrobin was frequently used for the control of *U. maydis*. The development of mutants resistant to *U. maydis* was observed in several countries. Mutants of *U. maydis* with high resistance to azoxystrobin, an inhibitor of mitochondrial transport at the cytochrome *bc1* complex, were isolated in a mutation frequency of 2.3×10^7 , after nitro-soguanidine mutagenesis and selection on media containing $1 \mu\text{g mL}^{-1}$ azoxystrobin in addition to 0.5 mM salicylhydroxamate (SHAM), a specific inhibitor of cyanide-resistant (alternative) respiration. Oxygen uptake in the whole cells was strongly inhibited in the wild-type strain by azoxystrobin ($1.5 \mu\text{g mL}^{-1}$) in addition to SHAM (1 mM), but not in the mutant isolates. Genetic analysis with nine such mutants led to the production of progeny phenotypes which did not follow Mendelian segregation, but satisfied the criteria of non-Mendelian (cytoplasmic) heredity (Ziogas et al. 2002).

Fungicides that were used regularly for managing gray mold disease of strawberry caused by *Botrytis cinerea* became ineffective in major strawberry production areas in China. *B. cinerea* isolates (486) collected from nine locations in Zhejiang Province were therefore screened for resistance to azoxystrobin, carbendazim, pyrimethanil, and fludioxonil. The majority of the isolates (72.6%) showed resistance to single or multiple fungicides,

whereas 52.3% of the isolates were simultaneously resistant to azoxystrobin, carbendazim, iprodione, and pyrimethanil. All 322 isolates resistant to azoxystrobin (Azo^R) showed the G143 point mutation. Populations of *B. cinerea* could be grouped into types based on the structure of the *cyt b* gene with (12.2%) or without (87.8%) the third introns Bcbi-143/144. The high frequency of *B. cinerea* isolates lacking Bcbi-143/144 intron indicated that populations of *B. cinerea* infecting strawberry possessed a high inherent risk for development of resistance to QoI fungicides (Yin et al. 2015).

Development of insensitivity to azoxystrobin in *Didymella bryoniae*, causal agent of cucurbit gummy stem blight disease, was observed in the eastern USA 2 years after commercial introduction of the fungicide in 1998. Azoxystrobin baseline sensitivity distribution was established in vitro for *D. bryoniae* isolates that had never been exposed to strobilurins (azoxystrobin), and the baseline values were comparable to those of other ascomycetes. The baseline sensitivities of isolates exposed to azoxystrobin for one or more seasons were compared. Sixty-one isolates of *D. bryoniae* collected before 1998 were sensitive. The median EC₅₀ was 0.055 mg L⁻¹ azoxystrobin with a range of 0.005–0.81 mg L⁻¹. Forty isolates collected after exposure during 1998 also were sensitive. On the other hand, 53 of 64 isolates collected in South and North Carolina between 2000 and 2006 were insensitive to 10 mg L⁻¹ azoxystrobin. Sensitive and insensitive isolates could be differentiated based on disease severity on *Cucumis melo* seedlings treated with azoxystrobin (20 or 200 mg L⁻¹). Insensitive isolates of *D. bryoniae* were identified in fields with a history of strobilurin applications (Keinath 2009).

The widespread and highly destructive occurrence of gummy stem blight (GSB) caused by *D. bryoniae* was observed in southeastern USA. The quinone-oxidase-inhibiting (QoI) fungicide azoxystrobin (AZO) initially suppressed the development of the GSB disease very effectively. Azoxystrobin inhibited mitochondrial respiration of the fungal pathogen by binding to the outer quinone-oxidizing pocket of the cytochrome bc1 (*cyt b*) enzyme complex. Resistance to AZO was later observed in *D. bryoniae* isolates from many watermelon-producing regions. Comparison of the DNA sequences of partial *cyt b* genes of four AZO-resistant (AZO-R) and four AZO-sensitive (AZO-S) isolates of *D. bryoniae* revealed the amino acid substitution of glycine by alanine at the 143 codon (G143A) in the AZO-R isolates tested. Allele-specific primers were designed to detect the resistant or sensitive allele at codon 143 of the *cyt b* gene which amplified a 165 bp PCR product from genomic DNA of nine AZO-R and nine AZO-S isolates of *D. bryoniae*, respectively. The primer pairs did not amplify DNA from other pathogens tested. The results indicated that the PCR assays developed in this investigation were specific in differentiating AZO-R and AZO-S isolates and the protocol might be employed for the detection of azoxystrobin resistance in *D. bryoniae* isolates (Finger et al. 2014).

Populations of *Ascochyta rabiei*, causal agent of *Ascochyta* blight of chickpea, resistant to QoI fungicides were detected in the Northern Great Plain of the USA. Assays were performed to assess the fungal sensitivity for two alternative fungicidal mode of action. Isolates (78) of *A. rabiei* collected between 1983 and 2007 were screened to determine the baseline sensitivity to the demethylation-inhibiting foliar fungicide prothioconazole, and 100 isolates collected between 1987 and 2007 were screened for their sensitivity to the methyl benzimidazole carbamate (MBC) fungicide, thiabendazole. Baseline EC₅₀ values for mycelial growth for prothioconazole ranged from 0.0526 to 0.2958 µg mL⁻¹, with a mean of 0.1783 µg mL⁻¹. Isolates of *A. rabiei* collected from 2007 to 2009 from North Dakota chickpea fields exposed to prothioconazole sensitivity were screened using the same assay. Mean EC₅₀ values for these isolates were 0.3544 µg mL⁻¹,

0.3746 $\mu\text{g mL}^{-1}$, and 0.7829 $\mu\text{g mL}^{-1}$ respectively. These values represent an approximate 2- (2007–2008) and 4.4-fold (2009) decrease in intensity from the baseline ranging from 1.192 to 3.819, with a mean of 2.459 $\mu\text{g mL}^{-1}$. No significant decrease in fungicide sensitivity was observed for thiabendazole against *Ascochyta* blight disease to chickpea (Wise et al. 2011).

Genetic modification in *A. rabiei* in response to the development of resistance to QoI fungicides was studied. Isolates of *A. rabiei* exhibiting resistance to QoI fungicides were collected in North Dakota, USA and a fragment of the cytochrome *b* gene from *A. rabiei* from the resistant isolates was characterized. The sequenced genome DNA fragment contained a group I intron immediately after codon 131. The size of the cytochrome *b* gene was estimated to be over 4.6 kb. Multiple alignment analysis of cDNA and protein sequences revealed a mutation that changed the codon for amino acid 143 from GLT to GTC, introducing an amino acid substitution from glycine to alanine (G143A) which is frequently associated with QoI resistance. Based on this mutation, a diagnostic PCR assay was developed using an approach called mismatch amplification mutation assay. This method was validated by testing 38 of 70 *A. rabiei* isolates which were resistant to QoI fungicide. The PCR protocol developed in this investigation could be applied for screening the isolates of *A. rabiei* for their sensitivity/resistance to QoI fungicide (Delgado et al. 2013).

The sensitivity of 50 isolates, including 25 each of the 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) genotypes (chemotypes) from a collection of *Fusarium graminearum* associated with Fusarium head blight (FHB) disease of wheat, to tebuconazole and metconazole was investigated. The effective concentration of tebuconazole leading to 50% reduction in mycelial growth (EC_{50}) was 0.28–8.09 mg L^{-1} , while metconazole had EC_{50} values of 0.05–0.86 mg L^{-1} . The mean sensitivity did not differ between the trichothecene genotype groups. The isolate Gz448NY11 showed the lowest sensitivity to tebuconazole (EC_{50} = 8.09 mg L^{-1}). The level of suppression of FHB and DON accumulation following application of the commercial rate of tebuconazole was significantly reduced in plants inoculated with tebuconazole resistant isolate compared with a sensitive isolate with known aggressiveness and toxigenicity on wheat. Application of metconazole did not have any effect on disease development and toxin accumulation. The number of the population of tebuconazole-resistant isolate recovered from spikes inoculated with an equal mixture of two isolates and sprayed with tebuconazole increased significantly. Monitoring of triazole-resistant isolates to determine the risk which these fungicides pose to disease management and food security is essential (Spolti et al. 2014).

FHB disease of wheat caused by *Fusarium culmorum* was effectively managed by applying azole fungicides, but the occurrence of insensitive strains of *F. culmorum* became a major problem. A tebuconazole-insensitive strain was produced in the laboratory by exposure to higher concentrations of tebuconazole, but the insensitive strain had a lower level of fitness in vitro. The tebuconazole-insensitive strain also showed cross-resistance to nine important azole fungicides. Further, plants inoculated with the insensitive *F. culmorum* strain showed no reduction of FHB symptoms and deoxynivalenol (DON) accumulation in wheat grains after tebuconazole application, compared to plants inoculated with the sensitive strain. Cultivation of wheat cultivars carrying high levels of resistance such as cultivar Toras was considered to be the most effective method of reducing FHB disease severity and DON accumulation. A fungicide mixture that could combine different mechanisms of action on the FHB pathogens might be

used in combination with resistant cultivars to tackle the problem of fungicide resistance in pathogens (Serfling and Ordon 2014).

The azole fungicide prochloraz has been found to be effective in controlling *Fusarium verticillioides* infecting corn in China when applied as foliar spray. The possible resistance risk due to prochloraz treatment was assessed under in vitro conditions. Four prochloraz-resistant strains were generated by successive selection on fungicide-amended media. The mycelial growth rates of the mutants were inversely related to the level of resistance. All four mutants showed cross-resistance to the triazole fungicides, triadimefon, tebuconazole, and difenconazole, but not to the multisite fungicide chlorothalonil or the MAP/histidine kinase inhibitor fungicide fludioxonil. Based on the Y123H mutation in FvCYP51B, the four resistant mutants were subdivided into two genotypes: PCZ-R1 mutants with wild-type FvCYP51B; and PCZ-R2 mutants with substitution Y123H and FvCYP51B. Wild-type FvCYP51B complemented the function of native ScCYP51 in *Saccharomyces cerevisiae* YUG37::erg11, whereas Y123H-mutated FvCYP51B did not. For the PCZ-R2 mutants, disruption of FvCYP51B function by the Y123H substitution caused constitutive upregulation of FvCYP51A expression and consequently to prochloraz resistance (Fan et al. 2014).

The triazole fungicides, sterol demethyl inhibitors, have been the primary systemic fungicides applied on cucurbits to reduce the incidence of gummy stem blight (GSB) disease caused by *Didymella bryoniae*. Isolates of *D. bryoniae* from South Carolina with no exposure to tebuconazole or exposed for several years were tested for sensitivity to tebuconazole and difenconazole. The parameters used were colony diameters, percentages of germination of ascospores, and conidia, and germ tube lengths were determined by growing the isolates (147) of *D. bryoniae* on agar amended with 0.10–10.0 mg L⁻¹ of tebuconazole and 0.01–1.0 mg L⁻¹ difenconazole. All isolates were sensitive to tebuconazole and difenconazole with mean EC₅₀ values of 0.41 and 0.054 mg L⁻¹, respectively. Retention of sensitivity of isolates of *D. bryoniae* to tebuconazole and difenconazole was attributed to the 2 year crop rotation and rotation of systemic and contact fungicides which prevented continuous exposure of the pathogen to tebuconazole and difenconazole. Despite exposure to tebuconazole for up to 9 years, isolates of *D. bryoniae* from South Carolina remained sensitive to triazole fungicides (Keinath and Hansen 2013).

Azoles such as epoxiconazole, propiconazole, prothioconazole, or tebuconazole and imidazoles such as prochloraz belong to the group of demethylation inhibitors (DMIs). Their mode of action is through inhibition of demethylation at 14- α carbon of lanosterol or 24-methylene dihydrolanosterol. Tebuconazole and prochloraz have been shown to be substrates for the cytochrome P450-dependent 14- α demethylase in the biosynthesis of fungal sterols (Gisi et al. 2002). Isolates (about 3000) of *Mycosphaerella graminicola* (anamorph *Septoria tritici*) were assayed in vitro for their resistance to QoI fungicide azoxystrobin and 46 isolates were found to be resistant. On subculturing, only 10 isolates remained resistant. Cross-resistance extended to other QoIs, but varied between different isolates. Application of azoxystrobin was not effective to the required level, especially at lower doses. Propylgallate, an inhibitor of alternative oxidase, potentiated the activity of azoxystrobin in vitro, so that resistance was no longer observed. The growth of resistant strains in the presence of azoxystrobin led to alternative oxidase activation. This increased flexibility in respiration allowed resistant strains to survive in the presence of a QoI fungicide. Under these conditions, selection for target-site mutations could occur. Using QoIs as preventive application reduced the risk of development

of resistance, since alternative oxidase cannot by itself generate all the energy required for spore germination and early infection (Miguez et al. 2004).

Resistance of *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) to QoI fungicides was observed in France and other European countries and reliable suppression of wheat blotch disease was considered to be dependent on inhibitors of sterol 14 α -demethylation (DMIs). Strains of *S. tritici* with reduced sensitivity to DMIs were found to be widespread. Sensitivity assays based on in vitro effects of fungicides on germ tube elongation allowed characterization of seven DMI-resistant phenotypes. In four of them, cross-resistance was not observed between all tested DMIs such as prochloraz, triflumizole, fluquinconazole, and tebuconazole. Further, the highest resistant factors to most DMIs were found only in recently occurring isolates. According to the responses of isolates to prochloraz, two categories were recognized. Molecular analysis showed that DMI resistance was associated with mutations in the *CYP51* gene encoding the sterol 14 α -demethylase. Changes at codons 459, 460, and 461 were related to low resistance levels, whereas at position 381 a valine instead of an isoleucine in combination with previous changes determined the highest resistance levels to all DMIs except prochloraz. Mutations in codons 316 and 317 were also found in some isolates exhibiting low-resistance factors towards most DMIs (Leroux et al. 2007).

The sensitivity of *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) to QoI fungicide trifloxystrobin, azoles propiconazole, tebuconazole, and prothioconazole and nonsystemic fungicides folpet and chlorothalonil was tested in a bioassay using strains isolated in 1999, 2004, or 2008 in the high-disease pressure and high-fungicide input areas between the North and Baltic seas. The percentage of sprays containing at least one demethylation inhibitor (DMIs, azoles) was around 60% at any given point of time in that region where *S. tritici* strains were sampled. The bioassay accurately detected the total loss of sensitivity of *S. tritici* towards trifloxystrobin used as a representative of the QoIs. In 2002, about 80% of the fungicide sprays contained QoIs in a wheat disease monitoring program in northern Germany. After the breakdown of QoI efficacy, use of QoI decreased to about 4% in 2006 and the sensitivity did not recover until 2008, indicating that QoI resistance was not coupled to a significant fitness penalty in *S. tritici*. DMI concentrations inhibiting pathogen growth by 50% decreased on average by $48 \pm 17\%$ between 1999 and 2004 and increased by $132 \pm 42\%$ between 2004 and 2008, compared to 1999. On the other hand, EC_{50} values of the multisite inhibitors folpet and chlorothalonil increased by $128 \pm 25\%$ between 1999 and 2004 and decreased by $40 \pm 11\%$ between 2004 and 2008, but changes over time were nonsignificant for the multiple inhibitors (Beyer et al. 2011). A high proportion of DMI-moderate resistance genotypes ($\geq 94\%$) of *S. tritici* was found in all samples. Distribution of DMI-resistant genotypes was not affected by different levels of resistance of wheat cultivars or by different periods of application of the fungicide prothioconazole. Treatment with prothioconazole significantly decreased the amount of pathogen DNA by 80% in preventive treatment, and they were still 70% efficient when applied curatively at 3 days postinoculation (dpi). The qPCR format was found to be an accurate, specific, and pre-symptomatic quantitative procedure for determining the pathogen population. Correlation between quantity of DNA estimated by qPCR assay and different levels of necrotic surfaces (0–100%) in leaf samples was investigated. *S. tritici* was detected in asymptomatic leaves and the DNA contents increased proportionally with an increase in leaf necrotic areas in the leaves. A high level of correlation ($CF=0.95$) was observed for up to 40% leaf necrotic surface. The DNA contents showed reduction beyond 50% leaf necrotic area and remained constant thereafter (Fig. 14.1) (Selim et al. 2014).

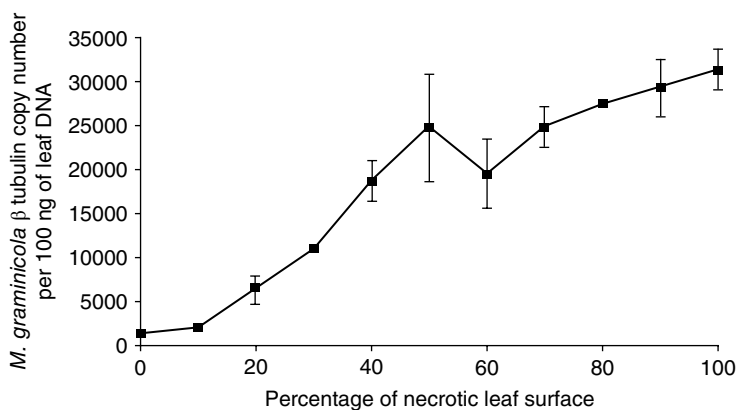


Figure 14.1 Positive correlation between the percentages of necrotic leaf surface on wheat cultivar Maxyl and the copy numbers of tubulin gene (DNA contents) determined by qPCR assay ($F=0.95$). Note the disease progress is proportional to the pathogen development. Source: Selim et al. (2014). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

Variations in the sensitivity to tebuconazole and metconazole of 50 isolates comprising 25 each of 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) from a contemporary collection of *Fusarium graminearum* associated with Fusarium head blight (FHB) disease of wheat were assessed. The effective concentration causing 50% reduction of mycelial growth (EC_{50}) for tebuconazole was $0.28\text{--}8.09\text{ mg L}^{-1}$ ($\mu = 1.12\text{ mg L}^{-1}$), whereas EC_{50} for metconazole was $0.05\text{--}0.86\text{ mg L}^{-1}$ ($\mu = 0.33\text{ mg L}^{-1}$). The mean sensitivity did not differ between the trichothecene genotype groups. Isolate Gz448NY11 of *F. graminearum* from Steuben county was first reported as the tebuconazole-resistant field isolate and had the lowest sensitivity to tebuconazole ($EC_{50} = 8.09\text{ mg L}^{-1}$). FHB suppression and DON production were significantly reduced following application at commercial rate of the fungicide in plants inoculated with tebuconazole-resistant isolate compared with those inoculated with sensitive isolate, which was aggressive and toxigenic. FHB disease and DON production were reduced by application of metconazole. Significantly greater numbers of individuals of the tebuconazole-resistant isolates were recovered from spikes inoculated with an equal mixture of the two isolates and sprayed with tebuconazole. The results indicated the need for constant monitoring of the appearance of triazole-resistant isolates for effective management of FHB disease using fungicides (Spolti et al. 2014).

Intensive application of DMI fungicides led to the selection of several fungal pathogens for different levels of insensitivity/resistance to these fungicides. *Fusarium graminearum* isolates (224) from 13 different German populations, 22 isolates from the UK, and 6 reference isolates from Italy and the USA were evaluated for their levels of sensitivity to propiconazole. Sensitivity within field population was about 80%, suggesting significant potential for selection of resistant isolates at the field scale. Sequence analysis of the pathogen isolates showed that the *CYP51A* and *CYP51C* genes carried several nonsynonymous substitutions, but the *CYP51B* protein was not polymorphic. The nonsynonymous substitutions were randomly distributed among field populations of *F. graminearum* and they showed no relationship with fungicide sensitivity. The results suggested that the contribution of genes outside *CYP51* family might be

more important for development of resistance to DMI fungicides in *F. graminearum* than those in *CYP51* family (Talas et al. 2015).

Phenylamide fungicides have been extensively applied for over three decades to reduce the incidence of oomycete-induced plant diseases. Mefenoxam application effectively suppressed the development of potato late blight disease caused by *Phytophthora infestans*. Isolates of *P. infestans* were collected from Wisconsin from 2009 to 2012. Distribution of clonal lineages, mating types, and sensitivity to the systemic fungicide mefenoxam were studied. The usefulness of analysis of the glucose-6-phosphate isomerase (Gpi) allozyme was assessed for predicting mefenoxam sensitivity to facilitate early transmission of information to the growers for taking appropriate disease management measures. Overall, 143 isolates were collected from 52 locations in 20 Wisconsin counties from 2009 to 2012. Three clonal lineages, US-22, US-23, and US-24 were identified. The US-22 was of the A2 mating type and sensitive to mefenoxam with Gpi 100/122. On the other hand, US-23 and US-24 belonged to A1 mating type and primarily intermediately sensitive to mefenoxam with Gpi 100/100 and 100/111, respectively. The mefenoxam sensitivity could be predicted based on the allozyme assay, because of the close correlation and the unique Gpi patterns for each lineage. The presence of new clonal lineages of *P. infestans* in Wisconsin indicated the need for continued close monitoring of late blight pathogen isolates (Seidl and Gevens 2013).

The insensitivity of isolates of *P. infestans* to phenylamide fungicide mefenoxam was investigated to determine the genetic basis of insensitivity. Crosses were effected between insensitive isolates and intermediate insensitive isolates of *P. infestans*. F1 progeny showed expected semi-dominant phenotypes for mefenoxam insensitivity and suggested the involvement of multiple loci, complicating the positional cloning of the gene(s) conditioning insensitivity to mefenoxam. The primary effect of phenylamide compounds was found to inhibit ribosomal RNA synthesis by the pathogen. The subunits of RNA polymerase I (RNA polI) were sequenced from sensitive and insensitive isolates and F1 progeny. Single nucleotide polymorphisms (SNPs) specific to insensitive field isolates were identified in the gene encoding the large subunit of RNA polI. In a survey of field isolates of *P. infestans*, SNP T1145A (Y382F) showed an association of 86% with mefenoxam insensitivity. Isolates lacking this association belonged predominantly to one genotype of *P. infestans*. Transfer of the allele governing sensitivity RPA190 to a sensitive line resulted in the generation of transgene lines insensitive to mefenoxam. The results demonstrated that sequence variation in RPA190 contributed to insensitivity to mefenoxam in *P. infestans* (Randall et al. 2014).

Development of insensitivity to other groups of fungicides in fungal pathogens has also been reported. The application of scytalone dehydratase inhibitors of melanin biosynthesis (MBI-D) on rice failed to suppress the incidence of rice blast disease caused by *Magnaporthe grisea* in Kyshu in Japan. Isolates of *M. grisea* were collected from Saga prefecture and analyzed for their sensitivity to MBI-D fungicides. Resistant isolates of rice blast pathogen were detected in all prefectures of Kyushu. DNA fingerprinting analysis showed that mutations causing resistance to MBI-Ds had arisen independently in each area. The data suggested that resistant isolates might occur in any area and become dominant under continuous selection pressure for MBI-Ds. However, it would be possible to manage the rice blast disease using reductase inhibitors of melanin biosynthesis (MBI-R) or commercial rice seed disinfectants (Sawada et al. 2004).

Loss of sensitivity to carpropamid in *Magnaporthe grisea* was investigated. The inhibitory activity of carpropamid on scytalone dehydratase (SDH) extracted

from a carpropamid-resistant strain of *M. grisea* was dramatically reduced in comparison with that on SDH extracted from the sensitive strain. A single-point mutation (G to A) located at the upstream region (233bp) downstream from the ATG codon, resulting in a one-amino acid substitution (valine (GTG) 75 to methionine (ATG0:V75M)) was found in the resistant strain. In order to examine whether the V75M mutation was the primary reason for the decrease in sensitivity of SDH to carpropamid, the SDH cDNAs of both the sensitive and the resistant strain were cloned into a GST-fused protein expression vector system. The recombinant SDHs of both strains exhibited the same levels of sensitivity to carpropamid as those extracted from the mycelia of the respective strains. The results revealed that the V75M mutation caused the low sensitivities of the SDHs of the carpropamid-resistant strains and strongly suggested that V75M mutation conferred resistance in these strains to carpropamid (Takagaki et al. 2004).

Resistance to isolates of *Magnaporthe grisea* may be due to a single-point mutation in the scytalone dehydratase gene. This mutation results in a replacement of valine with methionine at codon 75 of the fungicide target protein. PCR-Luminex, a novel system developed for high-throughput analysis of single nucleotide polymorphisms (SNPs), was effectively employed for diagnosis of MBI-D resistance using specific oligonucleotide probes coupled with fluorescent beads. In addition, the PCR-Luminex system was tested for its potential in identifying fungi involved in wheat Fusarium head blight disease. *Fusarium graminearum* (= *F. asiaticum*), *F. culmorum*, *F. avenaceum*, and *Microdochium nivale* were subjected to PCR-Luminex analysis successfully and their involvement in FHB was established in this disease complex in Japan (Ishii et al. 2008).

A novel cyanoacrylate fungicide JS 399-19 (2-cyano-3-amino-phenylacrylic acetate) exhibited specific activity in inhibiting mycelial growth of plant pathogenic *Fusarium* spp. and was evaluated for its efficacy against *Gibberella zeae* (anamorph *Fusarium graminearum*), one of the principal pathogens inducing wheat Fusarium head blight (FHB) disease. Field isolates (43) of *G. zeae* sensitive and 45 isolates resistant to JS 399-19 were evaluated. The resistant mutants were grouped into moderate-level resistance (MR) and high-level resistance (HR) based on the EC₅₀ values. Of the 45 isolates, 17 were MR and 28 were HR. Eight isolates representing three sensitivity-level phenotypes (S, MR, and HR) were randomly selected to investigate the inheritance of resistance in *G. zeae* to JS 399-19 by analyzing the sensitivity of hybrid F1 progeny. A nitrate-nonutilizing mutant (*nit*) was used as a genetic marker to confirm that individual perithecia were the result of outcrossing. Genetic analysis of the crosses indicated that resistance to JS 399-19 was governed by a single major gene in *G. zeae*. The two levels of resistance could be conferred by mutations at two sites or one site with two different mutations in the gene. There was a consistent 1:1 ratio of segregation in the crosses between parents with different resistance levels. The segregation pattern for the two levels of resistance to JS 399-19 in *G. zeae* indicated polymorphism in a single Mendelian gene. There was no positive cross-resistance between JS 399-19 and fungicides belonging to other chemical classes such as ergosterol biosynthesis inhibitors, including tebuconazole and prochloraz, and strobilurins (azoxystrobin). Combining or alternating applications of JS 399-19 with tebuconazole, prochloraz, or azoxystrobin might therefore be an effective strategy for the management of FHB disease of wheat (Chen et al. 2009). The efficacy of JS 399-19 (cyanoacrylate) was assessed for the management of rice bakanae disease caused by *Fusarium verticillioides* (= *F. moniliforme*). The EC₅₀ values for JS 399-19 for inhibiting mycelial growth of the

wild-type *F. verticillioides* strain ranged from 0.162 to 0.559 $\mu\text{g mL}^{-1}$ and the average value was $0.316 \pm 0.072 \mu\text{g mL}^{-1}$. Germination of healthy seeds of selected rice varieties was not affected by treatment with JS 399-19, which could be used as a disinfectant of rice seeds infested by *F. verticillioides*. The results suggested that there was little shift in the sensitivity of *F. verticillioides* isolates to JS 399-19 fungicide (Chen et al. 2012a).

The mechanism of development of resistance to the fungicide JS399-19 in *Fusarium graminearum* was investigated. Fimbrin is an actin-bundling protein and its homolog Sacbp was shown to have a critical role in endocytosis and other cellular process in *Saccharomyces cerevisiae*. *F. graminearum* Y2021A strain was highly resistant to JS399-19. The function of *FgFim* in *F. graminearum* was studied by constructing a deletion mutant of *FgFim*, designated $\Delta FgFim-15$, which showed a dramatic reduction in resistance to JS399-15 in addition to decrease in mycelial growth and other cultural characteristics compared to the wild-type strain. Virulence of the *FgFim* deletion mutant on flowering wheat heads was impaired, while sensitivity to agents that damage cell walls and cell membranes was increased. Reduced virulence was consistent with reduced production of deoxynivalenol (DON) in wheat tissues. All these defects could be restored by genetic complementation of the mutant with the parental *FgFim* gene. Quantitative real-time PCR assays showed that the basal expression of three *Cyp51* genes which encoded sterol 14 α -demethylase was significantly lower in the mutant than in the parental strain. The results indicated the critical role of *FgFim* in the regulation of resistance to JS399-19 fungicide and in other cellular processes occurring in *F. graminearum* (Zheng et al. 2014).

Pepper (*Capsicum* spp.) crop grown in the commercial plastic houses in Calabria (southern Italy) was regularly applied with metalaxyl for suppressing Phytophthora blight disease caused by *Phytophthora capsici*. Single-hyphal isolates of *P. capsici* isolated from the pepper plants were tested for their sensitivity to metalaxyl using different concentrations from 0.1 to 1000 mg L^{-1} incorporated in the potato dextrose agar (PDA) medium. The ED_{50} values for inhibition of mycelial growth of *P. capsici* isolates ranged over 1.41–44.46 $\mu\text{g mL}^{-1}$. More than 80% of the *P. capsici* from plastic house crops showed a moderate level of resistance to metalaxyl (Agosteo et al. 2000).

In a later study, extensive application of metalaxyl was found to result in widespread resistance in *P. capsici* to this fungicide in China. Several fungicides were therefore evaluated for their efficacy in suppressing the development of metalaxyl-sensitive and -resistant *P. capsici* isolates by determining the EC_{50} values. The carboxylic acid amide (CAA) fungicide mandipropamid showed high inhibitory activity against *P. capsici*, leading to suppression of Phytophthora blight disease by 70%. A new fungicide mixture (625 g L^{-1} fluopicolide + 625 g L^{-1} propamocarb) was still more effective in controlling the pepper blight disease caused by metalaxyl-resistant isolates. The results indicated that no cross-resistance between metalaxyl and other tested fungicide apparently existed. These fungicides might therefore be alternatives to metalaxyl for the management of pepper Phytophthora blight disease (Qi et al. 2012).

Phytophthora capsici, causing pepper Phytophthora blight disease, has been difficult to control because the pathogen produces different kinds of propagules capable of initiating infection. The fungicide should therefore be able to inhibit sporangium formation, zoospore germination, and mycelial growth. Carboxylic acid amides (CAA) dimethomorph, flumorph, and pyrimorph were evaluated for their efficacy in reducing the Phytophthora blight disease of pepper (chilli) caused by *Phytophthora capsici*. Isolates (572) of *P. capsici* were collected during 2006–2008 from seven provinces in China. Of these, 90 isolates

without a history of exposure to CAA fungicides were used to determine the baseline sensitivity. The baseline EC_{50} values ranged over $0.122\text{--}0.203\mu\text{g mL}^{-1}$ for dimethomorph, $0.301\text{--}0.487\mu\text{g mL}^{-1}$ for flumorph, and $0.557\text{--}0.944\mu\text{g mL}^{-1}$ for pyrimorph. Four CAA-resistant mutants were generated by repeated exposure to dimethomorph in vitro. The mutants showed similar fitness in hyphal growth, sporulation in vitro, and pathogenicity in vivo. Resistant mutants induced visible lesions on pepper stems or roots treated with recommended dose of dimethomorph. Two cholinephosphotransferase (CPT) genes (*AAPT1* and *AAPT2*), the primary target of CAAs, were sequenced and analyzed. As there was no difference between the amino acid sequences of *AAPT1* and *AAPT2* genes of wild-type and mutants, it was considered that resistance to dithiomorph was not due to mutations in the amino acid sequence of these target genes (Sun et al. 2010).

The effects of three compounds – mandipropamid, dimethomorph, and cyazofamid – on different stages of *P. capsici* life cycle were determined. In addition, the sensitivity and resistance levels of *P. capsici* to these fungicides were also assessed. Forty isolates of *P. capsici* were sensitive to mandipropamid and dimethomorph based on mycelial growth, zoospore germination, and sporangial production. The EC_{50} values for arresting these processes averaged 0.03, 5.70, and $0.02\mu\text{mL}^{-1}$ respectively for mandipropamid, while the average EC_{50} values for dimethomorph were 0.24, 0.10, and $0.46\mu\text{g mL}^{-1}$, respectively. The majority of isolates of *P. capsici* were either resistant or intermediately sensitive to cyazofamid at $500\mu\text{g mL}^{-1}$ or lower concentrations, based on mycelial growth or sporangial production. The results showed that mandipropamid and dimethomorph were effective in suppressing *P. capsici* at all stages of pathogen development. Mandipropamid inhibited mycelial growth and sporangial production at lower concentrations than dimethomorph, while dimethomorph inhibited zoospore germination at lower concentrations than mandipropamid. *P. capsici* populations in Georgia were sensitive to mandipropamid and dimethomorph. On the other hand, the majority of *P. capsici* isolates showed resistance to cyazofamid, indicating the need to select appropriate fungicides for application against this disease (Jackson et al. 2012).

Development of resistance to single-site fungicides occurs more frequently in fungal pathogens. Boscalid is a broad-spectrum fungicide included in the class of succinate dehydrogenase inhibitors (SDHIs). This group, including carboxim, flutolanil, and fluopyram, interferes with the activity of succinate ubiquinone reductase (complex II) or succinate dehydrogenase (SDH) complex, which is a functional part of the tricarboxylic acid (TCA) cycle in mitochondrial electron transport chain (Keon et al. 1991). Boscalid (a pyridine-carboxamide) lost its effectiveness against *Didymella bryoniae* (anamorph *Stagonosporosis cucurbitacearum*), incitant of gummy stem blight disease of cucurbits. Boscalid inhibits fungal respiration by binding to the enzyme succinate dehydrogenase in mitochondria. Boscalid in combination with pyraclostrobin (Pristine) was used against cucurbit gummy stem blight disease, because boscalid component was effective against strobilurin-insensitive isolates of *D. bryoniae* (Seebold and Langstrom Jr 2004). However, several isolates of *D. bryoniae* became insensitive to boscalid at 10 mg L^{-1} and resistant isolates were detected in South Carolina. A total of 201 isolates collected between 1998 and 2009 were tested for sensitivity to boscalid by determining percentages of germination of ascospores and conidia on media amended with boscalid at $0.01\text{--}10.0\text{ mg L}^{-1}$. All isolates collected in 1998, 2002, or 2005 were sensitive to boscalid. Of the 170 isolates collected in or after 2006, 84.7% were insensitive to boscalid including 19 of 30 isolates recovered from greenhouse-grown seedlings. The ascospores

were less sensitive than conidia. Twelve isolates that were insensitive, moderately insensitive, or highly insensitive based on conidial germination did not differ in relative virulence on boscalid-treated muskmelon seedlings, when inoculum suspensions contained only ascospores or mixture of ascospores and conidia. Since *D. bryoniae* produces conidia and ascospores on infected plants, it is essential that both types should be used to determine the sensitivity to the fungicide(s) of pathogens such as *D. bryoniae* that produce different spore forms on their host plants (Keinath 2012).

In another investigation, of the 103 isolates of *D. bryoniae* tested, 82 and 7 were found to be very highly resistant (B^{VHR}) and highly resistant (B^{HR}) to boscalid, respectively. Cross-resistance studies with the new SDHI penthiopyrad showed that the B^{VHR} isolates were only highly resistant to penthiopyrad ($B^{VHR}-P^{HR}$), while B^{HR} isolates appeared to be sensitive to penthiopyrad ($B^{HR}-P^S$). The molecular mechanisms of fungicide resistance in these two phenotypes ($B^{VHR}-P^{HR}$ and $B^{HR}-P^S$) were studied. In addition, their sensitivity to another SDHI fungicide fluopyram was also assessed. A 456 bp cDNA amplified fragment of the succinate dehydrogenase iron sulfur gene (DbSDHB) was initially cloned and sequenced from two sensitive (B^S-P^S), two $B^{VHR}-P^{HR}$, and one $B^{HR}-P^S$ isolate of *D. bryoniae*. Comparative analysis of the DbSDHB protein revealed that a highly conserved histidine residue involved in the binding of SDHIs present in wild-type isolates was replaced by tyrosine (H277Y) or arginine (H277R) in the $B^{VHR}-P^{HR}$ and $B^{HR}-P^S$ variants, respectively. Further, the H/Y and H/R substitutions were present in the remaining $B^{VHR}-P^{HR}$ and $B^{HR}-P^S$ variants, respectively. Analysis of the sensitivity to fluopyram of representative isolates showed that both SDHB mutants were as sensitive to this fungicide as the wild-type isolates. A selection of fungicides for effective management of *D. bryoniae* has to be based on the genotype-specific cross-resistance relationships between the SDHI boscalid and penthiopyrad, and the lack of cross-resistance between these fungicides and fluopyram (Avenot et al. 2012).

Resistance to fungicides may develop in fungal pathogens due to exposure to high-risk fungicides or the introduction of resistant isolates from external source(s). In order to assess the possibility of watermelon seeds being a potential source of fungicide-resistant inoculum of the fungal pathogen *Didymella bryoniae*, causing of gummy stem blight (GB) disease of watermelon and other cucurbits, direct plating, sweat box, and blotter assays were employed. Seeds from commercial watermelon seed lots were visually examined for symptoms of GSB disease. Three isolates of *D. bryoniae* were isolated from two seed lots and were evaluated for their levels of sensitivity/insensitivity to boscalid, tebuconazole, azoxystrobin, and Thiophanate-methyl, which were commonly used for GSB disease management. The isolates were resistant to thiophanate methyl, indicating the existence of fungicide-resistant isolates of *D. bryoniae* in naturally infested commercially distributed watermelon seeds. The results highlight that a new approach for fungicide resistance management in watermelon production is needed (Thomas et al. 2014).

Boscalid was found to be very effective in suppressing the development of *Sclerotinia sclerotiorum*, causing stem rot in oilseed rape. Five boscalid-resistant (BR) mutants were generated by culturing 10 field isolates of *S. sclerotiorum* for 2 weeks on PDA amended with boscalid at $50\mu\text{g mL}^{-1}$. The BR mutant had EC_{50} values of $>9\mu\text{g mL}^{-1}$, while the EC_{50} values of their parents were $<0.3\mu\text{g mL}^{-1}$. The mutants showed cross-resistance between boscalid and carboxin, whereas negative cross-resistance was detected between boscalid and kresoxim-methyl. All laboratory-induced BR mutants formed smaller colonies on fungicide-free potato dextrose agar (PDA) culture plates

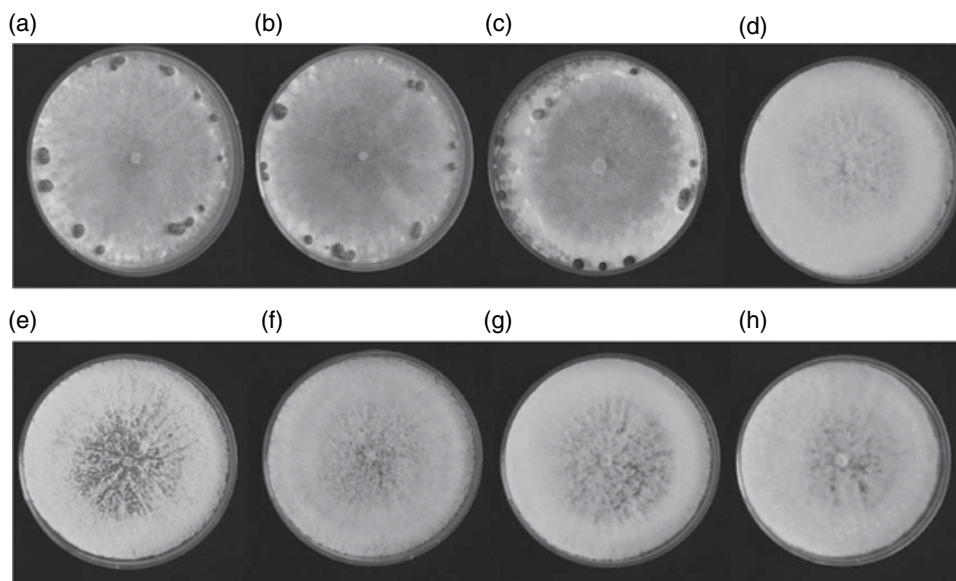


Figure 14.2 Comparative abilities of Boscalid-resistant (BR) mutants and their parental isolates in producing sclerotia on PDA medium at 40 days after inoculation. (a–c) Parental isolates; (d–h) BR mutants. Source: Wang et al. (2015). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

and less biomass in potato dextrose broth (PDB). The parental lines produced sclerotia at 40 days in PDA cultures, whereas the BR mutants failed to produce the sclerotia that may be required for their survival during adverse environmental conditions. BR mutants had a slower rate of mycelial growth and lacked the ability to produce sclerotia (Fig. 14.2). In addition, they had lower virulence and oxalic acid content, but they had higher mycelial respiration and SDH activity. DNA sequence analysis revealed that all BR mutants had the same point mutation AIIV (GCA to GTA) in the sulfur protein subunit (SDHB). The expression of the cytochrome *b* (*cyt b*) gene was reduced to varying degrees in BR mutants. The results indicated that the risk of development of resistance in *S. sclerotiorum* to boscalid was low to moderate. Further, the results suggested that boscalid should not be combined or alternated with carboxin because of the cross-resistance between these fungicides (Wang et al. 2015).

14.2 Application of Chemicals Against Bacterial Diseases

Bacterial disease management systems primarily include preventive methods such as use of disease-free seeds and propagules and eradication measures. The effects of physical and chemical treatments on the elimination of *Acidovorax avenae* subsp. *citrulli*, causal agent of watermelon bacterial fruit blotch (BFB) disease, were assessed. Healthy triploid watermelon seed lots were spiked with naturally infected watermelon and melon seeds harvested from three separate diseased fruits. Treatments with acidic electrolyzed water for 30 min eradicated the pathogen most effectively without reducing seed germination or seedling establishment. Treatment with Clorox at 50°C for 20 min, Tsunami 100 for 30 min, or acidified cupric acetate at 50°C for 20 min eradicated the

bacterial pathogen, but significantly reduced seed germination ($P=0.05$) and/or seedling establishment (emergence) (Feng et al. 2009). Common bacterial blight pathogen infecting dry bean is seedborne, which is difficult to control. The viability of the bacterial pathogen after treatment with microwave radiation on navy (cultivars Navigator and Envoy) and pinto (cultivar Ac Ole) bean was tested. The microwave treatment reduced the incidence of seed infection. Under field conditions, microwave and chemical treatments were combined. Cultivars Modern, MB, Ridgtown, and Exter seeds were exposed to microwave radiation and then treated with copper hydroxide (53.8%) and pyroclostrobin + fluxapyroxad + metalaxyl before planting. Microwave treatment reduced emergence of seedlings by up to 7%, but did not affect yield and profit margin. Chemical treatment alone or in combination with microwave treatment provided effective elimination of seed infection, leading to higher yields (Friesen et al. 2014).

Chemicals available for application to seeds and propagules are limited. Copper compounds and antibiotics have been used with limited success. Use of copper compounds may result in reduction in intensity of symptoms, such as in bacterial canker disease of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*. Seven antibacterial compounds (bronopol, copper sulfate, kasugamycin, oxolinic acid, oxytetracycline, streptomycin, and 8-hydroxyquinoline), three chemical formulates (Antibak RZ, an oligoelements mixture containing copper plus zinc; Orthopol, a potassium soap; and Param, a resistance inducer), and a combination of these compounds were tested for their efficacy in reducing the tomato bacterial canker disease. Treatments containing copper sulfate substantially reduced disease severity in plants inoculated with the pathogen, whereas streptomycin was less effective. In two experiments, the percentage of leaves showing symptoms was significantly lower (2.4% and 11.9%) after treatment with copper sulfate combined with 8-hydroxyquinoline at half-dose, than in inoculated controls (75% and 59.6%). The disease suppressive effect of combination was greater than copper sulfate alone. The results indicated that copper sulfate combined with 8-hydroxyquinoline might be effective in reducing the amount of copper applied to tomato crops (De León et al. 2008).

Copper compounds were applied for the management of cabbage black rot disease caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) in Trinidad. Native isolates (56) of *Xcc* collected from 12 farming districts and 7 reference strains were evaluated for their resistance to copper in buffered (pH 7.0) and unbuffered (pH 5.6) nutrient agar media. Most of the isolates showed resistance to copper buffered (34) and unbuffered (33) media. All reference strains were highly sensitive to copper. The mean minimum inhibition concentration for the 56 native isolates was 224.6 ppm copper, indicating high levels of copper resistance in *Xcc* isolates. There was a strong association between the length of time of continuous application of copper formulations to treat cabbage against black rot disease and the proportion of the native *Xcc* isolates with copper resistance (Lugo et al. 2013).

Copper compounds were widely applied for the management of citrus canker disease caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), resulting in the emergence of strains showing resistance to copper. Multiple and independent introductions of copper resistance genes into strains of *Xcc* and also in strains of *X. alfalfae* subsp. *citrumelonis* from Florida were investigated. The partial nucleotide sequences of principal copper resistance genes, *CopL*, *CopA*, and *CopB* from *X. citri* subsp. *citri* and *X. alfalfae* subsp. *Citrumelonis*, as well as of strains of other *Xanthomonas* spp. resistant to copper isolated

from 12 different countries and territories, were determined. The *CopLAB* gene cluster was detected in many species of *Xanthomonas* from different parts of the world. Alignment of partial nucleotide sequences of copper resistance genes among the copper-resistant (Cu^{R}) strains of *Xanthomonas* detected homology of >92, >96, and >91% for *CopL*, *CopA*, and *CopB*, respectively. It appeared that the plasmid had been horizontally transferred and various populations mutated based on selection pressure unique to geographic regions. Although high homology of the genes among the strains indicated that the copper resistance in xanthomonads had a common origin, the slight difference in the nucleotide sequences within groups of strains showed that Cu^{R} genes could be independently exchanged among species of *Xanthomonas* throughout the world (Behalu et al. 2013).

Streptomycin sulfate has been the antibiotic recommended for the management of bacterial diseases affecting many crops, including rice bacterial leaf blight (BLB) and streak (BLS) diseases caused by *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively. Inhibition of protein synthesis by the bacterial pathogens resulting in the interference with translational proofreading was the principal mechanism of action of streptomycin. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* were isolated from southern China. Single colony isolates (534 and 827, respectively) of these pathogens were tested for their sensitivity to streptomycin. Four strains of *Xoo* isolates from the same county of Yunnan Province were resistant to streptomycin and the resistant factor (the ratio of the mean median effective concentration inhibiting the growth of resistant isolates to that of sensitive isolates) was about 226. Resistance of these isolates in vivo was also observed. Less sensitive isolates of *Xoo* were also recovered in this county. On the other hand, none of the isolates of *X. oryzae* pv. *oryzicola* was found to be insensitive to streptomycin. Mutations in the *rpsL* (encoding S12 protein) and *rrs* genes (encoding 16S rRNA) and the presence of the *strA* gene accounting for resistance to streptomycin in other phytopathogens or animal and human pathogenic bacteria were examined on the sensitive and resistant strains of *X. oryzae* pv. *oryzae* by polymerase chain reaction (PCR) amplification and sequencing. As no mutation in the *rpsL* or *rrs* genes and presence of the *strA* gene could be detected, different mechanism(s) might be involved in the development of resistance to streptomycin in rice BLB pathogen (Xu et al. 2010).

In order to determine the molecular resistance mechanism of *X. oryzae* pv. *oryzicola* to streptomycin, seven streptomycin resistant mutants were obtained by UV irradiation or streptomycin selection on appropriate media. The mutants could grow at $100\mu\text{g mL}^{-1}$ of streptomycin, while the wild-type strain (RS105) could not survive at $5\mu\text{g mL}^{-1}$. Sequence analysis was applied for the detection of the *rpsL* gene encoding ribosomal protein S12, which had 125 amino acid residues. In all resistant strains, a mutation in which AAG was substituted for AGG (Lys $\rightarrow$ Arg) occurred either at codon 43 or 88. Two plasmids pUFRRS and pUFRRX were constructed by ligating the *rpsL* gene into the cosmid pUFR034. The plasmids pUFRRS and pUFRRX containing the Lys $\rightarrow$ Arg mutation of the *rpsL* gene conferred streptomycin resistance to the sensitive wild-type strain by electroporation. Both transformants RS1 and RS2 were able to develop in the medium amended with streptomycin at $50\mu\text{g mL}^{-1}$. A mutation at codon 43 or 88 in *rpsL* might result in resistance of *Xanthomonas oryzae* pv. *oryzicola* to streptomycin (Zhang et al. 2013b).

Development of resistance in the rice bacterial leaf blight (BLB) disease pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) to bismethiazol was investigated. Three

bismerthiazol-resistant mutants (1-1-1, 2-1-1, and 4-1-1) of *Xoo* were characterized in comparison to their parental strain ZJ173. After repeated subculturing of the mutants on bismerthiazol-free medium, they were pathogenic to rice plants treated with $300\mu\text{g mL}^{-1}$ of bismerthiazol, indicating the stability of the mutants. Bismerthiazol showed protective and curative activities against the wild-type strain ZJ173, but not against the three mutants. In order to determine the extent of natural occurrence of bismerthiazol-resistance in field isolates, 701 isolates of *Xoo* were obtained from 1419 leaf samples collected from eight provinces of China from 2007 to 2009. Rice plants were sprayed with bismerthiazol at $300\mu\text{g mL}^{-1}$ and inoculated with the isolates of *Xoo*. Of the 502 isolates, 62 were considered to be resistant (showing $>70\%$ inhibition relative to the wild-type strain). Frequencies of resistant strains among field populations ranged from 0 to 21%. Resistance to bismerthiazol in field isolates was not related to resistance determined in vitro. Determination of the extent of presence of bismerthiazol-resistant field populations of *X. oryzae* pv. *oryzae* would be useful for rice farmers to select the chemical(s) for application to reduce rice bacterial leaf blight disease (Zhu et al. 2013).

Emergence of strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) resistant to bismerthiazol indicated the need to find an alternative bactericide to suppress the development of rice bacterial leaf blight (BLB) disease in Anhui Province in China. Zinc thiazole was evaluated for its effectiveness against 109 strains of *Xoo* isolated from four counties in Anhui Province. The EC_{50} values of zinc thiazole for inhibition of pathogen growth were $0.53\text{--}9.62\mu\text{g mL}^{-1}$ with average values of $4.82 \pm 1.86\mu\text{g mL}^{-1}$. The minimum inhibitory concentration (MIC) for inhibition of bacterial growth ranged over $5.0\text{--}40.0\mu\text{g mL}^{-1}$ (Fig. 14.3). The protective and curative efficiency of zinc thiazole was determined under field conditions along with bismerthiazol. Zinc thiazole protected rice plants more effectively by providing over 88% control efficacy when applied at $300\mu\text{g mL}^{-1}$ at 1 and 3 days before or after inoculation, compared with bismerthiazol. Zinc thiazole at 375 g ai ha^{-1} provided over 70% control efficacy in 2012 and over 80% control efficacy in 2013 in four field trials conducted at two sites. Zinc thiazole at 250 g ai ha^{-1} was more effective in all field trials than bismerthiazol at 250 g ai ha^{-1} , revealing its potential to replace bismerthiazol against which *X. oryzae* pv. *oryzae* strains had developed resistance (Chen et al. 2015).

The systemic fungicide amicarbazol (2-amino-4-methylthiazol-5-carboxanilide) has been applied against bacterial pathogens to reduce disease incidence. Inhibition of

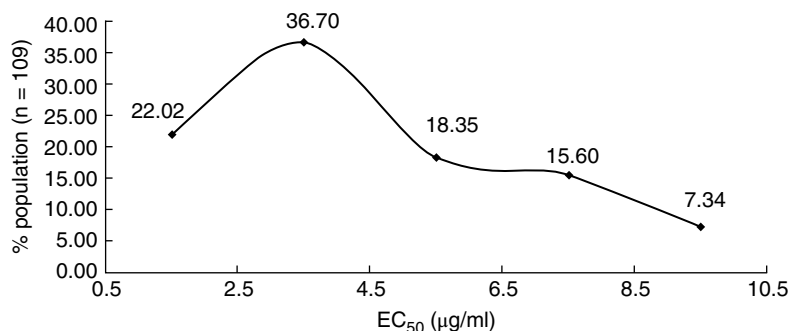


Figure 14.3 Baseline sensitivity frequency distribution of strains of *Xanthomonas oryzae* pv. *oryzae* to zinc thiazole. Source: Chen et al. (2015). Reproduced with permission of John Wiley & Sons/Blackwell Publishing Ltd, UK.

succinate dehydrogenase (SDH) activities of *Xanthomonas axonopodis* pv. *citri* by amicarbazol was observed. Three amicarbazol mutants – Xuv10, Xuv20, and Xuv40 – were produced by exposure to UV irradiation. The minimal inhibitory concentrations (MICs) of the mutants and the wild-type isolate were 400 and 100 $\mu\text{g mL}^{-1}$, respectively. Assays using triphenyltetrazolium chloride (TTC) showed that the SDH activities of these mutants were significantly lower than that of the pathogen. The complete nucleotide sequences of four subunits (SdhA, SdhB, SdhC, and SdhD) of succinate-ubiquinone oxidoreductase (SQR) were cloned by PCR amplification. An amino acid mutation (His₂₂₉ → Leu₂₂₉) in *sdhB* was found to confer resistance in *X. axonopodis* pv. *citri* to amicarbazol. The mutation might alter the SDH complex in some way that prevents binding of amicarbazol (Li et al. 2006).

In a later study, the molecular mechanism of resistance to amicarbazol in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was investigated. The activity of SDH in the wild-type *Xoo* was strongly inhibited by amicarbazol while UV-induced resistant mutants were insensitive, although their SDH activity was decreased, compared to the wild-type sensitive strain without amicarbazol. A mutation of Histidine₂₂₉ (CAC) to Tyrosine₂₂₉ (TAC) was identified in *sdhB* which encoded the iron-sulfur protein subunit of SDH. The *sdhB* from the mutant was ligated into a cosmid pUFR034 to generate pUF034RAX which conferred resistance to amicarbazol when transformed into the wild-type sensitive strain. A mutation of His₂₂₉ (CAC) to Tyr₂₂₉ (TAC) in SdhB was responsible for determining amicarbazol resistance (Zhang et al. 2010).

The formation of biofilms by *Xanthomonas axonopodis* pv. *citri* (*Xac*) was considered to be an important virulence factor playing a critical role in the early stages of infection in *Citrus* spp. The effectiveness of two organic compounds D-leucine and 3-indolylacetoneitrile (IAN) in restricting infection of citrus plants by *Xac* by inhibiting biofilm formation was assessed. D-leucine and IAN prevented biofilm formation by *Xac* on different abiotic surfaces and leaves of citrus at a concentration lower than the minimum inhibitory concentration (MIC). In addition, D-leucine and IAN increased the susceptibility of *Xac* cells to copper by a factor of c. 10 compared with untreated pathogen cells. Spray application of D-leucine (10 mM) or IAN (100 $\mu\text{g mL}^{-1}$) suppressed canker symptom development in grapefruit leaves when applied prior to or along with pathogen inoculation. The effectiveness of D-leucine and IAN was compatible to copper sulfate applied at 100 $\mu\text{g mL}^{-1}$ concentration. D-leucine and IAN appeared to act on *X. axonopodis* pv. *citri* by preventing biofilm formation, resulting in impaired infection and subsequent symptom development (Li and Wang 2014).

Citrus huanglongbing (HLB) disease caused by “*Candidatus Liberibacter asiaticus*” was reported to have affected an estimated 100 million citrus trees in 40 countries, threatening the survival of the US\$ 9 billion citrus industry (Sutton et al. 2005). As no curative treatment for eliminating the HLB pathogen was available, a new combination of antibiotics was evaluated for its efficacy using periwinkle and citrus trees. The combination of penicillin and streptomycin (PS) was effective in eliminating or suppressing “*Ca. L. asiaticus*” and provided a therapeutically effective level of control for a much longer period of time than when the antibiotics were applied separately in periwinkle cuttings. Young grapefruit seedlings (2 years old) were treated by root soaking in solutions of penicillin and streptomycin separately or in combination. HLB-affected 6 year old citrus trees were injected with the antibiotics. The antibiotic combination (PS) effectively eliminated the HLB bacterium from grapefruit seedlings and provided therapeutically effective level of control for 6 months, in contrast to treatment with penicillin (P) or streptomycin (S) alone. “*Ca. L. asiaticus*” population at 30 days after treatment

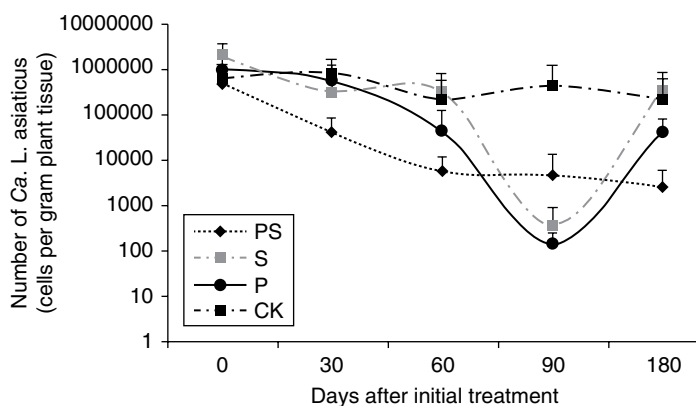


Figure 14.4 Effect of application of penicillin G (P) or streptomycin (S) individually or in combination (PS) on populations (cells g^{-1} of plant tissue) in huanglongbing (HB-) affected citrus seedlings under greenhouse conditions. Penicillin applied at 1 g L^{-1} and streptomycin at 100 mg L^{-1} ; CK: control (water). Source: Zhang et al. (2011). Reproduced with permission of the American Phytopathological Society, MN, USA.

(dat) for PS (36,733 cells g^{-1} of plant tissues) was significantly lower than the individual (P or S) treatment alone (282,188 and 500,766 cells g^{-1} of plant tissue, respectively). The pathogen population returned to relatively high level at 180 dat (Fig. 14.4). A synergistic and greater prolonged activity was observed due to the combined application of antibiotics. When the 6 year old citrus plants (mandarin x tangelo) were injected with PS, the HLB population decreased from 1,080,349 cells g^{-1} of plant tissue to 78,346 cells g^{-1} of plant tissue, indicating a 13-fold reduction in pathogen titer by PS. At 6 months after treatment, the number of bacterial cells was reduced below the level that could be detected by qPCR assay (<100 cells g^{-1} of plant tissue). The pathogen population was kept at <1000 cells g^{-1} of plant tissue until 14 months after PS treatment (Fig. 14.5). The results demonstrated the curative effects of the new combination of penicillin and streptomycin on HLB-infected citrus plants (Zhang et al. 2011).

The effect of chemical treatment via trunk injection in reducing the incidence/severity of huanglongbing (HLB) disease caused by "*Candidatus Liberibacter asiaticus*" (Las) was assessed. A mixture of streptomycin (250 mg L^{-1}), ampicillin (2.5 g L^{-1}), penicillin G (2 g L^{-1} and BacicureR (2 g L^{-1}) was injected into the trunk of Las-infected pomelo (*Citrus maxima*) trees. The bacterial pathogen DNA in treated trees was quantified by employing quantitative real-time polymerase chain reaction assay. This mixture of chemicals provided the most effective suppression of the pathogen population, as indicated by the assay. The pathogen population, as reflected by the mean threshold (Ct) values, was reduced from 20.8 ($=7.2 \times 10^7$ cells g^{-1} of plant tissues) to 36.6 ($=2140$ cells g^{-1} of plant tissues) in the greenhouse experiments and from 19.4 (c. 1.8×10^8 cells g^{-1} of plant tissues) to 37.8 (c. 969 cells g^{-1} of plant tissues) in the field trials. The effectiveness of the antibiotic treatment persisted for 1 year. Transmission electron microscope observations revealed the response of Las-infected pomelo to treatment with chemicals (Puttamuk et al. 2014).

The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is a common herbicide applied to reduce the weed populations that compete with crops for nutrients applied primarily for better growth and yield. Application of 2,4-D to foliage of potato plants

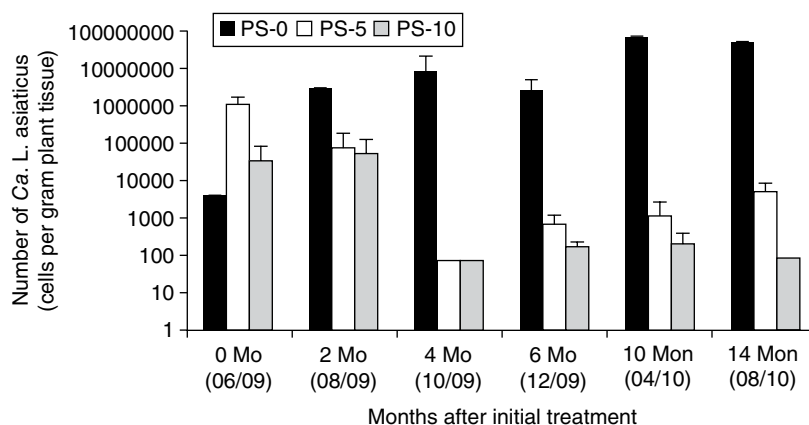


Figure 14.5 Effect of application as trunk injection of combination of penicillin and streptomycin (PS) at different ratios on bacterial pathogen titers (cells g^{-1} of plant tissues) in huanglongbing (HLB)-affected citrus plants under field conditions. PS-5: penicillin 5 g + streptomycin 0.5 g/100 mL; PS-10: penicillin 10 g + streptomycin 1 g/100 mL; PS-0: water control. Source: Zhang et al. (2011). Reproduced with permission of the American Phytopathological Society, MN, USA.

was found to reduce common scab disease caused by *Streptomyces scabies*. Phytotoxic effects of 2,4-D were observed when applied at a concentration of $200 mg L^{-1}$, and the yield of tubers was also reduced. With the minimal significant threshold rates of $8.3\text{--}23.6 mg L^{-1}$ of 2,4-D, the incidence of common scab disease was reduced in pot experiments. 2,4-D application at $10.8\text{--}41.0 mg L^{-1}$ minimized disease severity in all pot and field trials, except in one pot trial. Disease control due to 2,4-D was associated with decreased sensitivity of tubers to thaxtomin A produced by the pathogen required for disease development. The amount of residual 2,4-D left in tubers at harvest varied with cultivar, Russet Burbank accumulating more 2,4-D than Désirée. The results showed that application of 2,4-D at low rates ($<100 mg L^{-1}$) could provide a commercially viable strategy for the management of potato common scab disease (Thompson et al. 2014).

Some bacterial pathogens have multiple modes of transmission; they may be transmitted through infected propagules and also through natural biological vectors. Citrus huanglongbing (HLB) disease caused by "*Candidatus Liberibacter asiaticus*" is transmitted through infected propagules and also by psyllid vector *Diaphorina citri*, while grapevine Pierce's disease incited by *Xylella fastidiosa* is vectored by the glass-winged sharpshooter *Homalodisca vitripennis* (formerly known as *H. coagulata*; Yamamoto et al. 2006; Choi et al. 2010). A three-component disease management system was advocated to reduce the incidence of bacterial diseases and spread by the biological vectors. Thorough eradication of infected plants and plant debris, use of disease-free propagules and intensive chemical control of the pathogen were aimed to reduce the sources of inoculum and pathogen titer in the infected plants from which the inoculum may be spread by the vectors. In addition, by reducing the vector population the rate of spread of disease may be reduced. Management of Asian citrus psyllid (ACP) *D. citri* was considered a vital component of the program targeted at reducing the incidence and spread of HLB disease caused by "*Ca. L. asiaticus*." Eight different insecticides were applied sequentially on a calendar-based interval in individual plots (0.2–0.4 ha), making it a complete program. A reduced insecticide program consisting of five calendar applications of traditional insecticides or a mineral oil program (oil application

every 3 weeks plus 1 dormant insecticide spray) were also adopted. The results indicated that even the complete program was ineffective in preventing the huanglongbing disease spread (Hall et al. 2013). A similar approach for the management of grapevine Pierce's disease caused by *X. fastidiosa* was suggested. Control of the glass-winged sharp shooter on a regional basis or national level could be an important consideration for reducing the disease incidence (Gergerich et al. 2015).

14.3 Application of Chemicals Against Virus Diseases

Various kinds of chemicals have been applied for the inactivation of viruses or reduction of vector populations, and the consequent lower disease spread rate.

14.3.1 Application of Antiviral Chemicals

A variety of chemicals such as dyes, antibiotics, synthetic analogues of the purine and pyrimidine bases, and other organic compounds have been evaluated for their efficacy in suppressing the development of symptoms and/or virus replication. Although some chemicals have been reported to be effective in reducing virus replication, none has reached the stage of commercial production for wide application under field conditions. The principal problems associated with virus disease management through chemicals are: (i) the intracellular location of viruses in the host plants; and (ii) the intimate relationship between virus replication and host synthetic machinery, leading to substantial adverse effects on the host plant development. However, chemicals have been applied either to successfully eliminate the virus from meristem cultures or as a curative treatment of intact plants to produce virus-free plants. Phytotoxicity due to chemicals has been observed in several investigations.

Among the chemicals tested for antiviral activity, synthetic analogues of purine and pyrimidine bases were found to be promising. Virazole (ribavirin), a riboside of guanine, inhibited replication of *Potato virus X* (PVX) in the callus tissue and also in intact plants (Lerch 1977). Application of virazole at 2 days prior to or 8 h after inoculation with *Apple chlorotic virus* resulted in complete inhibition of virus replication (Hansen 1979). When injected into the branches of apple trees, virazole suppressed the symptoms of apple mosaic disease but the virus was not completely inactivated (Cheplick and Agrios 1983). Incorporation of virazole in the medium used for regeneration of orchids to free them of *Cymbidium mosaic virus* and *Ondotoglossum ringspot virus* by meristematic culture or thin-section culture effectively eliminated these viruses, as confirmed by enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) assays (Lim et al. 1993).

Potato virus S (PVS) could be eliminated by a combination of thermotherapy treatment of plants and apical meristem culturing in the medium amended with ribavirin. Several subcultures were needed to eliminate PVS from the potato cultivar Red Pontiac. Potato plantlets generated from the meristem cultures were free of PVS, as confirmed by ELISA test (Kim et al. 1996). Treatments with auxins and antiviral chemicals were shown to be effective for elimination of *Plum pox virus* (PPV). Infected shoots were grown in Murashige-Skoog (MS) medium supplemented with indole-butyric acid (IBA), gibberellic acid (GA), and different concentrations of ribavirin. Regenerated and rooted plants were raised under greenhouse conditions for 2 years. Freedom from PPV was ensured by indexing the plants using DAS-ELISA and IC-RT-PCR procedures.

Phytotoxicity symptoms due to ribavirin at higher concentrations (80 and 100 mg L⁻¹) were recorded (Paunović et al. 2007).

The effect of ribavirin on elimination of *Potato virus A* (PVA), *Potato leafroll virus* (PLRV), *Potato virus S* (PVS), *Potato virus X* (PVX), and *Potato virus Y* (PVY) was assessed. Potato plantlets were cultured on MS medium amended with different concentrations of ribavirin. The plant height and fresh weight were reduced with increasing concentrations of ribavirin from 0 to 150 mg L⁻¹; most of the plants could not survive when the concentration reached 200 mg L⁻¹. Ribavirin was more efficient in eliminating PVA, PVM, PVS, and PVX than PVY and PLRV from the surviving plants. Healthy plants could be obtained with high frequency (up to 100%) by culturing with 75–150 mg L⁻¹ of ribavirin after 2–3 subcultures. On the other hand, only 33–66% of PVY- and PLRV-infected plantlets were found to be virus-free after 3 subcultures with 75–150 mg L⁻¹ of ribavirin. ELISA tests indicated the freedom of plants from viruses, whereas quantitative RT-PCR assays showed that ribavirin could effectively reduce virus content in plantlets. No virus could be detected after eradication stage. No apparent genetic variation in plants following ribavirin treatment was discernible. The results demonstrated that ribavirin treatment could be used for the effective elimination of potato viruses (Yang et al. 2014).

Some systemic fungicides have been shown to reduce the symptom severity of virus diseases. Carbendazim is a fungicide that has been reported to be effective against several fungal diseases. When tobacco plants were watered with aqueous solution of carbendazim, development of mosaic symptoms induced by *Tobacco mosaic virus* (TMV) was suppressed. However, the virus titer was not reduced in the treated plants. The chlorophyll content of leaves of untreated control plants was drastically reduced, whereas chlorophyll content of the carbendazim-treated leaves was not reduced (Tomlinson et al. 1976). A similar ameliorative effect on intensity of symptoms was observed in rice tungro disease (Thomas and John 1980) and peanut green mosaic disease (Gopal and Srinivasulu 1988). Commercial formulations of strobilurins – azoxystrobin, kresoxim-methyl, trifloxystrobin, and pyraclostrobin – were evaluated for their efficacy in reducing *Bean common mosaic virus* (BCMV) under greenhouse and field conditions. Highest seed germination percentage and seedling vigor was recorded in seeds treated with pyraclostrobin (2 g L⁻¹). In the greenhouse trials, 76% protection against BCMV was observed. When pyraclostrobin was applied as seed treatment at 10 g L⁻¹ under field conditions with natural BCMV inoculum, the fungicide treatment resulted in 65% protection against BCMV infection. ELISA assays were used to determine the infection in bean plants and the immunoreactive values were recorded in common bean seedlings raised from seeds treated with pyraclostrobin and kresoxim-methyl. Strobilurins exerted a direct positive physiological effect on common bean plants and also protected bean plants against BCMV infection in greenhouse and field. The fungicides with reduced risk may be useful as potential inducers of resistance against BCMV and growth enhancers, and they could be a beneficial component of integrated disease management system for common bean (Udayashanker et al. 2012).

14.3.2 Chemicals Applied Against Vectors of Viruses

Managing virus diseases through the application of chemicals to reduce the populations of vectors has frequently been reported to be ineffective. Achieving zero population of vectors is not always possible. Further, no insecticide can exert an instant knockdown

effect on aphids and other vectors of nonpersistent viruses which may be acquired and transmitted with short acquisition/transmission feeding periods on virus-infected plants and healthy plants, respectively. A small population of the vectors left after chemical application may be sufficient to spread the virus disease and cause significant crop losses (Simmons and Zitter 1980). On the other hand, the spread of persistent viruses which require longer feeding periods by the vectors for both acquisition and transmission can be significantly reduced by the application of insecticides. For example, the spread of *Potato leafroll virus* (PLRV, persistent virus) could be markedly reduced by application of insecticides, whereas spread of *Potato virus Y* (PVY, nonpersistent virus) could not be reduced by chemical application, despite the fact that the viruses are transmitted by the same aphid species *Myzus persicae* (Webley and Stone 1972; Olubayo et al. 2010; Hansen and Nielsen 2012). Systemic and persistent chemicals have been found to be more effective than contact insecticides, which are easily washed away by rains. Protection of plants by chemicals in the nursery or in the early stages of crop growth may be more important, since young plants are more susceptible to viruses and older plants are likely to develop adult-plant resistance to virus infection (Narayanasamy 2002).

Transmission of plant viruses by their vectors may be prevented or reduced by the application of mineral oils. Aphids (*Myzus persicae*) carrying the nonpersistent (styletborne) *Potato virus Y* (PVY) was unable to transmit the virus when it was fed through a film of oil on the membrane surface. As the infective aphids probed through the film of oil, infectivity was substantially reduced (Bradley et al. 1966). The application of mineral oils has become common practice to provide protection to potato crops against virus diseases, especially to PVY (Olubayo et al. 2010; Fageria et al. 2014). The efficacy of two mineral oils (Superior 70 and Vazyl-Y) in reducing the spread of PVY was assessed by using three concentrations of mineral oils under three spray regimes. The presence of PVY in tubers was determined by employing ELISA tests. In the plots sprayed with Superior 70, PVY spread in treated plots ranged over 2.1–12.2%, while in control plots it ranged over 20.4–37% across three cultivars. In the case of Vazyl-Y, PVY spread in treated plots ranged over 2.1–26.5%, while in the control plots the infection ranged over 49.9–85.7% across three cultivars. The results showed that the PVY incidence was significantly reduced due to application of mineral oils (Fageria et al. 2014). Greenhouse and field trials were conducted to shed light on the mechanism of action of mineral oils bringing about reduction in the incidence of disease caused by *Potato virus Y*. The local and systemic accumulation of PVY^O in susceptible cultivars Shepody and Russet Burbank was investigated. The results suggested that the mineral oils did not influence PVY^O levels in mechanically inoculated leaves or tubers of plants with a secondary infection. However, a reduction in systemic PVY^O titer was recorded in mineral-oil-treated older plants but not in younger plants, suggesting that the mineral oil might inhibit PVY^O movement in an age-dependent manner (Al-Daoud et al. 2014).

The effects of application of mineral oil Sunspray 850 EC alone or in combination with commonly applied aphidicides on the incidence of potato viruses *Potato virus Y* and *Potato virus M* (PVM) were assessed under field conditions. The aphidicides, Mospilan 20SP (acetamprid), Primor 500 (primicarb), and Karate Zeon 050CS (lambda-cyhalothrin) were used for control of aphid population. Mineral oil application was the most effective in reducing the incidence of PVY and offered better protection to potato plants than the aphidicides, although Mospilan 20SP reduced the aphid populations

most effectively. Mineral oils, when used alone or in a mixture with Primor 500WG, were the most effective in limiting PVY infection by 64% relative to the control. Reduction in PVM incidence by mineral oil application was not significant because of low disease pressure. Application of insecticide and mineral oil mixtures was not always as effective as the mineral oil alone. Further, the use of mixtures might not be acceptable to growers since due to the extra cost (Wróbel 2014).

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15

Crop Disease Management

Integration of Strategies

Depending on the nature of microbial pathogens and existing environmental conditions favoring disease development and spread, crop diseases may cause qualitative and quantitative losses of varying magnitudes in cultivars with differing levels of susceptibility/resistance to the disease(s) concerned. In order to limit yield losses, compatible disease management strategies have been integrated to achieve yield levels approaching the biological potentials of the crop cultivars if other crop requirements are satisfied. Integrated disease management systems, involving the use of disease-free seeds and propagules and other strategies in different combinations, have been developed to provide effective protection against various seed- and propagule-borne pathogens.

15.1 Development of Integrated Disease Management Systems

The effectiveness of different strategies is known to vary, so the search for new disease management strategies has been pursued over several years. Prior to the nineteenth century, cultural methods were the mainstay for the control of crop diseases. From the latter half of the twentieth century, chemicals with antimicrobial properties became increasingly available in large numbers and were used either in conjunction with or instead of cultural practices. With the advent of the Mendelian era, the development of cultivars with built-in resistance through breeding provided a strong foundation for effective crop protection against some crop diseases. The limitations of genetic improvement of resistance of cultivars were realized because of the nonavailability of dependable sources of resistance and the rapid development of new race strains/races of pathogens capable of adapting to the resistance genes incorporated into the cultivars. The possibility of employing biological control agents (BCAs) to achieve acceptable levels of disease control have therefore been explored. Commercial formulations containing fungal and bacterial BCAs have been developed for efficiently tackling some crop diseases. The usefulness of biotechnological methods has been demonstrated in improving the levels of resistance of cultivars by incorporating genes from diverse sources which could not be utilized by conventional breeding methods (Narayanasamy 2002, 2011).

Research and extension efforts of plant pathologists provided direction to the integration of different compatible strategies or tactics to improve the effectiveness of management of crop diseases. The term “integrated pest management” (IPM) was first used by the entomologists R.E. Smith and R. van den Bosch (Smith and van den Bosch 1967) based on the concept of Stern et al. (1959). IPM was defined as “applied pest control which combines and integrates biological and chemical control used as necessary and in a manner which is least disruptive to biological control.” On the other hand, from the very inception of plant pathology crop disease management has relied upon integrated disease management (IDM) using basic information on the loss potential, pathogen biology, ecology, and epidemiology. This is clearly demonstrated by searching the USDA-CRIS project database for IDM-related projects from 1985 to 1995; 1755 projects in plant pathology and 1426 in plant entomology were listed in this database (Jacobsen 1997).

The concept of economic injury level (EIL) and economic threshold (ET) introduced by Stern et al. (1959) provided workable bases for insect (pest) control since the populations of insects could be easily estimated. On the other hand, plant pathologists have to assess the populations of microbial pathogens with great difficulty and the assessments do not always result in predictable differential damage. However, in some pathosystems damage may exceed the ET even before symptoms become visible. The ET levels prescribed for insects as pests cannot be used for insects functioning as vectors of viruses. Populations remaining below ET levels are sufficient to spread virus diseases, and it is not economical to maintain zero populations of vectors by application of insecticides to restrict virus disease spread. It has therefore not been possible for plant pathologists to adopt the EIL and ET concepts of IPM meant for insect management. Furthermore, crop disease management generally did not depend at any time on levels of chemical uses that were commonly associated with insect control. Many destructive diseases were managed successfully by integrating various strategies such as sanitation, certification, crop rotation (sequence), selection of suitable sowing/planting date, and use of resistant cultivars if available. The use of fungicides or other chemicals was kept at minimal levels. Although these strategies were commonly employed for insect pest control, greater emphasis was given to chemical control. The term integrated disease management (IDM) has been increasingly used in plant pathology investigations, while the term “pest” is largely confined to insect control. With basic knowledge of pathogen biology and ecology, sources of inoculum, and epidemiological factors favoring disease development, different strategies have to be integrated to effectively reduce the incidence and spread of crop diseases to derive maximum economic benefit to the growers.

15.1.1 Selection of Strategies for Integration

The effects of individual strategies on pathogen development and disease spread were discussed in Chapter 10 (exclusion of pathogens), Chapter 11 (reduction of pathogen inoculum), Chapter 12 (enhancement of genetic resistance of crops), Chapter 13 (biological management of crop diseases), and Chapter 14 (chemical control of crop diseases). These strategies/tactics may be grouped into four categories based on their effects on the pathogen and the development of diseases which may vary due to the prevailing environmental conditions in different agroecosystems.

- i) Programs aimed at preventing the introduction of pathogens into a country, geographic location, or field have to be carried out at different levels by various agencies such as certification agencies, plant quarantines, national or provincial governments, plant protection advisers, extension pathologists, and growers.
- ii) Close coordination, constant surveillance, and efficient technical support for rapid detection and identification of new pathogens and feedback on the effectiveness of corrective measures taken to restrict the spread of the pathogens to neighboring states/fields may ensure the effectiveness of this approach, which is vital for all kinds of diseases caused by microbial plant pathogens.
- iii) The reduction of inoculum buildup may be achieved by choosing suitable geographic locations for planting sites where sources of inoculum are absent or at minimal levels.
- iv) In addition, suitable planting dates, use of disease-free seeds/propagules, treatment of seeds/propagules, modification of cultural practices such as crop rotation, application of organic amendments and mulches, soil solarization, eradication of alternate and alternative host plant species, proper disposal of infected plants and plant debris, and tillage practice may reduce buildup of pathogen inoculum, which has a direct influence on the disease incidence and spread in a newly planted crop. Growers must adopt selection practices that may avoid or reduce inoculum buildup and consequently restrict the use of chemicals.

The rate of spread of crop diseases has to be reduced by adopting effective tactics. Use of resistant cultivars is the most important strategy to reduce the rate of disease spread. Disease development is retarded depending on the levels and durability of resistance possessed by a cultivar to be grown. Cultivars with horizontal resistance may be preferred to cultivars with vertical resistance. Use of cultivar mixtures and multilines has been shown to be effective in reducing disease incidence and/or severity in certain pathosystems. Although several biological control agents (BCAs) have been shown to be effective in some locations, only a few have been shown to have the potential for widespread application under field conditions. Cost-effectiveness and comparative performance, along with commonly used standard fungicides, have to be demonstrated convincingly. Many fungicides have the potential to restrict the spread of crop diseases. However, continued and indiscriminate use of fungicides has resulted in the development of insensitivity/resistance in pathogens against frequently applied single-site systemic fungicides. The cost of using alternative disease management strategies is reported to be far less than the cost of chemical control (Pimentel et al. 1991). Consequently, the approaches to chemical application emphasize not only the efficiency of fungicides and reducing risks of development of resistance, but also their specificity and biodegradability (Maloney 1995). This trend is due to environmental concerns about chemicals in general. Nonchemical disease management methods involving traditional farming systems, use of biological control agents, and induction of resistance in crop plants which are otherwise susceptible have been accepted by all concerned with crop production.

Integration of disease management strategies may result in an increase or decrease in the effectiveness of disease suppression. It is therefore vital to select the strategies that are compatible with each other and do not have any negative influence on other strategies. Efforts to assess the effectiveness of integrated disease management system(s)

have been far less than desirable. The possibility of integrating different management strategies for effective reduction in the incidence and spread of some of the economically important diseases of various crops is discussed in the remainder of this chapter.

15.2 Management of Fungal Diseases

15.2.1 *Fusarium* Head Blight Disease

Fusarium head blight (FHB) disease is caused by different species of *Fusarium* forming a complex, the components of which may vary at different locations. *F. graminearum* (teleomorph *Gibberella zeae*), *F. avenaceum*, *F. culmorum*, *F. poae*, and *Microdochium nivale* are commonly associated with the FHB disease. Crop residues left on the soil surface after harvest forms the principal source of inoculum and infection of spikes takes place at the time of heading. Crop rotation, including soybean prior to wheat crop, resulted in lower incidence of FHB disease. The incidence and severity of FHB were lower in moldboard-ploughed plots than in either chisel-ploughed or no-till plots and the yield also increased (Dil-Macky and Jones 2000). The favorable effect of moldboard ploughing was also observed in a later investigation for reducing the incidence of FHB disease incited by *Fusarium* spp. (Steinkellner and Langer 2004). Application of nitrogenous fertilizer (type and quantity of N applied) had a significant influence on the incidence of FHB and mycotoxins produced by the pathogen. A significant increase in FHB severity and deoxynivalenol (DON) accumulation in wheat grains was observed with an increase in N dosage from 0 to 80 kg ha⁻¹. At higher input levels of N, FHB and DON concentrations did not show appreciable variations (Lemmens et al. 2004). Variable effects of N application on the ear rot disease incidence in maize were recorded. Effects of urea and a slow-release fertilizer did not show significant variations in the contamination levels of grains for zearalenone, fumonisin B1, or ochratoxin A. Ear rot disease incidence and severity were generally higher in ears from plants fertilized with insufficient N, but application of high N fertilizer doses (>300 kg ha⁻¹) significantly increased zearalenone accumulation in maize kernels. In contrast, concentrations of fumonisins were related to nitrogen deficiencies (Blandino et al. 2008). The effect of heat treatment on elimination of *Fusarium graminearum* from wheat and barley seeds, with 84% and 23% infection respectively, was assessed. The pathogen was eliminated from Canadian Western spring wheat seeds treated at 60°C for 15 days, at 70°C for 5 days, or at 80°C for 2 days. In a similar manner, barley seeds were disinfected by heat treatment at 60°C for 21 days, at 70°C for 9 days, or at 80°C for 5 days. Seed germination was not adversely affected by heat treatments (Clear et al. 2003).

The effectiveness of fungal biocontrol agents in reducing *Fusarium* head blight (FHB) disease was demonstrated by some investigations under field conditions. The yeast species, *Cryptococcus* sp. OH71.4 and *C. nodaensis* OH182.9, reduced the FHB disease severity by 57%, but the yeast antagonists did not have any significant effect on the extent of mycotoxin concentration of grains (Schisler et al. 2002). *Fusarium equiseti* strain G, selected as the most effective biocontrol agent, was tested for its ability to reduce FHB disease incidence and the production of mycotoxin deoxynivalenol by *F. culmorum* and *F. graminearum* in wheat grains under field conditions. The BCA strain could reduce the percentage diseased grains by 92% and DON contents by 94%, indicating potential for reducing the disease incidence and mycotoxin production by the pathogen in grains (Dawson et al. 2004). In another investigation performed under

field conditions, *Clonostachys rosea* (IK726) significantly reduced FHB severity. Dried and stored conidia of *C. rosea* suppressed disease development as effectively as freshly harvested conidia of the BCA (Jensen et al. 2000). A mixture of *C. rosea* strain 47 and *Trichoderma atroviride* strain 312 with carboxin, thiram, and triticonazole applied to wheat seeds significantly suppressed the development of *F. culmorum*. The effectiveness of BCA application in reducing FHB incidence was comparable to that of triticonazole under field conditions (Roberti et al. 2006). The plant growth-promoting rhizobacteria (PGPR) pseudomonads showed significant disease suppressive potential against *F. culmorum* under both greenhouse and field conditions. The disease severity and loss in 1000-grain weight were significantly reduced when *Pseudomonas fluorescens* strains MKB158 and MKB249 and *P. frederiksbergensis* strain 202 were applied. In the *F. culmorum* infested fields, treatment with strain MK158 or MKB 249 reduced FHB disease severity in wheat and barley. Further, levels of deoxynivalenol (DON) were also reduced in wheat and barley grains by 74–78%. Protective application of *P. fluorescens* has the potential for large-scale application to reduce FHB incidence and DON accumulation in grains (Khan and Doohan 2009).

The efficacy and stability of integrating fungicide with cultivar resistance to reduce the incidence of Fusarium head blight (FHB) disease of wheat and accumulation of deoxynivalenol (DON) in grains was determined. Data from over 40 trials in 12 US states were analyzed using means of FHB index (index) and DON from up to six resistance-class-fungicide management combinations per trial as parameters. Multivariate meta-analysis and mean log-response ratios across trials were estimated and transformed to estimate mean percent control (C) due to management combinations relative to the susceptible-untreated (S-UT) treatment. All combinations significantly reduced index and DON ($P < 0.001$). Moderate resistance of the cultivar with fungicide treatment (prothioconazole + tebuconazole) was the most effective combination with C of 79% for index and 71% DON reduction relative to S-UT. The combination of fungicide application and resistance to disease was additive in terms of percent control for index and DON contents of grain. Moderate resistance of wheat cultivar treated with fungicide had the mean rank among treatment combinations relative to susceptible without fungicide protection. The results showed that although the magnitude of percent control may vary among environments, integrating a single tebuconazole + prothioconazole application at anthesis with cultivar resistance could be a more effective and stable management practice for both disease index and DON content reduction than either strategy applied alone (Willyerd et al. 2012).

15.2.2 Rice Blast Disease

Rice blast disease pathogen *Magnaporthe grisea* (anamorph *Pyricularia grisea*) occurs as numerous races with varying levels of virulence (aggressiveness) differentiated based on their interactions with differential varieties/genotypes of rice. Cultural practices such as changing planting dates (when disease incidence is less), the application of optimal levels of nitrogenous fertilizer, and maintaining optimum levels of irrigation have been shown to reduce disease incidence and severity. Use of resistant/tolerant cultivars may be a single strategy that may be more effective in reducing crop losses than others. Application of silicon (Si) as sodium silicate in Si-deficient soils reduced the blast disease severity and increased grain yield by about 20–35% compared to nonamended soils. Fungicide edifenphos in combination with Si application increased yield levels. When blast disease pressure was high, Si application followed by treatment with

tricyclazole resulted in reduced loss due to blast disease (Seebold et al. 2004). *Pseudomonas fluorescens* strain Pf1 applied as seed treatment, followed by three foliar applications, significantly reduced the blast disease incidence and severity (Vidhyasekaran et al. 1997) and also increased grain yield (Karpagavalli et al. 2001). The effectiveness of plant activators that stimulate defense responses of plants to develop local/systemic resistance to several diseases has been demonstrated. Exogenous application of salicylic acid (SA) might enhance concentrations of defense-related compounds in treated rice plants, resulting in inhibition of rice disease development (Daw et al. 2008). Likewise, treatment with benzothiadiazole (BTH) enhanced the resistance of cultivar Nakdong (susceptible to blast disease). The PR-genes involved in the development of resistance to *M. grisea* were activated by application of BTH (Ahn et al. 2005). It may be desirable to select the suitable strategies and integrate them to achieve the required level of disease suppression and enhancement of yield, keeping the application of chemicals at a minimum.

15.2.3 Rice Sheath Blight Disease

Rice sheath blight disease caused by *Rhizoctonia solani* is widespread in all rice-growing countries. As the pathogen is soilborne and has a wide host range, adoption of crop rotation including noncereal crops has limited use. Other cultural practices such as proper spacing and application of optimal doses of nitrogen may not favor the incidence of sheath blight disease. Silicon fertilization in silicon-deficient soils (as calcium silicate) was reported to reduce the severity of the disease (Rodriguez et al. 2001). Extensive screening of rice genotypes for identifying resistance sources has not yielded encouraging results. Suppression of sheath blight disease using biotic and abiotic biocontrol agents (BCAs) appears to be a feasible approach. Talc-based formulations containing *Pseudomonas fluorescens* strains PF1 and PF7 either individually or in combination reduced the sheath blight disease incidence to different levels, depending on the strain or strain mixture. Treatment with PF strains, in addition to suppression of disease symptoms, also increased the yield of grains by up to 40.4% (Nandakumar et al. 2001). Furthermore, talc-based formulations of *P. fluorescens* strains PF1 and PF7 in combination with chitin amendment were more efficient in reducing sheath blight disease incidence up to 62% and enhancing the grain yield up to 21% (Radjacommare et al. 2002).

Mixtures of fungal BCAs *Trichoderma harzianum*, *T. viride*, and *Gliocladium virens* provided effective protection against infection by *Rhizoctonia solani* (Tang et al. 2002). The effectiveness of disease suppression offered by the BCAs *P. fluorescens* or *T. viride* was comparable to the level achieved by application of the fungicide carbendazim. The additive effect of combining both BCAs was only marginal on disease reduction and yield increase (Mathivanan et al. 2005). *Bacillus megaterium*-based water-soluble granular formulation applied as seed treatment followed by three sprays at seedling, tillering, and flowering stages reduced the development of rice sheath blight and blast diseases. With proper weed control and an optimal dose of nitrogenous fertilizer, yield of grains was increased compared to untreated control treatment (Kanjamaneesathian et al. 2009). In another investigation, the effectiveness of application of *Bacillus subtilis* strain NJ-18 alone or in combination with a fungicide half-dose of kresoxim-methyl at 225 g ai ha⁻¹ to reduce the incidence of rice sheath blight disease was assessed. The sheath blight disease was more effectively suppressed by the combination of the

bacterial BCA and the fungicide than kresoxim-methyl (50%) alone or the antibiotic Jingtangmycin at 125 g ai ha⁻¹. Treatment with *B. subtilis* alone was also effective in suppressing the development of *R. solani* (Yang et al. 2009). Resistance-inducer BTH, when applied as soil-drench or foliar spray, reduced sheath blight disease development on inoculated leaf sheath and upwards spread to younger leaves (Rohilla et al. 2002). Various fungicides have been tested for their efficacy, but the economic feasibility of applying chemicals seemed to be limited. The results of various experiments indicate that if the effectiveness of the biocontrol agents could be established under different ecological environments, use of BCAs might be the best option for integration with cultural practices.

15.2.4 Late Blight Diseases of Tomato and Potato

Phytophthora infestans, incitant of late blight diseases of tomato and potato, is intensively investigated because of the extensive damage caused by this fungal pathogen in all countries. The pathogen produces different kinds of spore forms and exists in the form of several races with varying pathogenic potential (virulence). The pathogen can infect all plant parts and also tubers in potato, causing tuber blight disease. It has been difficult to suppress disease development in greenhouse tomato crops because of the limited possibility of manipulating the environmental conditions of prolonged periods of leaf wetness and temperature favoring development of the disease. Polyethylene mulch was shown to provide consistent, effective, and highly significant suppression of late blight disease development in tomato, the disease control efficacy being 83.6 ± 5.6%. The combination of polyethylene mulch and fungicide Kocide 2000 plus Neemguard showed an additive effect, the effectiveness being greater than that provided by polyethylene mulch alone. The suppressive effect of polyethylene mulch was due to many factors such as reduction in leaf wetness duration and reduction in sporulation by the pathogen. Additional benefit of using polyethylene mulch was reduction in weed infestation and the consequent saving of irrigation water by up to 30% (Shtienberg et al. 2010).

The effectiveness of organic compounds in suppressing development of late blight disease in potato has been demonstrated. Allicin, present in garlic extract, suppressed seedborne infection of *Phytophthora infestans* in tomato seeds and tuber blight disease development in potato (Slusarenko et al. 2008). Bacterial biocontrol agents *Bacillus pumilus* strain SE34 and *Pseudomonas fluorescens* strain 89B61 protected tomato plants by inducing systemic resistance, the level of which was similar to what could be achieved by application of resistance inducer β-aminobutyric acid (BABA) (Yan et al. 2002). BABA induced different levels of systemic resistance in potato tubers against tuber blight, when potato plants were treated with BABA. In the resistant cultivar Ovatio, the activation of systemic resistance was faster and stronger than in susceptible cultivar Bintje (Liljertoth et al. 2010; Bengtsson et al. 2014).

Among different types of plastic mulches, black polyethylene film and copper hydroxide-treated agricultural textile used to cover the hills reduced the incidence of potato tuber blight compared to controls. The effectiveness of black polyethylene mulch was considered to be due to blocking of water movement through potato hill and the alteration of soil environment within the hills by making hills drier and slightly warmer. However, preventing/reducing foliar epidemics through host resistance strategies have to be applied wherever it is possible (Glass et al. 2001). Potato late blight disease management system possibly represents the culmination of various disease

management strategies to achieve the level of effectiveness that is yet to be fully achieved. Different disease management strategies have been applied alone or in combination to restrict the disease incidence and spread through forecasting models, genetic resistance, cultural practices, biological control agents, and chemical application. Varying degrees of success have been obtained under different environmental conditions by integrating the approaches suitable for the geographical locations concerned. Use of disease-free certified seed tubers is the basic requirement to effectively eliminate the initial sources of inoculum of *Phytophthora infestans*. Production of disease-free tubers depends on the prevention of tuber infection in the previous crop to a great extent. Crop sanitation, selection of appropriate planting date, monitoring of disease incidence, and immediate elimination of all infected plants, including volunteer plants, were effective in reducing the late blight disease incidence (Basu 2009). Strip-cropping of potatoes with cereals or a grass-clover mix was effective in reducing disease incidence (Bouws and Finckh 2008).

Several fungal and bacterial biocontrol agents (BCAs) have been evaluated for their efficacy in suppressing the development of *Phytophthora infestans* under in vitro or greenhouse conditions. The fungal BCAs *Penicillium aurantiogriseum* and *Stachybotrys atra* suppressed the lesion development in greenhouse-grown potato plants to the extent of 93% and 84%, respectively (Jindal et al. 1988). *Pseudomonas putida* and *Serratia plymuthica* suppressed the development of *P. infestans* US-8 clonal lineage, a highly virulent strain, through induction of resistance and antibiosis, respectively, indicating the different mechanism of action by the bacterial BCAs (Daayf et al. 2003). Tuber infection by *P. infestans* could be reduced significantly by treating the potato tubers with strain mixtures of *Pseudomonas fluorescens* (Slininger et al. 2007). Treatment of potato tubers with *Trichoderma harzianum* (5 g kg^{-1}) and *P. fluorescens* (2.5 g kg^{-1}) protected the plants effectively and increased the yield from 13.8 t ha^{-1} in control plots to 19.5 and 19.0 t ha^{-1} , respectively, in treated plots (Basu 2009). A formulated product of the fungal BCA, *Chaetomium globosum*, was evaluated for its efficacy in suppressing the development of late blight on potato plants and tuber blight in tubers. The BCA was applied as tuber, soil, and foliar treatment at 1 mL kg^{-1} of tubers, 1 mL kg^{-1} farmyard manure (FYM), and 0.7%, respectively, under field conditions. Incidence of disease was reduced by 28%. Application of *C. globosum* increased the tuber yield in two field experiments, indicating the effectiveness of the commercial product of *C. globosum* (Shanthiya et al. 2013).

Management of potato late blight continues to be largely dependent on the use of different fungicides in many countries, although emergence of isolates insensitive/resistant to commonly applied fungicides and the possible environmental pollution due to excessive application of fungicides are known. Application of copper-based fungicides even in organic production system has increased, despite the existing ceiling of 6 kg of elemental copper per hectare (Ghorbani et al. 2004). Several organic compounds that can induce resistance in potato plants as alternatives to these fungicides were therefore evaluated for their efficacy. The protective effect of β -aminobutyric acid (BABA) against potato late blight pathogen *Phytophthora infestans* was demonstrated in two cultivars Bintje and Pampeana with different levels of horizontal resistance. Pretreatment of potato plants with BABA at 30 days after emergence provided about 60% protection in cultivar Pampeana against *P. infestans*. Foliar application of BABA stimulated the expression of molecules associated with induction of resistance to diseases (Altamiranda et al. 2008). The degree of enhancement of resistance by BABA depended on the natural level of genetic resistance of potato cultivars to different races of *P. infestans* (Olivieri et al. 2009). BABA treatment could compensate for a 20–25% reduction in the dosage of

fungicide Shirlan needed as the combination of the fungicide and BABA was as effective as the full dosage of Shirlan alone, indicating the possibility of using BABA as a component of potato late blight disease management system (Liljeroth et al. 2010).

Phosphites (Phi), alkali metal salts of phosphorus acid, showed potential for protecting potatoes against fungal diseases. Treatment of seed tubers with calcium or potassium phosphite (CaPhi or KPhi) at 1% commercial product facilitated earlier plant emergence. When sprayed on foliage of two cultivars Shepody and Kennebec, CaPhi or KPhi provided significant protection (2–4 times) to the plants against late blight disease. The cultivar Kennebec was more responsive to Phi application, which was more effective against *Phytophthora infestans* than against *Fusarium solani* (causing dry rot) and *Rhizoctonia solani* (inducing black scurf). Phi application did not induce any apparent adverse effects on growth of potato plants, indicating that Phi had the potential to protect potato plants against *P. infestans* and other fungal pathogens of potato. In addition, leaves in Phi-treated plants had a darker green color and showed delay in setting of leaf senescence compared to untreated control plants (Lobato et al. 2008).

Different levels of multisite- (contact) and single-site (systemic) fungicides have been applied as protective/curative treatments on seed tubers, soil, and potato plants against infection by late blight disease caused by *Phytophthora infestans*. Seed tuber treatment with thiophanate-methyl + mancozeb increased plant emergence and uniform stand of potato crop. Dimethomorph + mancozeb, cymoxanil + mancozeb, or propamocarb + chlorothalonil were effective as tuber treatment and also for foliage application at later stages of crop growth (Singh and Pundhir 2004). Cyazofamid with translaminar and curative activity was specific against *P. infestans* and reduced the formation of zoosporangia. The fungicide did not show cross-resistance with other commonly used fungicides under field conditions, providing high levels of disease suppression (Mitani et al. 2002). Several fungicides were found to be effective against potato late blight disease in the initial years of their introduction. However, *P. infestans* isolates resistant to the fungicides have been recovered after some years. For example, phenylamide fungicide mefenoxam reduced the disease development for over three decades. However, isolates of *P. infestans* insensitive to mefenoxam were collected in Wisconsin from 2009 to 2012. The isolates of US-23 and US-24 clonal lineages belonging to A1 mating type were found to be intermediately sensitive (reduced) to mefenoxam. The sensitivity of the isolates was determined using the analysis of the glucose-6-phosphate isomerase (Gpi) allozyme for predicting the mefenoxam sensitivity of isolates of *P. infestans*. The presence of new clonal lineages of *P. infestans* in Wisconsin emphasized the need for constant monitoring of the development of insensitive strains of the pathogen (Seidl and Gevens 2013). Insensitivity to mefenoxam was governed by the allele RPA190 in the isolates of *P. infestans*. Transfer of this allele RPA190 to a sensitive isolate resulted in generation of transgenic lines insensitive to mefenoxam. Sequence variation in RPA190 was shown to contribute to insensitivity to mefenoxam in *P. infestans* (Randall et al. 2014).

15.3 Management of Bacterial Diseases

15.3.1 Rice Bacterial Leaf Blight Disease

Xanthomonas oryzae pv. *oryzae* (Xoo), incitant of rice bacterial leaf blight (BLB) disease, has the potential for causing considerable losses when environmental conditions are favorable in SE Asian countries. The bacterial biocontrol agent *Lysobacter antibioticus*

strain 13-1 isolated from rice rhizosphere reduced rice BLB disease index by 78.3% and 59.1% in 2005 and 2006, respectively, compared to the control. The bacterial antagonist was more effective than the antibiotic zhongshengmycin with disease suppression efficiencies of 69.4% and 47.7% in 2005 and 2006, respectively. The strain 13-1 was able to colonize rice leaves and stems at 3 weeks after application and reduce the disease development in most rice cultivars, irrespective of their susceptibility levels (Ji et al. 2008). Harpins produced by phyto bacterial pathogens possess the capacity for multiple functions, including induction of resistance to bacterial diseases. The effectiveness of two fragments of harpin (from *X. oryzae* pv. *oryzicola*) HpaG₁₀₋₄₂ and HpaG_{xoo} was assessed for suppression of BLB disease development under field conditions. The activity of HpaG₁₀₋₄₂ was greater than that of HpaG_{xoo} and stimulated resistance to three important rice diseases: rice blast, sheath blight, and bacterial leaf blight disease. The grain yields were increased in 672 experimental plots with nine rice cultivars planted at three locations. HpaG₁₀₋₄₂ applied at 6 µg mL⁻¹ once at nursery seedling stage and three times in the main field after transplanting was the most effective. Incidences of BLB, blast, and sheath blight were reduced by 61.6, 93.6, and 93.3% and 56.4, 76.0, and 55.05% in indica and japonica cultivars, respectively, relative to the untreated controls. Further, grain yields were increased by 22–27% compared to control plots. Application of harpin HpaG₁₀₋₄₂ was as effective as the local management practices, including the use of chemicals. The results revealed the suitability of HpaG₁₀₋₄₂ protein fragment for the efficient management of three major rice diseases and enhancement of the yield levels of rice crops (Chen et al. 2008).

Streptomycin sulfate has been the most frequently used antibiotic against bacterial diseases of crops, but strains showing resistance to streptomycin sulfate have been recovered from different locations. *Xanthomonas oryzae* pv. *oryzae* (Xoo), causal agent of rice bacterial leaf blight (BLB), and *X. oryzae* pv. *oryzicola*, incitant of rice bacterial leaf streak (BLS) disease, were isolated in Yunnan Province, China where streptomycin sulfate had been applied. Four strains of Xoo were found to be resistant to streptomycin. The resistant factor (the ratio of the mean median effective concentration inhibiting the growth of resistant isolates to that of sensitive isolates) was about 226. Less sensitive isolates of Xoo were also present in this region. In contrast, none of the isolates of *X. oryzae* pv. *oryzicola* was found to be insensitive to streptomycin under field conditions. The PCR amplification and sequencing of *rpsL* gene (encoding S12 protein) and *rrs* genes (encoding 6S rRNA) showed a lack of any mutation in these genes considered to be responsible for resistance to streptomycin, indicating the involvement of a different mechanism for development of resistance to streptomycin in *X. oryzae* pv. *oryzae* (Xu et al. 2010). In all strains of *X. oryzae* pv. *oryzicola* showing resistance to streptomycin sulfate a mutation on the *rpsL* gene encoding ribosomal protein S12 had occurred. The amino acid sequence analysis showed that in the mutant strains, AAG was substituted for AGG (Lys → Arg) either at codon 43 or 88, resulting in the development of resistance to streptomycin sulfate in *X. oryzae* pv. *oryzicola* (Zhang et al. 2013). Bismethiazol suppressed the development of rice bacterial leaf blight (BLB) disease caused by *X. oryzae* pv. *oryzae* (Xoo). Bismethiazol showed protective/curative activity against the wild-type strain ZJ173, but not against the mutants showing resistance. Frequencies of resistant strains arising among field isolates ranged over 0–21.1%. Resistance to bismethiazol in field isolates was not related to response induced in vitro using bismethiazol-amended medium (Zhu et al. 2013).

15.3.2 Tomato Bacterial Canker Disease

Various disease management strategies have been evaluated for limiting the incidence of tomato bacterial canker disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). No commercial tomato cultivar with substantial levels of resistance, to ensure the zero-tolerance level emphasized in commercial seed trade to prevent introduction of *Cmm* through seeds (Sen et al. 2015), is available. Soil solarization using transparent polyethylene sheets for covering soil in commercial tomato production areas was applied for 6 weeks in plastic houses. Bacterial canker disease severity was drastically reduced by soil solarization/mulching throughout the tomato-growing season. Soil solarization with polyethylene sheets did not affect the population of *Pseudomonas*, *Bacillus*, and the actinomycete *Streptomyces* antagonistic to *Cmm*, and they could survive in the tomato rhizosphere even in the increased temperature conditions prevailing during soil solarization. The fluorescent pseudomonads isolated from the rhizosphere soil were able to induce resistance to *Cmm* in plants growing from seeds treated with pseudomonads (Antoniou et al. 1995). The biocontrol agents *Bacillus subtilis*, *Trichoderma harzianum*, and *Rhodosporidium diobovatum* were found to be effective in suppressing the development of tomato bacterial canker disease to varying degrees (Utkhede and Koch 2004).

The effectiveness of plant activators capable of inducing resistance to crop diseases have been assessed for their efficacy in reducing the incidence and severity of tomato bacterial canker disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). Pretreatment with acibenzolar-*S*-methyl (ASM) reduced the severity of canker disease. The development of resistance to canker disease depended on the provision of an interval of 1–7 days between challenge inoculation with the bacterial pathogen and application of ASM. An interval of 3 days between ASM application and pathogen inoculation resulted in the highest level of protection (Baysal et al. 2003; Soylyu et al. 2003). Another disease-resistance inducer DL- β -aminobutyric acid (BABA) was able to induce resistance in tomato against *Cmm*. Foliar sprays of BABA (500 g L⁻¹) reduced canker disease development by up to 54% at 14 days after inoculation. Bacterial populations were reduced by 84% in planta by treatment with BABA, which enhanced the activities of defense-related enzymes in tomato plants (Baysal et al. 2005).

Managing tomato bacterial canker disease using copper compounds was carried out with some success. Antibacterial compounds such as bronopol, copper sulfate, kasugamycin, and 8-hydroxyquinoline, three commercial formulates (Antibak RZ, an oligoelements containing copper plus zinc; orthopol, a potassium soap; and Param, a resistance inducer), and combinations of these compounds were evaluated for their efficacy in suppressing bacterial canker disease development. Treatments containing copper sulfate significantly reduced disease severity in inoculated plants. Streptomycin was less effective. Copper sulfate combined with 8-hydroxyquinoline (at half dose) was more effective than copper sulfate alone in reducing bacterial canker disease in greenhouse-grown tomato plants. This combination of treatments appeared to be ecofriendly since the amount of copper applied could be reduced (de León et al. 2008).

15.3.3 Potato Scab Diseases

Streptomyces scabies and *S. turgidiscabies* cause potato scab and common scab diseases, respectively. These bacterial pathogens are soilborne and can survive in the soil for long periods. Cultivation and incorporation of specific green manure crops have been

suggested as a disease management strategy because of their ability to improve the soil conditions, favoring the buildup of microbial populations antagonistic to these bacterial pathogens. The effects of buckwheat and canola as green manures and rotation crops on potato scab disease (*S. scabies*) were investigated in 2 year field trials. Potatoes grown in soil planted with corn or alfalfa in the previous year had lower incidence of scab and higher yield levels than potatoes grown in soil previously planted with potato. Streptomycetes from soil collected from green manure (GM-) treated plots tended to have greater pathogen inhibitory potential (determined in vitro) than those from fallow-treated plots (without GM). The antagonist populations also showed increases in the GM-treated plots. GM crops appeared to selectively enrich the abundance or activity of antibiotic producers within the soil microbial community (Wiggins and Kinkel 2005). Incorporation of green manures had a favorable effect on potato common scab disease caused by *Streptomyces turgidiscabies*. The number of diseased tubers harvested from soil incorporated with lopsided oat or wooly pod vetch was significantly reduced, compared with tubers obtained from soil planted to oat or continuous potato cultivation in a planter experiment. Under field experiments conducted during 1999–2000, treatment with lopsided oat followed by lopsided oat or wooly pod vetch was significantly more effective in suppressing the disease severity than oat and continuous potato cultivation ($P < 0.001$). A significantly greater increase in marketable tuber ratio was also observed in plots treated with lopsided oat compared with plots with oat or potato as previous crop. The results suggested that lopsided oat might be grown as a green manure crop and incorporated prior to cultivation of potato to reduce disease severity and enhance marketable potato tubers (Sakuma et al. 2011).

Among organic compounds of animal origin, chitosan has been demonstrated to be effective in suppressing the development of many crop diseases. The effectiveness of chitosan on the development of *Streptomyces scabies* applied either alone or in combination with *Streptomyces melanosporofaciens* strain EF76, capable of producing the antibiotic geldanamycin, was assessed. Combined application of chitosan and the strain EF76 was the most effective in reducing the scab disease incidence under greenhouse conditions. Under field conditions, the number of geldanamycin-resistant actinomycetes (GRA) on harvested potato tubers was significantly increased in treatments containing chitosan than in other treatments. The results suggested chitosan might promote the establishment of antagonistic actinomycetes on progeny tubers and induce resistance in tubers against infection by *S. scabies*. On the other hand, the strain EF76 might suppress pathogen development through production of the antibiotic geldanamycin (Prévost et al. 2006).

15.3.4 Citrus Canker Disease

Xanthomonas axonopodis (campestris) pv. *citri* causes cankers in all plant organs, including fruits, reducing the market value of fruits of *Citrus* spp. Enforcement of quarantine regulations prevented the introduction and establishment of the canker disease to varying levels in different countries. In the USA, State and Federal regulatory personnel were involved in the eradication of 20 million citrus trees. Infected trees and all plant materials within 125 feet were burnt as per quarantine regulations. The clothes of worker, tools, machinery, and vehicles were thoroughly disinfected (Schumann 1991). Planting disease-free seedlings and plant sanitation are very important to reduce inoculum buildup, in addition to the management of citrus leaf minor, the feeding

injuries of which make way for the easy entry of the citrus canker pathogen. Various kinds of organic compounds have been evaluated for their efficacy in suppressing the development of canker disease.

The protective potential of imidacloprid, a neonicotinoid insecticide which breaks down in the plants into 6-chloronicotinic acid, a compound related to the SAR inducer isonicotinic acid (INA), was assessed. Soil application of imidacloprid effectively suppressed the development of citrus canker. Swingle citrumelo seedlings (*Citrus paradisi* x *Poncirus trifoliata*) were treated with imidacloprid, isonicotinic acid (INA), and acibenzolar-*S*-methyl (ASM), proven SAR inducers, as soil drenches or with ASM as foliar spray at 1 week prior to challenge inoculation of immature leaves with *X. axonopodis* pv. *citri*. Seedlings were reinoculated with the pathogen four times over a period of 24 weeks. Soil application of the SAR inducers (plant activators) reduced disease severity (number and size of lesions) by 70% relative to the untreated control plants (Francis et al. 2009). In a later investigation, the efficacy of imidacloprid, thiamethoxam, and ASM for the suppression of canker disease development on Ray Ruby grapefruit trees was assessed. Foliar infection of *Xac* was significantly suppressed by all treatments on combined spring–summer fall flushes. The recommended practice of applying copper hydroxide and streptomycin (11 sprays at a 3 week interval) reduced the canker disease incidence on shoot flushes produced throughout the season compared with untreated control. The soil-applied SAR inducers also reduced foliar infection depending on the rate, frequency, and timing of application. During a tropical storm, SAR inducers were generally not effective. The protective efficiency of SAR inducers differed depending on the concentration and timing of their application (Graham and Myers 2011).

Copper compounds have been applied widely for the management of citrus canker disease for a long time, resulting in the development of *Xanthomonas axonopodis* pv. *citri* (*Xac*) strains resistant to these chemicals. Multiple and independent introduction of copper resistance genes into sensitive strains of *Xac* and also into strains of *X. alfalfae* subsp. *citrumelonis* from Florida were investigated. The principal copper resistance genes *copL*, *copA*, and *copB* were identified in both pathogens. The *copLAB* gene cluster was present in many species of *Xanthomonas* from different parts of the world. It appeared that the plasmid had been horizontally transferred and various populations had mutated, based on selection pressure unique to the geographic locations concerned. Although high homology of the genes among strains indicated that the copper resistance in *Xanthomonas* had a common origin, the slight differences in the nucleotide sequences within groups showed that Cu^R gene could be independently exchanged among species of *Xanthomonas* throughout the world (Behalu et al. 2013). The need to restrict the use of copper-based chemicals is underscored and adoption of other options for reducing the citrus canker disease incidence may be the feasible approach.

15.4 Management of Virus Diseases

Virus diseases affecting various crops are managed primarily by applying strategies aimed at reduction/prevention of virus infection and spread. Use of virus-free seeds and propagules is the basic requirement to prevent the plants growing from infected seeds/propagules, serving as the potential sources of inoculum for the secondary spread of the disease through the natural biological vectors. Eradication of all sources

of inoculum present within the field/farm as infected volunteer plants or weed species has to be followed strictly. If ignored, these strategies targeting the virus inoculum may result in considerable yield losses in quantity and quality. Protecting the crops against virus infection by using mild strains against severe strain has been successful more frequently in the case of viruses transmitted through propagules than viruses carried by true seeds.

15.4.1 Management of Seedborne Virus Diseases

Soybean mosaic virus (SbMV) is seedborne and is also transmitted by aphids under natural conditions. SbMV occurs in the form of different strains. The effectiveness of using attenuated strains of SbMV to protect soybean plants against severe strains of the virus was assessed. An attenuated strain of SbMV was generated by exposing the infected plants to 15°C for 14 days or 30 days (Kosaka and Fukunishi 1993). The attenuated SbMV isolate Aa15-M2 generated by exposure to 15°C for 14 days was used to cross-protect the susceptible soybean plants. Black soybean seedlings (20,000 in 1990 and 78,000 in 1991) were planted 1–3 days after immunization with the attenuated strain in seed farms in Kyoto Prefecture, Japan. Infection by the severe strain of SbMV was effectively suppressed in the immunized plants. The seeds obtained from the cross-protected plants were virtually free of the virus. As the next step, seedlings growing from the seeds of protected plants were transplanted in the farmers' fields. The incidence of virulent strains of SbMV up to pre-flowering stage (about 40 days after transplanting) was 1.3% in the first year and 0.8% in the second year. The effectiveness of preimmunization of soybean plants by an attenuated strain of SbMV was demonstrated by Kosaka and Fukunishi (1994).

Attenuated strains of *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), and *Watermelon mosaic virus* (WMV-2) were produced by exposing the plants infected by the respective viruses to low temperatures. The attenuated strains effectively protected the plants against respective severe strains and no difference in the effectiveness of the attenuated strains in protecting plants was observed. The degree of cross-protection was correlated to the concentration of the protecting strain in the plants. The WMV-mild strain provided a higher level of cross-protection to plants against severe strains than CMV-mild strain and ZYMV-mild strain (Kosaka and Fukunishi 1997). An attenuated strain CM95 of CMV efficiently protected cucumber plants under field conditions. The effectiveness of cross-protection by attenuated strain was assessed by symptom expression assay and RT-PCR-RFLP procedure after challenge inoculation with severe strain. The RT-PCR-RFLP method indicated the level of protection much earlier than the bioassay, which required several days to provide results (Nakazono-Nagaoka et al. 2005). The attenuated strain of *Zucchini yellow mosaic virus*-2002 (ZYMV) obtained after repeated low temperature treatments at 12.5–15°C was purified by five cycles of single-plant transfer. The attenuated isolate ZYMV-2002 protected cucumber plants efficiently and the protected plants yielded as high as healthy plants. They showed very mild or no symptoms following inoculation with ZYMV-2002 under field conditions. The results revealed the potential of the attenuated strain in protecting cucumber plants under field conditions (Kosaka et al. 2006).

Another strategy that has been shown to be effective in restricting the incidence of virus diseases in cucurbits is the use of reflective, metalized plastic mulch with a thin coat of aluminum (Summers et al. 2004). The effectiveness of disease suppression by

integrating the UV-reflective plastic mulch and commercially available resistance-enhancing compound BioYield was assessed. The incidence of *Watermelon mosaic virus* (WMV) infecting summer squash (*Cucurbita pepo* var. *meloepo*) was significantly reduced in plants grown on silver-on-black (UV-reflective mulch) compared to the plants grown on black mulch (control) in the trial conducted during spring. The virus concentration (as determined by ELISA test) was significantly reduced in plants from treated plots compared to controls. The fall trial including highly UV-reflective silver mulch showed that the highly UV-reflective silver mulch treatment was more effective in reducing incidence of WMV compared to silver-on-black and black mulch treatments. However, the yield of squash was greater in plants covered with silver-on-black mulch treatment than from the other two treatments. The application of BioYield was ineffective in either reducing the disease incidence or increasing the yield of squash plants (Murphy et al. 2008).

Some fungicides such as carbendazim have been reported to alleviate the symptom severity in virus-infected plants. The extent of discoloration induced by *Tobacco mosaic virus* (TMV) was reduced by carbendazim by preventing the destruction of chlorophyll in infected plants (Tomlinson et al. 1976). Similar ameliorative effect of fungicides on bean plants infected by *Bean common mosaic virus* (BCMV) was observed. Commercial formulations of strobilurins, namely azoxystrobin, kresoxim-methyl, trifloxystrobin, and pyraclostrobin, were evaluated for their efficacy under screenhouse and field conditions. Seed treatment with pyraclostrobin (20 g L^{-1}) resulted in the highest seed germination percentage and seedling vigor and provided 76% and 65% protection against BCMV under screenhouse and field conditions, respectively. Infection by BCMV in plants growing from seeds treated with pyraclostrobin or kresoxim-methyl was indexed using ELISA tests which showed the protective ability of these fungicides. Strobilurin exerted a direct positive physiological effect on common bean plants. These fungicides with reduced risk could be useful as potential inducers of resistance against BCMV and growth promoters (Udayashankar et al. 2012).

15.4.2 Management of Propagule-Borne Virus Diseases

Viruses transmitted via propagules have the potential to ruin a production system almost entirely, as in the case of *Banana bunchy top virus* (BBTV) and *Citrus tristeza virus* (CTV), if adequate attention is not bestowed upon the selection of healthy propagules. Introduction of the viruses via propagules has to be efficiently prevented by implementing certification and quarantine programs. Several rapid and sensitive diagnostic techniques for detection and identification of virus(es) present in the planting materials are available (Chapter 7). The production of virus-free planting materials through tissue culture techniques and establishment of virus-free nuclear stocks are essential so that growers can begin with clean planting materials to ensure healthy crops and high levels of yield. Virus disease management strategies have the aim of preventing the incidence of diseases, rather than curing infected plants. Restricting the spread of the virus diseases by reducing the populations of biological vectors has limited application, since a small population of vectors left after chemical application may be able to spread the disease to a significant number of healthy plants. All sources of virus infection should be eradicated thoroughly to reduce incidence of new infections. Spread of *Citrus tristeza virus* (CTV) was effectively contained by planting virus-free citrus on citrus-tolerant or -resistant rootstocks, along with eradication of CTV-infected trees by the Central California

Tristeza Eradication Agency (CCTEA) (Yokomi and De Borde 2005). Monocropping generally increases the risk of providing conditions for the virus disease to become endemic. Plant diversification through mixed cropping including cover crops, border plants, intercrops, and barrier (trap) crops along with the primary crop has therefore been found to be useful to reduce the incidence and subsequent spread of virus diseases. The incidence of *Potato virus Y* (PVY) in potato could be reduced by raising sorghum, soybean, or wheat as border crops, which might reduce the movement of aphid vectors (Di Fonzo et al. 1996).

Immunization of crop plants with mild strains against infection by severe strains of the same virus or related viruses has been demonstrated to have practical utility to reduce the adverse effects of severe strains on plant growth and yield. Mild strains are selected from naturally occurring strains or are generated artificially by exposure to chemicals, UV-irradiation, or unusual temperatures (high or low). Cross-protection (immunization) as a virus disease management strategy has been most successfully employed for the protection of citrus plants against *Citrus tristeza virus* (CTV). In Brazil, 5,000,000 preimmunized plants were planted over a period of 10 years with no evidence of breakdown of the level of protection offered by the mild strain (Costa and Miller 1980). This approach for management of CTV was adopted in several other countries such as Australia, Japan, South Africa, and India. Constant monitoring of the strain composition in a geographical location has to be carried out to sustain the effectiveness of this strategy (Garnsey et al. 2005; Sieburth et al. 2005).

The usefulness of cross-protection as a disease management strategy applicable for *Citrus tristeza virus* (CTV) depends on the early detection and accurate identification of CTV isolates. The CTV isolates are usually dispersed to new areas by propagation of infected buds, and the virus is largely spread by different species of aphids in a semipersistent manner. The lack of knowledge of actual genetic determinants of CTV pathogenicity, the presence of mixed populations in field trees, and the lower levels of sensitivity of detection techniques pose considerable problems to be solved (Sieburth et al. 2005). The real-time RT-PCR procedure could be employed for precise identification of 56 biologically characterized CTV isolates from 20 different countries. All mild strains of CTV were identified accurately and differentially from the severe strains causing seedling yellows (SY) and stem-pitting (SP) symptoms. This protocol showed the potential for use by quarantine and certification personnel to avoid introduction or nursery propagation of potentially dangerous isolates in areas where only mild isolates might be prevalent (Ruiz-Ruiz et al. 2009).

Chinese yam necrotic mosaic virus (CYNMV) causes considerable yield losses in Chinese yam (*Dioscorea opposita*) crops. An attenuated strain KM3 of CYNMV was selected because it produced only mild mosaic symptoms on leaves of Chinese yam cultivar Nagaimo. Aphids were used to inoculate the attenuated strain KM3 to protect healthy yam plants against severe strains YS117 and IW5. The strain KM3 provided effective protection against severe strains of CYNMV under field conditions (Kondo et al. 2007). Another attenuated strain T-3 of *Japanese yam mosaic virus* (JYMV) was able to protect the Chinese yam against severe strain of JYMV. The native strains were detected in only 1 of 50 plants grown from tubers preimmunized with T-3 strain during a period of 6 years. The preimmunized plants yielded significantly more marketable tubers than the unprotected plants. The attenuated strain T-3 showed potential for effective protection of yam plants against *Japanese yam mosaic virus* (Kajihara et al. 2008).

Severe strains of *Sweet potato feathery mottle virus* (SPFMV) cause russet crack and discoloration of storage root skin of sweet potato (*Ipomoea batatas*), making them unacceptable to consumers. A mild strain of SPFMV 10-0 rarely induced skin discoloration and russet crack symptom, and this strain was used to protect the sweet potato plants against severe symptoms. The mild strain did not reduce the yield. The strain 10-0 was rarely present in sweet potato plants under natural conditions, so the mild strain was maintained in *Ipomoea nil* by graft inoculation. This mild strain 10-0 showed potential for protecting the sweet potato plants against severe strains of SPFMV (Yamasaki et al. 2009). Nitrous acid treatment procedure was applied for generating attenuated strains (mutants) of *Potato virus Y* (PVY). Among the 15 attenuated mutants of PVY generated, two mutants M-MY10 and N-NA10 were effective in providing protection against the wild-type strain NTND6 which induced necrotic symptoms in potato tubers (Ogawa et al. 2013).

Management of virus diseases in perennial crops requires constant monitoring using sensitive diagnostic assays, eradication of all infected plants and sources of inoculum, replanting with certified virus-free plants/planting materials, and applying chemicals to reduce vector populations to restrict secondary spread of the viruses. A comprehensive integrated management system was applied for the management of *Grapevine leafroll-associated virus 3* (GLRaV-3) in South Africa. The management strategies included planting certified material tested free of detectable viruses, application of herbicide and subsequent removal of infected plants and debris, adoption of fallow periods during which time all volunteer host plants were eradicated, application of systemic and contact insecticides to reduce populations of viruliferous mealy bugs, and consequent reduction in disease spread. Incidence of GLRaV-3 was reduced from 100% infections on 111,431 vines planted in 41.26 ha during 1989–1992 to 0.027% infection (58 of 209,626 vines) in 2012. The reduction in infection by GLRaV-3 could be achieved by replacing fully infected vineyards and rouging out 3105 young infected vines in new vineyards. In the majority of vineyards, growers did not adopt the GLRaV-3 control strategies earlier; the disease was therefore widespread in these areas (Pietersen et al. 2013).

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