P. Narayanasamy

Molecular Biology in Plant Pathogenesis and Disease Management:

Microbial Plant Pathogens

Volume 1

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Contents

Table of Contents for Volumes 2 and 3

Volume 2

Volume 3

Preface

Molecular biology has emerged as one of the most important branches of biological sciences, helping them in achieving rapid and significant advancements. This branch has been able to provide the essential information on the basic cell behavior patterns such as growth, division, specialization, movement and interaction in terms of various molecules responsible for them. In the early stages of molecular biological research, simple organisms such as viruses and bacteria formed the preferred test materials. The realization that the function of whole organism can be fully understood, only when it is dissected and analyzed at molecular levels became widespread. Hence, the structure and function of nucleic acids (DNA and RNA) and proteins were extensively examined. It has been well demonstrated that individual molecules function generally as components of complex gene expression mechanisms in metabolic pathways or as structural elements invariably in concert with other molecules. Although the concept of the gene as a unit of hereditary information was introduced by Gregor Mendel, the term "gene" was coined later by Wilhelm Johansen to describe a heritable factor responsible for the transmission and expression of a particular trait. The one gene-one enzyme model paved the way for the finding that the gene is a length of DNA in the chromosome and it encodes the information required to produce a single enzyme. These early discoveries laid the foundation for the initiation of scientific inquiries to understand the structure and functions of genes in higher organisms including plants and mammals.

Interactions between biotic and abiotic factors and crop plants determine their health and consequent reproductive capacity. Plants, as they develop from seeds or propagative materials, encounter various kinds of microorganisms capable of favoring or adversely affecting their development. Microbial plant pathogens have evolved, over a period of time, strategies to overcome the defense responses of and to breach successfully different barriers formed by plant hosts, leading to initiation of infection and subsequent colonization of tissues of a compatible plant species. However, the plants also step up their defense-related activities, as soon as the presence of the pathogen is perceived, at different stages of plant-pathogen interaction by activating defense-related genes, leading to the formation of structural barriers and biosynthesis of antimicrobial compounds. Plant defense responses involve complex biochemical pathways and functions of multiple signal molecules.

Studies on molecular biology of plant pathogens, infection process and disease resistance have provided information essentially required to understand the vulnerable stages at which the microbial pathogens can be effectively tackled and to workout novel strategies to incorporate disease resistance genes from diverse sources and/or to enhance the levels of resistance of cultivars with desirable agronomic attributes. New molecules, without any direct inhibitory effect on the pathogen, but capable of eliciting plant defense responses have been developed or discovered. Transgenic plants with engineered genes encoding viral coat proteins, HR-elicitors, such as harpins or overexpressing *R* genes or PR proteins, such as chitinases with enhanced levels of resistance to pathogens show promise for effective exploitation of this approach. It is possible to complement or replace the chemicals- fungicides and bactericides- by adapting new disease-management technologies emerging from the basic knowledge of plant-pathogen interactions at molecular and cellular levels. Constant, cooperative and comprehensive research efforts undertaken to sequence the whole genomes of plants and pathogens can be expected to result in the development of better ways to manipulate resistance mechanisms enabling the grower to achieve higher production levels and the consumer to enjoy safer food and agricultural products.

This book presents updated and comprehensive information in an easily understandable style, on the molecular biology of plant-pathogen interactions in three volumes: 1. Microbial plant pathogens, 2. Molecular biology of plant disease development and 3. Molecular biology in crop disease management. The usefulness and effectiveness of molecular techniques to establish the identity of pathogens precisely, to have a better understanding of the intricacies of the success or failure of pathogen infection respectively in compatible and incompatible plant species and to develop more effective crop disease management systems is highlighted with suitable examples. Appendices containing protocols included in appropriate chapters will be useful for students and researchers of various departments offering courses and pursuing research programs in molecular biology in general and plant pathology in particular.

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Chapter 1
Introduction

Agricultural and horticultural crop production is hampered by several limiting factors, of which diseases caused by microbial pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses and viroids – still remain a formidable one. Losses due to diseases are estimated to be about 30% in developing countries, whereas European and North and Central American countries may lose about 15–25% of the produce (James 1981; Pimentel 1997). A later assessment by Pimentel et al. (2000) indicated that losses caused by natural introduction of invasive plant pathogens and insect pests accounted for about $10 billions in the USA alone annually. The imperative need to save the crops from the attack of potential pathogens was realized and research efforts were intensified in the later half of the twentieth century. Various aspects of the pathogens and the diseases induced by them were investigated. The conventional methods involving cultural and microscopic methods provided the basic knowledge on plant-pathogen interactions leading to development of disease symptoms on compatible host plant species or cultivar. In contrast, the pathogen development was partially or entirely inhibited depending on the levels of resistance in incompatible interaction. Furthermore, epidemiological factors favoring disease incidence and spread under experimental and natural conditions were determined to develop disease prediction models and forewarning systems. Short-term and long-term strategies were planned based on the results of conventional techniques. However, the inherent limitations of the conventional methods necessitated to look for techniques with higher levels of precision and reliability (Narayanasamy 2002, 2006).

1.1 Molecular Biology as a Research Tool

Studies relating to molecular biology and biotechnology have provided an impetus to the rapid development of different branches of biological sciences, resulting in accumulation of information which could not have been gathered using conventional procedures. The importance of discovery that *Tobacco mosaic virus* (TMV) causing tobacco mosaic disease could be crystallized, was duly recognized by the award of Nobel Prize to Stanley (1935) and this work signaled the commencement

P. Narayanasamy, *Molecular Biology in Plant Pathogenesis and Disease Management: Microbial Plant Pathogens, Vol. 1*.

of application of molecular methods in Plant Pathology. Equally important was the finding of Bawden et al. (1936) that TMV was a nucleoprotein, opening up the field of Plant Virology and of research on virus diseases of humans and animals for molecular analysis. The presentation of crucial evidence for gene-for-gene interaction in flax-rust pathosystem by Flor (1942, 1946) established the concept that the genes conditioning the reaction of the host may be identified by their interaction with specific strains of the pathogen. On the other hand, those genes that condition pathogenicity may be identified by their interaction with specific host varieties. The concept of Flor, demonstrated to be true in several pathosystems, was hailed as one of the most important contributions during the last century, providing a firm footing for studying the host plant-pathogen interactions more critically.

Development of techniques for isolation, cloning and sequencing the deoxyribose nucleic acid (DNA) of various organisms including plants and pathogens marked the period of significant revolution in biological sciences. Braun and Pringle (1959) demonstrated that the crown gall bacterium *Agrobacterium tumefaciens* induced permanent transformation of plant host cells resulting in the autonomous and rapid growth of the transformed cells in culture. The *A. tumefaciens*-mediated transformation system is still widely adopted for transforming plants with genes from diverse sources. *Arabidopsis thaliana*, a small dicotyledonous cruciferous plant species has been adopted as a model species for most aspects of plant biology, because of its small genome size, short life cycle, small stature and ability to produce large number of progenies. These attributes have made this plant species ideally suited for genetic and mutational analyses. *Arabidopsis* has been shown to have a relatively high proportion of genes involved in metabolism and defense. Many of the physiological processes investigated in *Arabidopsis* and crop plants appear to share many common features, especially related to disease and pest resistance and salt tolerance. As the genome of *A. thaliana* has been fully sequenced, it provides the ideal tool to have a more clear insight into the molecular bases of various physiological processes, including resistance to diseases caused by microbial pathogens. However, the conclusions arrived at based on experiments performed on *A. thaliana* need to be translated into practical outcomes in various crops. Application of genomics to molecular resistance breeding is considered as the most important area of promise, leading to development of crop cultivars with built-in resistance to one or more diseases (Slater et al. 2003; Lucas 2004).

Some of these early breakthrough-findings obtained by applying the molecular techniques revealed the effectiveness and potential of these approaches. Evidences in support of numerous suggestions and hypotheses proposed earlier have been obtained by using molecular methods. These methods have been shown to be very useful to investigate various aspects of plant-pathogen interaction resulting in either disease progress or restriction due to effective elicitation of host defense responses nullifying the adverse effects of the pathogen-derived products. Interaction between avirulent strains of pathogens and resistant hosts generally results in hypersensitive response (HR). Bacterial pathogens and their host plant species have been shown to be preferable systems for molecular approaches. An avirulence gene *avrA* from a race 6 strain of *Pseudomonas syringae* pv. *glycinea* was cloned. This avirulent

gene, when transferred to other races, conferred the ability to elicit a resistance response on soybean cultivars with *Rpg 2* gene for resistance (Alfano and Collmer 1996). By cloning of virulence and *hrp* genes from bacterial pathogens, significant progress was made in understanding various phenomena relating to pathogenesis such as virulence, plant recognition and host range in many pathosystems. The discovery of genes involved in the production of host-specific toxins (HSTs) by fungal pathogens elucidated the molecular bases of symptom induction and resistance to the pathogens producing the HSTs (Durbin 1981; Baker et al. 2006). Furthermore, the effectiveness and reliability of the molecular techniques in hastening the application of different disease management strategies particularly for the development of cultivars with built-in resistance to diseases caused by microbial pathogens has been well recognized as a significant advantage over conventional methods.

1.2 Application of Molecular Techniques

The characteristics of microbial pathogens have been studied to identify and to classify them into different classes, families, genera and species based on morphological variations and this approach was primarily applied in the case of oomycetes and true fungi. Biochemical studies are also required for classifying bacterial pathogens, since the variations in the morphological features alone are not enough to differentiate genera and species. As the viral pathogens are extremely small in size, the virus particle morphology offers no dependable basis for differentiation, necessitating the use of molecular biological approaches for their detection and differentiation. Even in the case of fungal pathogens differentiation based on morphological characteristics may not be possible, if their development is affected by environmental conditions and the presence of other fast-growing saprophytic microorganisms. The effectiveness and applicability of molecular techniques in studying the pathogen characteristics, disease development and formulation of suitable crop disease management systems are discussed in three volumes that include ten chapters, in addition to this introductory chapter.

In Volume 1, the information on the molecular techniques to study the characteristics of microbial pathogens is presented. Rapid detection, precise identification and unambiguous differentiation of various microbial pathogens or variants of a pathogen species are of paramount importance to initiate effective strategies of crop disease management. The effectiveness of molecular techniques to meet this requirement is discussed in Chapter 2. The genetic diversity of plant pathogens has to be assessed to understand the different levels of pathogenic potential (virulence) of strains, races or biotypes of a pathogen species, so that the occurrence of more virulent strain(s) can be detected, identified and quantified rapidly (Volume 1, Chapter 3).

Various phases of disease development in susceptible plants under in vitro and factors influencing disease incidence and spread under in vivo resulting in occurrence of epidemics have been examined in detail by using molecular techniques.

The intricacies of host-pathogen interactions at molecular and cellular levels are elucidated in two chapters in Volume 2 of this treatise. It has been possible to visualize and monitor various steps from pathogen adhesion to tissue colonization and symptom expression during different phases of pathogenesis by applying suitable and sensitive molecular methods (Volume 2, Chapter 2). Factors influencing plant disease incidence and spread have been studied using conventional methods for identification and quantification of pathogen populations in epidemiological investigations. With the availability of molecular techniques and specific monoclonal antisera or primers that can amplify specific sequences of pathogen DNA to precisely identify pathogens up to strain/varieties level, it has been possible to determine the distribution and components of pathogen populations and spatial and temporal variations in pathogen populations, in addition to locating the different sources of inoculum (Volume 2, Chapter 3).

Management of crop diseases successfully is the ultimate aim to provide reasonable margin for the grower for his efforts to produce food to meet the requirements of the consumers who need food free of pathogens and their toxic metabolites or chemical residues. Volume 3 encloses six chapters which provide information on the short- and long-term disease management strategies that can be made effective by application of appropriate molecular methods. It has been well demonstrated that molecular assays are highly efficient in detecting and identifying pathogens present in plant materials that may or may not exhibit symptoms of infection by pathogens of quarantine importance. Application of these techniques can effectively prevent introduction of exotic pathogen(s) that may find suitable conditions for development and spread in new geographical location(s) (Volume 3, Chapter 2).

Use of crop cultivars with built-in resistance to different diseases is considered as the economical and ecologically safe strategy of disease management. Locating disease resistance genes not only in plants, but also in diverse sources including insects and rapid selection of resistant genotypes or lines using genetic markers at early growth stage, have been possible due to the application of appropriate molecular methods. Furthermore, the visual scoring methods to assess the disease intensities exhibited by different genotypes have not provided consistent results, because environmental factors are likely to influence the symptom expression. In contrast, molecular methods employed to determine the quantum of pathogen DNA have proved to be more accurate, reliable and rapid (Volume 3, Chapter 3).

It is very difficult to transfer resistance gene(s) into cultivars from distantly related or unrelated plant species, because of the sterility of progenies associated with interspecific or intergeneric crosses. But biotechnological approaches have offered the possibility of transferring resistance gene(s) from plants and also from diverse sources such as fungi, insects, and frogs. Genetic engineering techniques have provided wide options which are not available, if conventional breeding procedures are to be followed. Pathogen-derived resistance (PDR) approach has been shown to be successful in developing virus disease resistant crop cultivars (Volume 3, Chapter 4).

Development of disease-resistant cultivars through genetic engineering technology has been attempted in the case of a few crops such as tomato, tobacco,

potato and rice, leaving out a large majority of crops untouched. Nevertheless, the possibility of enhancing the levels of resistance of desired cultivars by the application of effective inducers of resistance has been demonstrated to be a practical proposition. Both biotic and abiotic inducers have been tested on a wide range of agricultural and horticultural crops. As resistance inducers activate the natural disease resistance (NDR) mechanisms existing in the plants, it is apparent that the plants treated with inducers, behave like genetically resistant plants. Furthermore, the inducers act on plants, but not on the pathogens. Hence, the chances of development of resistance to these agents are remote, indicating the usefulness and practicability of this disease management strategy (Volume 3, Chapter 5).

Various fungal and bacterial species existing in the rhizosphere, phyllosphere or spermosphere have been found to have the potential of protecting plants against microbial pathogens. The molecular bases of protection to plants against microbial pathogens by the activities of biocontrol agents (BCAs) have been investigated. The genes controlling the production of antibiotics and enzymes capable of inhibiting the growth and development of the pathogen have been isolated, cloned and characterized. Efforts have been made to enhance the biocontrol potential of the selected BCAs by transferring genes from other microorganisms. In addition, monitoring the spread, persistence and survival of the introduced BCAs has been effectively carried out by using appropriate molecular techniques (Volume 3, Chapter 6).

Various kinds of chemicals have been used at different stages of crop growth and storage for the control of microbial pathogens causing different diseases in agricultural and horticultural crops and the produce. Although chemicals are able to reduce the disease incidence and spread significantly, the emergence of resistant or less sensitive strains of fungal and bacterial pathogens has been of great concern for the growers and the industry. Further, the growing awareness of the general public of the possible effects of pollution and persistence of residues, due to chemical application resulted in considerable difficulty in marketing the produce with higher levels of chemical residues. This situation led to restricted use or withdrawal of certain fungicides/chemicals from the market. Rapid identification and differentiation of resistant and sensitive strains and monitoring of appearance of new strains resistant to fungicides may be possible using molecular techniques that can detect the changes in the sequences of specific gene(s) of the pathogens (Volume 3, Chapter 7).

This book aims to provide the latest information to gain comprehensive knowledge on various aspects of plant-pathogen interaction leading either to development of symptoms induced by the pathogen or restriction of development and consequent elimination of the pathogen. The study of plant-pathogen interaction at cellular and molecular levels needs no emphasis, since molecular biological investigations have opened up the avenues that could not be accessed through conventional procedures. The information presented in this volume is expected to be useful for researchers, teachers and upper level graduate students, pursuing investigations in biological sciences in the Departments of Plant Pathology, Molecular Biology and Biotechnology, Microbiology, Biochemistry, Plant Physiology and Plant Breeding and Genetics and also personnel of Plant Quarantine and Certification Programs.

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Chapter 2
Molecular Techniques for Detection of Microbial Pathogens

Abstract Detection, identification and differentiation of microbial plant pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses and viroids – infecting various crops constitute the basic step for the development of effective crop disease management systems. The conventional cultural methods involving isolation and studying the morphological characteristics using microscope are labor intensive and cumbersome, yielding sometimes, inconclusive results. The molecular techniques, on the other hand, are able to provide precise, reliable and reproducible results rapidly, facilitating early disease management decisions. Biochemical, immunological and nucleic acid-based assays have been preferred, because of the distinct advantages over the conventional methods. The molecular techniques have been very useful in the identification of obligate pathogens causing diseases such as downy mildews, powdery mildews and rusts and also fungi that grow very slowly in the culture media, taking several weeks to produce spore forms that can be used for identification. The availability of antisera, primers and commercial kits has led to widespread application of the molecular techniques for on-site detection during field surveys for assessing the distribution of existing pathogens, occurrence of new/introduced pathogens or strains and for preventing introduction of new pathogens through seeds or planting materials. Furthermore, molecular techniques have been demonstrated to be useful in breeding programs to identify sources of resistance to disease(s). Several protocols for molecular techniques, useful for researchers and students are presented in the Appendix.

Depending on the levels of resistance/susceptibility of plant species to different microbial pathogens, the nature of the interaction between host and pathogen may vary. In a compatible (susceptible) interaction, development of symptoms characteristic of the disease may be observed, facilitating the identification of the pathogen inducing such symptoms. On the other hand, in an incompatible interaction (resistant/immune), the pathogen development may be hampered to different degrees resulting in the production of small necrotic (hypersensitive) flecks or complete absence of any visible symptom. In another type of interaction, the plant species is susceptible to the pathogen in question, but no symptom of infection may be discernible. This type of interaction known as latent infection is frequently seen

P. Narayanasamy, *Molecular Biology in Plant Pathogenesis and Disease Management: Microbial Plant Pathogens, Vol. 1*.

in the case of plants infected by viruses. These plants are known as symptomless carriers, posing potential danger for crop production. In yet another type of interaction designated quiescent infection, the pathogen remains dormant in infected immature fruits till they ripen. Such quiescent infections are seen in bananas and mangoes frequently. Nevertheless, the characteristic symptom may provide, however inaccurate it may be, a basis for the identification and differentiation of some microbial pathogens.

Disease diagnosis relates to the identification of the nature and cause of the disease, whereas detection deals with establishing the presence of causative target organism with the test sample. It has been found to be very difficult, frequently unsuccessful, if the symptom expression in a pathosystem is not clear. Further, if a host plant is subject to attack by many pathogens simultaneously or successively one after another, the symptom picture may be very different from those symptoms induced by pathogens individually. Reliability on symptoms alone may lead to erroneous identification of the causative agent(s). Rapid and precise identification of the cause of the disease is the basic requirement for the development of effective disease management systems. A wide range of diagnostic procedures has been employed for identification, differentiation and quantification of microbial plant pathogens. The sensitivity, reliability and reproducibility of the techniques have been constantly improved by the intensive research efforts in different countries.

Identification of fungi and fungi-like, oomycetes infecting plants has been made primarily on the basis of morphological characteristics such as type, shape and color of sexual and/asexual spore forms. Oomycetes lack taxonomic affinity with true fungi. The taxonomic position of the oomycetes with a unique lineage of eukaryotes unrelated to true fungi, but closely related to brown algae and diatoms has been established based on molecular phylogenetic and biochemical investigations. Oomycetes are included in the Kingdom Stramenopila, group Stramenopiles which encloses golden-brown algae, diatoms and brown algae such as kelp (Sogin and Silberman 1998; Baldauf et al. 2000). The fungi-like oomycetes are included under different sections for fungal pathogens for discussion of various aspects. Tests to determine physiological and biochemical characteristics were the basic tools used to identify and differentiate between bacterial pathogens for several decades from 1930s. These tests were occasionally applied for the identification of filamentous fungi which in general exhibit greater phenotypic plasticity than bacteria (Bridge 2002). The physiological and biochemical tests applied for the bacterial and fungal pathogens cannot be employed for the identification and differentiation of plant viruses, since they are extremely small and do not have any detectable physiological activity.

The physiological and biochemical properties vary widely between different groups of bacterial pathogens. No single standard set of tests can be used for all bacteria. Hence, different sets of tests have to be employed to identify isolates of different bacteria. However, commercial kits have been developed for Gram-positive and Gram-negative bacteria based on assimilation tests by Biolog Inc., USA. As some metabolites like mycotoxins (aflatoxin, ochratoxin) are produced only by a narrow range of fungal species, this property may be of significance in the systematics of

certain filamentous fungi like *Aspergillus* spp. and *Penicillium* spp. (Frisvad et al. 1998). Production of mycotoxins may be more precisely detected by using serological methods (Narayanasamy 2005).

The conventional methods depending on the isolation of microbial pathogens from infected plant tissues, multiplication on suitable media and determination of morphological characteristics and physiological and biochemical features require substantially long periods. Furthermore, the results are significantly influenced by cultural conditions and the interpretation of observations requires considerably long experience. Attempts were made to develop methods that depend on the intrinsic characteristics of the microbial pathogens. In addition, the identification of the isolates, strains, races/biotypes has to be done very rapidly and precisely, if the incidence of a new disease is seen. This will facilitate initiation of measures to prevent or restrict further spread of the disease(s). Diagnostic techniques based on the molecular characteristics of the microbial pathogens have been demonstrated to fulfill these requirements.

During the three decades and more, rapid advances made in the study of molecular biology of microbial pathogens have provided adequate information for the development of several sensitive and rapid methods for characterization of microbial pathogens and determination of molecular variability of and relationship between fungal, bacterial and viral pathogens. Molecular techniques have been shown to be very useful in studying various aspects of plant-pathogen interactions, epidemiology of crop diseases and to assess the effectiveness of different disease management strategies. A wide range of techniques has been employed to suit the pathosystem (Narayanasamy 2001, 2005). The relative usefulness of some of the basic molecular techniques widely applied, are presented in this chapter.

2.1 Detection of Microbial Pathogens by Biochemical Techniques

2.1.1 Electrophoresis

Microbial pathogens may be detected and identified based on their specific physiological and biochemical activities in the case of some fungal and bacterial pathogens. Manipulating and analyzing DNA are fundamental procedures in the study of molecular biology of organisms. DNA is isolated intact and treated with restriction enzymes to generate pieces small enough to be resolved by electrophoresis in polyacrylamide or agarose. Separating complex mixtures of DNA into different sized fragments by electrophoresis has been a well established method for over three decades. Analysis of total protein profiles generated by separating whole cell protein extracts by electrophoresis has been shown to be useful. Isozyme electrophoresis has been found to be effective in species/strain differentiation. Isozyme electrophoresis of enzymes such as esterases, phosphatases and dehydrogenases of fungal and bacterial origin provides different patterns according to their relative mobility. Each band is considered as an allele of a specific locus in the pathogen genome. The bands are labeled alphabetically from the slowest to the fastest.

2.1.1.1 Fungal Pathogens

Isozyme electrophoresis can be used for defining groups within species of oomycetes and fungi. Based on the analysis of isozyme patterns, *Phytophthora cambivora, P. cinnamomi* and *P. cactorum* could be clearly separated. Further, it was possible to further subdivide each species into electrophoretic types (ETs). By using cellulose acetate electrophoresis (CAE) for fractionation of phosphoglucose isomerase malate dehydrogenase and lactate dehydrogenase, intraspecific diversity and interspecific relatedness of different papillate species of *Phytophthora* were assessed. A very close genetic relatedness between *P. medii* and *P. botryosa* that clustered together was evident. Likewise, *P. katsurae* and *P. heveae* were together in a cluster, while *P. capsici* and *P. citrophthora* formed another cluster (Oudemans and Coffey 1991a, b).

Within a morphologic species of *Leptosphaeria maculans,* highly virulent and weakly virulent strains inducing black leg or stem canker disease in canola (*Brassica napus*) were differentiated based on the fast or slow movement of isozymes of glucose phosphate isomerase (GPI). The highly virulent strains contained fast isozyme and they were placed in electrophoresis Type 1 (ET1). The isozymes of weakly virulent strains moved only short distance and they formed a distinct group (ET2) (Sippell and Hall 1995). The presence of GPI in leaf lesions induced by *L. maculans* was also detected by CAE which could be also employed to differentiate the pathogen from *Pseudocercosporella capsellae*. In addition to ET1 and ET2 electrophoretic patterns of highly virulent and weakly virulent strain, ET3 allozyme was present in a few typical and atypical lesions caused by *L. maculans*. The lesions induced by *P. capsellae* had the fastest allozyme ET4 (Braun et al. 1997). The isolates (726) of *Phytophthora infestans*, causing late blight disease of potato and tomato collected in Canada were classified into eight genotypes depending on the allozyme patterns at two loci GPI and peptidase (Pep) with markers for mating type, metalaxyl sensitivities and cultural morphology. Differences in the banding patterns for the allozymes of the GPI locus were significant leading to differentiation of seven of the eight genotypes (Peters et al. 1999). Four allozyme genotypes at GPI and Pep loci were distinguished among the 85 isolates of *P. infestans* present in North Carolina. These isolates predominantly belonged to US-7 genotype and US-8 genotype (Fraser et al. 1999).

Isozyme polymorphisms among different isolates of closely related species of *Fusarium* such as *F. cerealis, F. culmorum, F. graminearum* and *F. pseudograminearum* occurring around the world, were investigated by using cellulose-acetate electrophoresis (CAE). Remarkably uniform isozyme patterns were obtained intraspecifically irrespective of the geographical origin of the isolates or the host/substrates from which they were isolated. Comparison of the elctrophoretic types (ETs) of adenylate kinase (AK), NADP-dependent glutamate dehydrogenase (NADPGDH) peptidase B (PEPB), peptidase D (PEPD) and phosphoglucomutase (PGM) proved to be diagnotic for at least one of the four species examined. However, PEPD alone was useful as a marker to distinguish the four taxa studied, providing a rapid and simple CAE-based diagnostic protocol. The results based

on similarity values indicated that *F. graminearum* was more closely related to *F. cerealis* and *F. culmorum* than to *F. pseudograminearum.* The morphological similarity of *F. graminearum* and *F. pseudograminearum* did not reflect their genetic relatedness, suggesting the need for supporting evidence(s), for classification of fungal pathogens (Làday and Szécsi 2001).

2.1.1.2 Bacterial Pathogens

The usefulness of SDS-PAGE technique for the differentiation of bacterial pathogens has been demonstrated. Silver staining of SDS-lysed cells of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) electrophoretically separated into dark gray bands with a MW of 32–35 kDa and 25–27 kDa. The band with larger MW designated α was present in 192 of 197 tomato race/strain and the band with smaller MW designated β, was present in all 55 strains of tomato race 2. Race/strains expressing an α band could not hydrolyse starch (Amy^-) and very few degraded pectate (Pec^-). In contrast, most of race 2 strains were Amy^+ and Pec^+. Silver staining of protein profiles and testing for amylolytic activity of *Xcv* may be useful to assign uncharacterized strains of *Xcv* to appropriate phenotypic group (Bouzar et al. 1994) [Appendix 1]. In the case of *Pseudomonas syringae* pv. *pisi* (*Psp*) the envelope protein profiles of bacterial cells formed the dependable basis for identification and differentiation of the strains. *Psp* strains showed three unique protein bands (60, 65 and 150 kDa) which were absent in 29 strains of *P. syringae* pv. *syringae* tested (Malandrin et al. 1996). Some of the bacterial pathogens have been identified using their isozyme profiles. The suitability of applying the patterns of enzymes of esterase (EST) and superoxide dismutase (SOD) for the identification, differentiation of strains and diagnosis of diseases caused by *Psp* was assessed. Two EST zymotypes specifically present in the profiles could be used for the identification of *Psp*. Furthermore, there was significant correlation between these EST patterns and race structure of this pathovar. Races 2, 3, 4 and 6 exhibited patterns similar to zymotype 1, whereas races 1, 5 and 7 were included in zymotype 2 based on the similarity of isozyme profiles (Malandrin and Samson 1998). The whole-cell protein analyses of *Xylella fastidiosa* (causing Pierce's disease of grapevine) strains by employing SDS-PAGE technique indicated variations in the protein banding patterns among the strains. It was possible to detect and identify the strains (75) based on the presence, absence or intensity of 10 protein bands and assign them to 6 different groups. This technique has been shown to be a rapid and consistent method of identifying the strains of *X. fastidiosa* (Wichman and Hopkins 2002).

A new procedure known as pulsed field gel electrophoresis (PFGE) to separate DNA was introduced by Schwartz and Cantor (1984). With further studies, PFGE has reached a level for routine application and commercial pulsed field units have been manufactured for large scale use. Now PFGE permits cloning and analysis of a small number of very large pieces of a genome. The genomic analysis of strains of *Erwinia amylovora,* (causing fire blight disease), from the Mediterranean region and European countries was performed. The PFGE patterns were determined by assaying the *Xba1* digests of bacterial genomic DNA. The strains from Austria and

Czech were grouped with the central European type (Pt1). The strains from the eastern Europe and Mediterranean region were placed in the second group Pt2. Italy has strains showing patterns of all three types (Zhang et al. 1998). The isolates of *Acidovorax avenae* subsp.*citrulli* infecting cucurbits in Israel were subjected to PFGE analysis which revealed 23 unique DNA fragments and five different profiles, each of which contains 9–13 bands. The isolates were grouped into two classes. The distinct advantage of PFGE over rep-PCR assay was the greater levels of reproducibility of results and the genetic diversity of the isolates may be reliably assessed PFGE analysis (Burdman et al. 2005).

2.1.1.3 Viroid Pathogens

The distinct nature of the causal agent of potato spindle tuber disease was first established by using PAGE system. The cellular nucleic acids from both healthy and infected plants were extracted and separated by PAGE. The causative agent *Potato spindle viroid* (PSTVd) appeared as a distinct band only in the samples from infected tissues and it was absent in the comparable healthy potato tissues. The mild and severe strains of PSTVd could be detected and differentiated. Further, PAGE was successfully employed to free several elite or basic seed stocks of potato in certification programs (Morris and Wright 1975). The differences in the electrophoretic mobility of isolates of *Coconut cadang-cadang viroid* (CCCvd) were used as the basis of differentiating the different isolates (Randles 1985). *Coconut tinangaja viroid* (TiVd) in coconut leaf samples was detected by analytical agarose gel electrophoresis (Hodgson et al. 1998).

The presence of citrus viroids – *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viorid* (CBLVd), *Hop stunt viroid* (HSVd) and *Citrus viroid III* (Cvd-III) – in citrus samples from greenhouse was detected by sequential polyacrylamide gel electrophoresis (sPAGE). Sample extracts (20 µl equivalent to 300 mg fresh weight) are subjected to nondenaturing 5% PAGE at 60 mA for 2.5 h. After staining the gel with ethidium bromide, a segment (1.5 cm) of the gel between CEVd and 7S RNA is excised and subjected to a second 5% PAGE containing 8 M urea. After silver staining, the viroid bands can be viewed (Barbosa et al. 2005).

2.1.1.4 Viral Pathogens

Electrophoretic techniques have been employed to a limited extent as a step in the process of virus purification from crude plant extracts or suspensions containing mixture of viruses or their strains. Plant virus suspensions in suitable buffers are layered into appropriate buffered density gradients of gels formed in a tube and separation of components of the suspension takes places over a period of several hours in a zonal density gradient. Other methods based on the principle of electrophoresis, using of pH gradient or paper curtains have been used for the purification of *Southern bean mosaic virus* and for separation of *Tobacco mosaic virus* strains. For unstable viruses like *Citrus infectious variegation virus*, electrophoretic technique is especially valuable (Narayanasamy and Doraiswamy 2003). The chitinase isozymes

patterns of healthy and virus-infected leaves may form a basis for the detection of virus infection by SDS-PAGE technique. The chitinase isozymes were detected by SDS-PAGE technique in the crude extracts from leaves of healthy and TMV-infected tobacco plants. There were eight dominant chitinase isozymes detected in tobacco extracts. One of them was present only in the TMV-infected leaves, while another accumulated at a greater concentration in TMV-infected than in mock-inoculated leaves (Pan et al. 1991).

SDS-PAGE technique was applied to detect the presence of a unique protein band (32–34 kDa) present in leaves of maize infected by a new virus designated *Wheat yellow head virus* (WYHV). This protein was not present in extracts from healthy maize leaves [Appendix 2]. The amino acid sequences of their protein was most closely related to the nucleoprotein of *Rice hoja blanca virus*, indicating that WYHV is a tenuivirus (Seifers et al. 2005). The relative molecular mass of *Apricot latent virus* coat protein (CP) was determined by using SDS-PAGE technique. The dissociated CP migrated as a single band with an estimated size of ca 50 kDa (Ghanen-Sabanadzovic et al. 2005) (Fig. 2.1). The nucleocapsid proteins (Nps) of *Calla lily chlorotic spot virus* (CCSV), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Watermelon silver mottle virus* (WSMoV) were dissociated from the purified nucleocapsids and subjected to SDS-PAGE analysis for the estimation of their relative molecular mass. CCSV NP had a molecular mass of 31 kDa which was similar to that of WSMoV, but slightly larger than those of TSWV and INSV which had molecular mass of 29 kDa (Lin et al. 2005).

SDS-PAGE technique was applied to determine the molecular masses of coat proteins in purified preparation of *Peanut chlorotic streak virus* that has two polypeptides with approximate relative molecular masses of 51 and 58 kDa (Reddy et al. 1993). The virus coat proteins may be expressed in the bacterium *Escherichia coli*. The expressed coat protein can be purified by SDS-PAGE technique for use as

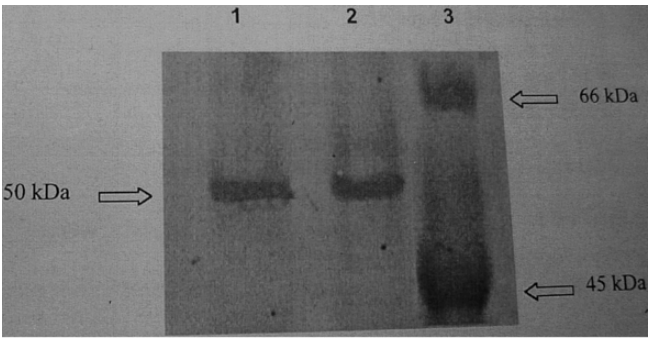

Fig. 2.1 Electrophoretic analysis of dissociated *Apricot latent virus* (ApLV)-strain Apr47 coat protein (CP)
Note the single band of 50-kDa protein in lanes 1 and 2 representing viral CP. Molecular markers [albumin egg (MW 45,000) and albumin bovine (MW 60,00)] are placed in lane 3. (Courtesy of 2005 2005; Journal of Plant Pathology, Edizioni ETS, Pisa, Italy)

immunogen. This procedure was followed for the production of antisera to *Bean golden mosaic virus* Brazil isolate (BGMV), *Cabbage leaf curl virus* (CabLCV), *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV) (Abouzid et al. 2002). The coat protein of *Grapevine leafroll-associated virus-8* (GLRaV-8) was purified using a Tricine-SDS-PAGE system and its MW was 37 kDa. This CP was serologically distinct from CP of GLRaV-1 in Western blot assay (Monis 2000). By using SDS-PAGE procedure, the bacterially expressed coat protein (BE-CP) of *Faba bean necrotic yellows virus* (FBNYV) was purified. BE-CP migrated as a protein of approximately 23 kDa and it was used for the production of polyclonal antibodies against FBNYV (Kumari et al. 2001).

2.2 Detection of Microbial Pathogens by Immunoassays

Immunodiagnostic assays have been applied for detection, differentiation and quantification of microbial pathogens. They have been found to be highly specific, sensitive, simple, rapid and cost-effective and can be automated for large scale applications. Hence, the immunoassays have largely replaced conventional analytical methods which are time-consuming, cumbersome and expensive. The comparative effectiveness and usefulness of several immuno-assays have been discussed earlier (Narayanasamy 2001, 2005). Improvements and modifications, however, have been made to suit different host-pathogen combinations. The advancements during the recent years have focused the attention of the researchers on the need for applying rapid, reliable and sensitive methods for the detection of microbial pathogens and diagnosis of diseases caused by them.

Immunoassays primarily depend on the visualization of the specific binding of antibody to the antigen, directly or indirectly. The formation of precipitate or precipitin lines indicating the binding between reactants, may be seen. These tests require large volumes of the reactants and longer time to provide results which may be inconclusive. On the other hand, the assays requiring labeling of antibodies are more sensitive and rapid with possibility of automation for large scale application. The antibodies may be labeled with enzymes such as alkaline phosphatase or fluorescent dyes.

Among the techniques using labeled antibodies, enzyme-linked immunosorbent assay (ELISA) has been most widely applied for studying various aspects of plant-pathogen interactions, in addition to detection and characterization of microbial pathogens. Three formats of ELISA viz., the double antibody sandwich (DAS)-ELISA, the triple antibody sandwich (TAS)-ELISA and the plate-trapped antigen (PTA)-ELISA are frequently employed for detection and disease diagnosis. Other immunoassays such as direct tissue blot immunoassay (DTBIA) and immunosorbent electron microscopy (ISEM) for in situ detection of viruses have also been employed. The development of monoclonal antibody technology has remarkably enhanced the specificity of immunoassays, resulting in the differentiation of strains, biotypes or races of microbial pathogens more precisely than by using polyclonal antibodies.

2.2.1 Viral Pathogens

2.2.1.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The advantage of employing immunoassays were clearly demonstrated in the case of grapevine which is infected by 44 different viruses (Martelli and Walter 1998). For certification programs depending on the biological indexing by grafting onto indicator hosts, it was very difficult to ensure freedom from viruses in the plant materials. Indexing required 2–3 years for symptom development on indicators. Biological indexing for detection of *Grapevine leafroll-associated viruses* (GLRaV) 1 to 4 was compared with detection by ELISA. Biological indexing gave positive results for only 49 of 57 grapevine plants infected by GLRaV-3, whereas ELISA test could detect the virus in all infected plants (Rowhani et al. 1997). Application of immunoassays for the detection and resolution of etiology of leaf roll and rugose wood complexes of grapevine progressively increased due to the availability of results rapidly (Martin et al. 2000).

Tospoviruses, belonging to the family Bunyaviridae, cause spotted wilt diseases that are agriculturally important because of the significant losses in different crops and flowers. ELISA has been frequently employed using PABs specific against viral nucleoprotein, due to the ease of the purification step, in preference to other methods that rely on detection of complete virus particle with envelope which require a different enveloped particle preparation (Hsu et al. 2000; Takeuchi et al. 2001; Tanina et al. 2001). A new-tomato-infecting tospovirus designated *Tomato yellow ring virus* (TYRV) occurring in Iran was serologically compared with other tospovirus species by employing DAS-ELISA format, using PABs directed against the nucleoprotein of each virus viz., *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INS), *Iris yellow spot virus* (IYSV), and *Watermelon silver mottle virus* (WSMoV). No significant cross-reaction could be noted for TYRV with antisera of other tospoviruses tested and vice-versa, suggesting that TYRV was serologically distinct from the viruses compared (Hassani-Mehraban et al. 2005). The occurrence of another new tospovirus was also detected by ELISA. Calla lilies (*Zantedeschia* spp.) were infected by a new tospovirus. By using ELISA, this new virus was found to be distantly related to WSMoV (Chen et al. 2005a). By using PABs and MABs specific to *Calla lily chlorotic spot virus* (CCSV) and WSMoV in indirect ELISA and immuno-blotting assays, CCSV was shown to be a distinct member in the genus *Tospovirus.* It was suggested that CCSV is a new species belonging to WSMoV serogroup (Lin et al. 2005) [Appendix 3].

Sweet pepper plants generated from apical meristems were tested for freedom from TMV and TSWV by applying ELISA test (Katoh et al. 2004). The usefulness of the indirect ELISA format in detection and differentiation of the new *Wheat yellow head virus* (WYHV) from *Wheat soilborne mosaic virus* (WSMV), *Wheat American striate mosaic virus* (WASMV), *Agropyron mosaic virus* (AgMV) and *High plains virus* (HPV) was demonstrated (Seifers et al. 2005) The sanitary status of stone fruits was assessed by employing ELISA to check the presence of *Plum*

pox virus (PPV), *Prunus necrotic ring spot virus* (PNRSV) and *Prune dwarf virus* (PDV). The survey showed that PDV was absent, whereas PNRSV and PDV were frequently detected in single and mixed infection (Michelutti et al. 2005). Infection of rose and lily plants by nepoviruses *Strawberry latent ringspot virus* (SLRSV) and *Arabis mosaic virus* (ArMV) was confirmed by applying DAS-ELISA test, using virus-specific antibodies. Only symptomatic leaves showed the presence of SLRSV and ArMV in rose cultivars Raktgandha, Landora, Sonia and Oriental Hybrid, whereas in lily cultivars Galeili, Star Gazer Max and White Merostar, SLRSV alone was detected (Kulshrestha et al. 2005).

From time to time, infection by viruses on host species which are not known as hosts, has been observed later. Viruses infecting vanilla in French Polynesia were detected by employing ELISA technique. Three distinct potyviruses *Vanilla mosaic virus*, *Watermelon mosaic virus* and a virus related to *Bean common mosaic virus* were detected and differentiated by this technique (Grisoni et al. 2004). During a survey of potato viruses, potato leaf samples were tested by ELISA for the presence of PVY. Among the PVY-positive samples (394), three samples reacted positively with the MAB 1F5 and they induced veinal necrosis symptoms in tobacco. Two of these isolates caused tuber necrosis in the potato cv. Yukon Gold. In addition, PVY and *Potato virus* S (PVS) were detected in a majority of samples as well as in mixed infections (Baldauf et al. 2006). The incidence of *Prunus necrotic ringspot virus* (PNRSV) on rose geraniums (*Pelargonium* spp.) was detected and its identity was established by ELISA format using antibodies raised against different Ilarviruses. The diagnosis was later confirmed by Northern hybridization, RT-PCR, restriction enzyme digestion and sequencing. There was high amino acid sequence homology (97%–99%) between the virus infecting geranium and other PNRSV isolates (Kulshrestha et al. 2005). During the survey to assess the incidence of *Iris yellow spot virus* (IYSV) in leek and onion, the ELISA format was applicable for the detection of the virus. The disease incidence was observed in 11 of 21 leek and 2 of 26 onion plantings. As the distribution of IYSV in leek was irregular, it was suggested that samples should include tissue subsection from the top and middle portions of several leaves from each plant for obtaining realistic estimate of disease incidence (Smith et al. 2006).

It is very difficult to obtain intact of *Rice black-streaked dwarf virus* (RBSDV) virions, as they are fragile. The major outer capsid protein P10 of RBSDV encoded by S10 gene was expressed in *Escherichia coli* cells as glutathione-S-transferase (GST) fusion protein. Polyclonal antibodies generated against the purified P10 protein, specifically recognized RBSDV from infected plant tissue and a planthopper vector in Western blotting assays. An indirect (ID)-ELISA procedure developed was able to detect RBSDV in very dilute wheat leaf extract in routine and large scale application (Wang et al. 2005).

Development of monoclonal antibody (MAB) technology has led to substantial enhancement of the sensitivity and specificity of immunoassays including ELISA tests. In an indirect protein A-coated plate (ACP)-ELISA format, the specific MAB 2B5 was used for detection of *Sugarcane mosaic virus* Zhejiang isolate (SCMV-ZJ) in maize leaf samples showing dwarf mosaic symptoms. Results of ACP-ELISA

with MAB 2B5 correlated well with that of RT-PCR findings using the same tissues. Hence, MAB 2B5 can be employed to detect isolates of SCMV in field samples (Chen et al. 2003). A highly specific MAB was shown to react only with citrus tissues infected by *Citrus tristeza virus,* but not with non-infected citrus plant extracts (Öztürk and Cirakolu 2003). Indirect ELISA using the universal potyvirus group-specific MAB, DAS-ELISA using *Bean Common mosaic necrosis virus* (BCMV) specific strains MAB-12 and *Beam common mosaic* (BCMV)-specific MAB-1-E5, the strains of these viruses were detected in infected plants in bean germplasm lines (Larsen et al. 2005). Thirty-six *Citrus tristeza virus* (CTV) isolates occurring in CTV-eradicative and noneradicative districts in central California did not react with MAB 13 that detected presumptive CTV severe strains as shown by double-antibody sandwich indirect (DASI)-ELISA format. On the other hand, standard control isolates of CTV, T36, T3 and VT reacted with MAB13. The results showed that the presence of CTV in citrus trees should be determined by a pathogen detection method such as ELISA rather than by symptom expression (Yokomi and DeBorde 2005). The presence of a recombinant isolate of *Plum pox virus* (PPV) in apricot trees was detected by DASI-ELISA using four strain-specific MABs and this report seems to be the first record of a recombinant isolate of PPV in Italy (Myrta et al. 2005).

2.2.1.2 Tissue Blot Immunoassay

Determination of the geographic distribution of viruses particularly viruses infecting perennial horticultural trees that are vegettively propagated is very important. This knowledge will facilitate control or eradication of the pathogen from the affected areas/country. Furthermore, this will also enable identification of safe or unsafe areas for budwood collection and prevention of inadvertent introduction of the pathogen (s) to other areas/countries. The comparative efficacy of double antibody sandwich (DAS)-ELISA, triple antibody sandwich (TAS)-ELISA and direct tissue blot immuno- assay (DTBIA) in detecting *Citrus psorosis virus* (CPsV) was assessed. All the three assay procedures detected CPsV readily in young shoots and leaves. However, DTBIA was less efficient in detecting the virus in old leaves. The presence of CPsV in nine different citrus varieties was efficiently detected by ELISA formats and DTBIA, indicating that CPsV accumulated to equivalent levels in the varieties tested. There was good correlation between results of immunoassays and that of biological indexing (Martin et al. 2002a) [Appendix 4]. The results showed that CPsV in infected trees was clearly detected in old hardened leaves in winter. By using a minimum of four leaves taken from around the trees, high reliability can be achieved in any season (Cambra et al. 2000). DTBIA showed that external and internal necrosis in Bently and Lennox cabbage was due to a mixture of eight of isolates of *Turnip mosaic virus* (TuMV) and that TuMV was unevenly distributed in the cabbage heads (Krămer et al. 2000). DTBIA is simpler, less expensive and faster than ELISA providing the same level of specificity and sensitivity, if young leaves are selected for testing. Furthermore, tissue prints can be prepared in the field and stored for long periods without loss of reactivity, making DTBIA more useful and

convenient technique for epidemiological studies. In addition, several membranes can be printed with the same shoots for future processing with new antibodies and the tissue-printed membranes may be mailed to a laboratory where the required antibodies are available (Martin et al. 2002a).

By employing MABs (3A2E3 and 2F7H5) in tissue blot immunoassay (TBIA), *Sugarcane yellow leaf virus* (ScYLV) could be detected routinely. The MAB 3A2E3 gave equivalent effectiveness as a polyclonal antiserum to purified virus in comparative testing using TBIA (Korimbocus et al. 2002). The antiserum raised against the bacterially expressed coat protein (BE-CP) of *Faba bean necrotic yellows virus* (FBNYV) gave strong FBNYV-specific reactions in TBIA assessment. The background reactions with control (non-infected) tissues were very weak, similar to those produced by monoclonal antibodies, indicating the usefulness of the approach of using polyclonal antibodies in place of monoclonal antibodies which require long period of time and expertise for production (Kumari et al. 2001). Tissue immunoblot analysis of tobacco cv. Xanthi nc inoculated with *Potato Virus Y* (T01 isolate) alone and also in combination with *Cucumber mosaic virus* (CMV) was made to visualize the distribution of viral antigens in cross-sections of stems or petioles. PVY was detected at high levels in almost all sections in stems, petioles and apical tissues in doubly infected plants. But in singly infected plants, PVY was not detected in shoot apex and the reaction was weaker in tissues from middle or young stems and petioles. However, CMV was detected in almost all sections of both singly and doubly infected plants (Fig. 2.2) (Ryang et al. 2004) [Appendix 5]. Tissue immunoblot technique was employed to study the distribution of *Turnip mosaic virus* (TuMV) and its chimeraic viruses in the cotyledons and leaves of cabbage and radish at 12 days post inoculation. Chimeric viruses showed very low systemic infectivity (Suehiro et al. 2004).

An improved DTBIA protocol for the detection of *Citrus tristeza virus* (CTV) involves printing of fresh young stems of healthy and infected plants by gently and evenly pressing the fresh cut surface of the stems onto a nitrocellulose membrane. After air-drying for 5 min, the tissue blots were incubated with prereaction solutions of CTV-specific antibodies and labeled secondary antibodies, goat antimouse Ig (H+L)-alkaline phosphatase conjugate or goat anti-rabbit IgG alkaline phosphatase conjugate, for up to 2 min, rinsed with PBST buffer for 5 min and immersed into an NBT-BCIP substrate solution for 15–20 min. After rinsing the blots in water for a few seconds to stop the reactions, results are recorded by observation under a light microscope. Both CTV decline-inducing isolate T-36 was detected by using CTV-specific PAB- PCA1212 and MABs 17G11 and MCA13, whereas PAB-PCA 1212 and MAB 17G11 detected non-decline-inducing isolate T-30. This TBIA was found to be as reliable as PCR for detecting CTV in field samples and this procedure could be completed within 1 h by having a pre-reaction of CTV-specific antibodies and labeled secondary bodies in solution prior to their application to the tissue blots (Lin et al. 2006).

Simplification increased the feasibility of application of immunoassays. For the detection of *Citus tristeza virus* (CTV), an in situ immunoassay (ISIA) was developed. Sections of stems, petioles or leaf veins of test plants (healthy/infected) were

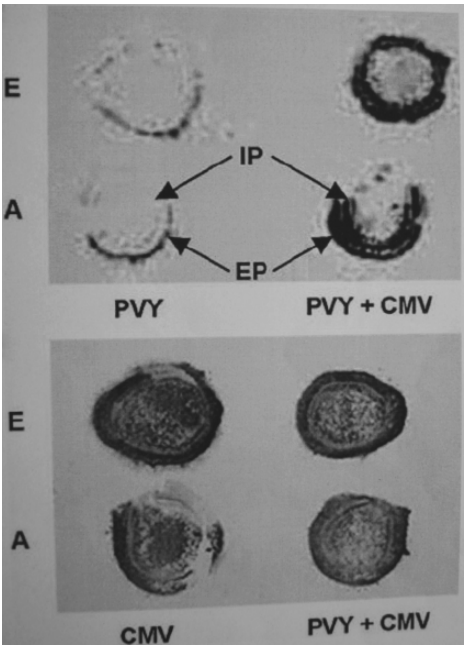

Fig. 2.2 Localization of *Potato virus Y* (PVY) in cross-sections of stem tissues of tobacco infected singly by PVY or doubly with *Cucumber mosaic virus* (CMV) by employing tissue immunoblot technique
Top: Stem tissues infected by PVY alone and PVY+CMV at two different positions; *Bottom*: Stem tissues infected by PVY alone and PVY+CMV at 7 days after inoculation. (Courtesy of Ryang et al. 2004; Society for General Microbiology, United Kingdom)

fixed with 70% ethanol and incubated with specific PABs or MABs followed by application of enzyme-conjugated with secondary antibodies and exposure to a substrate mixture consisting of nitroblue tetrazolium and 6-bromo-4-chloro-3-indolyl phosphate. Development of a purple color indicating a positive reaction for the presence of viral antigen, can be visualized by light microscopy. The presence of CTV antigens could be detected directly and precisely by ISIA in both fresh tissues and samples stored in plastic bags at 4°C or frozen for 4 weeks. ISIA providing reliable results comparable to DTBIA, is simple, rapid and specific has potential for wide applicability (Lin et al. 2000) [Appendix 6]. The mild and severe isolates of CTV in grapefruits trees could be detected and differentiated by employing ISIA which was more sensitive than ELISA. Two MABs capable of reacting differentially with CTV isolates were used for their detection in infected grapefruit trees (Lin et al. 2002). Hammer blotting is another simple technique for detecting virus infection in plants. Different patterns of distribution of *Turnip mosaic virus* (TuMV) in two ecotypes of *Arabidopsis thaliana* (Col.0 and Ler) that react to TuMV differently were observed (Kaneko et al. 2004). TBIA technique is simple, sensitive, rapid and less labour-intensive than ELISA and it is applicable during field survey for analyzing

1000–2000 samples per day. Kits for detection of several viruses, are available. The International Center for Agricultural Research in the Dry Areas has developed kits for 19 viruses of legumes (Webster et al. 2004).

Certification of plant materials represents the first line of virus disease management systems. ELISA is considered to be the technique of choice for certification or field surveys to assess the disease incidence levels for the detection of *Prune dwarf virus* (PDV), a major virus affecting stone fruits. The results of DAS-ELISA tests showed that PDV could be efficiently detected as in the case of reference commercial kit (Abou-Jawdah et al. 2004). Variants of ELISA have been demonstrated to be efficient in the detection of viruses in various host plant species. Plate-trapped antigen (PTA)-ELISA was used to screen the hybridoma cells producing monoclonal antibodies against *Sugarcane yellow leaf virus* (ScYLV) and for routine detection of the virus in infected plant tissues (Korimbocus et al. 2002). PTA-ELISA was employed to detect *Cucumber mosaic virus* (CMV) in various crops and different regions of Bulgaria using individual MABs (Hristova et al. 2002). By using the MAB 2B5 in indirect antigen-coated plate-ELISA, *Sugarcane mosaic virus* was detected in maize leaf samples showing dwarf mosaic symptoms in China (Jiang et al. 2003).

Seeds infected by viruses are the most important sources of infection spreading the viruses into new areas or countries. Viruses of quarantine importance may be different and specific regulations for importation of seeds are being enforced in many countries. *Cowpea aphid borne mosaic virus* (CAMV) was detected efficiently in peanut seeds by ELISA which was equally sensitive as reverse transcription-polymerase chain reaction (RT-PCR) assay (Gillapspie Jr. et al. 2001). In the recent years, the incidence of *High plains virus* (HPV) has been reported from Australia, Brazil, Chile, China, Israel and New Zealand in addition to several states in the United States of America (Jensen 1999; Lebas et al. 2005b). By applying ELISA, 0.015%–4% of the sweet corn seeds were found to be HPV-positive (Forster et al. 2001; Blunt and Hill 2004). In New Zealand, sweet corn plants raised from seeds imported from the US in a quarantine level 3 glass house were tested by a F(ab') 2-ELISA and also by Agadia Diagnostic Services. The results of ELISA tests were similar to that of reverse-transcription (RT)-polymerase chain reaction (RT-PCR) assay confirming the transmission of HPV through sweet corn seeds (Lebas et al. 2005). This study indicates the effectiveness of ELISA determination for indexing seeds in post-entry quarantines to prevent introduction of new virus(es). The seed transmissibility of *Wheat streak mosaic virus* (WSMV) in wheat seeds was demonstrated for the first time by application of ELISA format along with RT-PCR assay ELISA with WSMV-specific antibodies and RT-PCR with WSMV-specific primers detected the virus in wheat seedlings infected by WSMV carried by the seeds of germplasm lines. The investigation showed 0.2–0.5% seed transmission across wheat breeding collection tested, with levels upto 1.5% in individual genotypes (Jones et al. 2005).

The possibility of apricot and peach fruits from trees infected by *Plum pox virus* (PPV) being the source of infection was examined. *Myzus persicae*, the natural aphid vetor of PPV could transmit French isolates of PPV after probing on PPV-infected fruits under controlled condition (Labonne and Quiot 2001). The ability

of *M. persicae* and *Aphis spiraecola* to acquire and transmit PPV from infected peach fruits was revealed by testing the peach fruits and peach seedlings to which PPV was transmitted by the vectors after acquisition feeding by applying standard DAS-ELISA format. This study indicated another mode of PPV movement (through fruits) allowing the virus to bypass natural barriers and invade new geographical locations (Gildow et al. 2004).

Plate-trapped antibody (PTA)-ELISA technique has been shown to be sufficient in detecting some plant viruses in infected tissues. By using specific antisera *Sugarcane yellow leaf virus* (SCYLV) (Korimbocus et al. 2002) *Tomato yellow leaf curl virus* (TYLCV), *Tomato mosaic virus* (ToMoV) (Abouzid et al. 2002), *Cucumber mosaic virus* (Hristova et al. 2002) and *Sugarcane mosaic virus* (Jiang et al. 2003) have been detected reliably. Antigen-coated plate (ACP)-ELISA format was employed to detect *Maize streak virus* (MSV) and *Maize mottle chlorotic stunt virus* (MMCSV) in leaf samples from germplasm in Nigeria. The widespread occurrence of the virus emphasized the need for use of lines with built-in resistance (Taiwo et al. 2006). Likewise, triple antibody-sandwich (TAS)-ELISA also has been reported to be useful in detecting virus-infected plants. The viruses that could be detected successfully by TAS-ELISA are CMV (Hristova et al. 2002) *Faba bean necrotic yellows virus* (FBNYV), *Potato virus Y* (Ounouna et al. 2002) and CMV (Yu et al. 2005).

Application of ELISA formats for the detection of viruses not only in plants but also in soils has been reported. DAS-ELISA was developed for the extraction of *Pepper mild mottle virus* (PMMOV) from soil taken from green pepper (*Capsicum annuum*) fields and optimized for its detection. The samples giving positive results by DAS-ELISA, were verified by inhibition testing using specific anti-PMMoV antibody, immuno-electron microscopy, reverse transcription-polymerase chain reaction (RT-PCR) and biological testing on assay hosts. By using this test, soils with PMMoV may be reliably identified to avoid possible damage to green pepper crops (Ikegashira et al. 2004). Another soilborne virus, *Beet necrotic yellow vein virus* (BNYVV) causes rhizomania disease of sugar beet. The detection of BNYVV was carried out by planting sugar beet baiting plants followed by ELISA to diagnose virus infection. By following this procedure, the varieties which exhibited resistance when grown in BNYVV-infested soil from Salinas, California (CA) became susceptible when planted in BNYVV-infected soil from Imperial Valley, CA as reflected by ELISA results. This study suggested the difference in the virulence in the isolates BNYVV occurring in different areas (Liu et al. 2005).

2.2.1.3 Dot-Immunobinding Assay

Dot-immunobinding assay (DIBA) and ELISA are similar in principle, the microtiter plate being substituted by nitrocellulose membrane. DIBA may help to minimize the nonspecific interference, but requires blocking of the free protein-binding sites present in the membrane using bovine serum albumin (BSA) or nonfat dry milk powder or gelatin. Among the several advantages over ELISA, the simplicity of the test, short duration, possibility of easy storage and mobility and being less expensive

are the important ones. In a comparative test DIBA was found to be more sensitive in detecting *Tomato spotted wilt virus* (TSWV) in asymptomatic leaves and stems of infected plants (Berger et al. 1985). Seedborne infection of *Barley stripe mosaic virus* (BSMV) in barley and *Bean common mosaic virus* (BCMV) in French bean single seed or even in flour could be detected by DIBA (Lange and Heide 1986). The presence of *Lily symptomless virus* (LSV), *Tulip breaking virus* (TBV-L) and *Cucumber mosaic virus* (CMV) in the scale segments of *Lilium spp.* was revealed by DIBA (Nümi et al. 1999). *Grapevine rupestris stem-pitting-associated virus* (GRSPaV) present in leaf petioles and cortical scrapings from dormant grapevine canes could be more effectively detected by DIBA than ELISA during the whole vegetative season. Hence, DIBA was recommended for use in large scale survey to assess the virus disease incidence (Minafra et al. 2000).

2.2.1.4 Immunoblot Assays

The transfer of electrophoresed viral proteins from the gel matrix onto a membrane and making them accessible for subsequent analysis by specific immunoprobes constitute the basic principle of immunoblot technique. It is possible to detect viral capsid protein or non-structural proteins present in infected plants by employing PABs or MABs in this technique. Further, the serological relationship between viruses and strains can be established by applying immunoblot analysis. The P1 protein and a protein of approximately 25 kDa (P1-C25) of *Potato leafroll virus* (PLRV) were detected by Western blot analysis using PABs and MABs in PLRV-infected plants. P1-C25 is a proteolytic cleavage product during P1 processing during virus replication (Prüfer et al. 1999). Immunoblots probed with *Soybean dwarf virus* dwarfing (SbDV-D) PAB antiserum showed that SbDV-D and *Soybean dwarf virus*-yellowing (SbDV-Y) isolates were serologically related. The close serological relationship was indicated by the presence of a single 26 kDa CP band (Damsteegt et al. 1999).

Western blot analysis was used to identify and characteristize a new *Potyvirus* naturally infecting chickpea in Bolivia. The total protein sample preparations of *Chickpea yellow mosaic virus* (CYMV) were probed with the *Potyvirus* MAB on Western blots. A single band ca 32 kDa was seen in the total protein sample, whereas two distinct bands ca32 and 28 kDa were produced from purified virus preparations. When purified virus proteins were subjected to SDS-PAGE and silver staining, similar results were obtained (Fig. 2.3) [Appendix 7] As there was no reaction with antisera raised against potyviruses *Bean Yellow mosaic virus, Clover yellow vein virus, Cowpea aphidborne mosaic virus, Pea seedborne mosaic virus, Bean common mosaic virus* or *Bean common mosaic necrosis virus,* the CYMV was considered as a distinct member of *Potyviridae* (Larsen et al. 2003). For the detection of *Barley yellow dwarf virus* (BYDV-PAV) polyclonal antisera against protein encoded by ORFs 1 and 2 of a German ASL-1 isolate was developed using recombinant antigens expressed in *E.coli*. In Western blot analysis with total protein extracts from BYDV-infected plants antisera effectively recognized the 99 kDa fusion protein expressed from ORF1 and 2 (Fomitcheva et al. 2005).

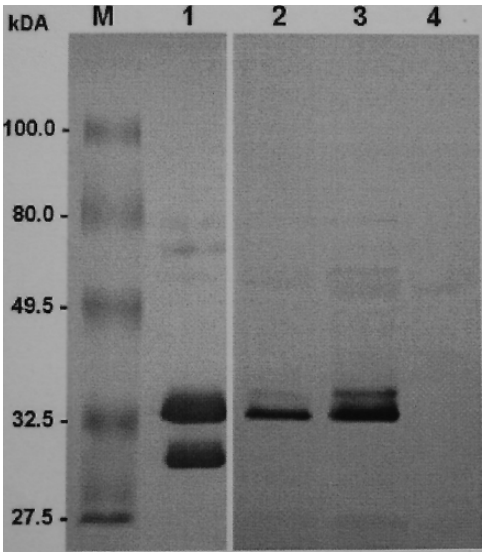

Fig. 2.3 Detection of new potyvirus *Chickpea yellow mosaic virus* (CYMV) infecting chickpea in Bolivia by Western blot using potyvirus group-specific monoclonal antibody
Lane M: Molecular markers (prestained); Lane 1: Nucleoprotein from purified CYMV virions; Lane 2: Total protein preparation form chickpea cv. Dwelley; Lane 3: Total protein preparation from chickpea cv. Dark Skin Perfection; Lane 4: Healthy chickpea. (Courtesy of Larsen et al. 2003; The American Phytopathological Society, St. Paul, MN, USA)

The coat proteins (CPs) of Begomoviruses such as *Bean golden mosaic virus* Brazil isolate (BGM-B) *Cabbage leaf curl virus* (CabLCV), *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV) may be expressed in the bacterium *Escherichia coli* and purified by SDS-PAGE technique. The polyclonal antisera prepared against PABs reacted positively in indirect (plate-trapping) – ELISA with extracts from Begomovirus-infected tissues. The polyclonal antisera prepared to expressed begomovirus CPs could be used for the detection of begomoviruses in an array of assays. Antisera to TYLCV and ToMoV reacted in indirect ELISA with extracts from begomovirus-infected tissues. The antisera to BGMV, CabLCV, TYLCV and ToMoV also reacted specifically with the test begomovirus antigens in leaf imprint blots and Western blots. CabLCV and TYLCV antisera could be used to detect BGMV antigens by immunogold labeling of thin sections of infected bean tissues. In tissue blot immunoassays, the TYLCV antiserum showed positive reaction with TYLCV antigens, but not with ToMoV antigens. But CabLCV antigens reacted well with ToMoV antigens and weakly with TYLCV antigens (Abouzid et al. 2002). A fragment of the CP and read-through domain of *Sugarcane yellow leaf virus* (SCYLV) was expressed in the bacterial expression systems. This protein after purification was used for the preparation of the monoclonal antibodies which were employed in PTA-ELISA and TBIA techniques for the detection of SCYLV (Korimbocus et al. 2002). Likewise, the polyclonal antibodies against the

LC5 isolate of *Little cherry virus* (LchV-LC5) coat protein produced in bacterial cells were employed for the detection of LchV-LC5 in infected cherry trees in the survey conducted in British Columbia, using ELISA (Theilmann et al. 2004).

2.2.1.5 Immunosorbent Electron Microscopy (ISEM) and Gold Labeling Techniques

The presence of plant viruses in infected plant tissues and crude extracts may be spot-checked rapidly by using ISEM. With the availability of MABs specific for viruses, it has become an useful technique not only for visualization of viruses in situ, but also to establish relationship between viruses. Greater sensitivity, rapidity, absence of cross-reactivity with plant proteins and the possibility of using crude antiserum without fractionation (in the case of polyclonal antisera) are some of the distinct advantages of ISEM compared with ELISA. *Banana streak virus* (BSV) forming a major constraint to banana production was detected in various samples collected from different locations in Uganda by employing ISEM (Harper et al. 2002). The usefulness of ISEM for the detection of *Citrus psorosis virus* (CPsV) in cross-protected citrus plants was reported by Martin et al. (2004). Furthermore, the location of viruses in tissues of vectors that transmit them from plant to plant can be observed as in the case of *Tomato spotted wilt virus* (TSWV) in thrips *Frankliniella occidentalis* (Nagata et al. 1999), *Rice ragged stunt virus* in planthopper *Nilaparavata lugens* (Lu et al. 1999), *Cucurbit aphid-borne yellows virus* (ABYV) in *Myzus persicae* and *Aphis gossypi* (Reinbold et al. 2003), *Tobacco rattle virus* in nematodes *Paratrichodorus anmones* (Karanastasi et al. 2000) and *Tomato ringspot virus* in *Xiphinema americanum* (Wang et al. 2002). The mealybug wilt in pineapple (MWP) is one of the most destructive diseases of pineapple. However, the exact cause(s) of the disease is yet to be resolved. One *Ampelovirus* species *Pineapple mealbug wilt-associated virus-2* is considered to be involved in the etiology of MWP. The relationship between an undescribed *Ampelovirus* sharing highest homology with PMWaV-1 and a putative deletion mutant sharing highest homology with PMWaV-2 was established by ISEM and reverse transcription polymerase chain reaction (RT-PCR) (Sether et al. 2005).

A new carmovirus infecting Angelonia plants (*Angelonia angustifolia*) causing flower break and mild foliar symptoms was isolated from plants grown in United States and Israel. Serological reactions in Western blot and decoration tests with *Angelonia flower break virus* (AnFBV) antisera were highly specific and provided clear and strong reactions with the polypeptide corresponding to the coat protein (Fig. 2.4). The antiserum did not react with the carmoviruses *Pelargonium flower break virus* (PFBV), *Saguaro cactus virus* (SgCV) and *Carnation mottle virus* (CarMV) in either ELISA or Western blots assays. Further, the antisera specific to these viruses reacted only with the homologous virus, but not with AnFBV. This indicated that these viruses were serologically distinct. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) technique revealed the presence of one major polypeptide band with an estimated molecular weight of 38 kDa.

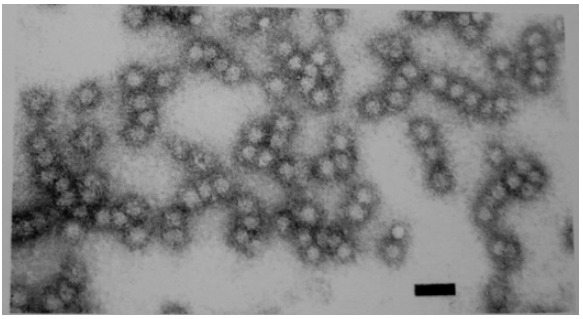

Fig. 2.4 *Angelina flower break virus* (AnFBV) particles immunodecorated with specific rabbit polyclonal antiserum raised against AnFBV
Bar = 50 nm. (Courtesy of Adkins et al. 2006; The American Phytopathological Society, St. Paul, MN, USA)

Immunoblots with AnFBV antisera provided a clear and strong reaction with the 38 kDa band establishing its identity as the AnFBV CP (Adkins et al. 2006).

2.2.2 Bacterial Pathogens

Bacterial plant pathogens are mostly single-celled organisms and more complex in structure compared with viruses. They have well-defined cell walls which have many kinds of specific antigenic determinants (epitopes). By employing specific antibodies as probes, variations in the composition of bacterial cell walls of different species, even pathovars within a species of bacteria may be recognized. The presence of pathogenic bacteria in seeds, clonal materials, plants and soil may be detected and differentiated by employing appropriate polyclonal and monoclonal antibodies. The immunoassays are especially useful for detecting latent/quiescent infections in plants and plant materials that do not express any visible symptoms. In addition, antisera may be prepared for specific recognition of components or metabolites of bacteria and they have been used for characterization of bacterial pathogens. It is possible to enhance the sensitivity and specificity of polyclonal antisera in two ways: (i) diluting the cross-reacting antibodies and (ii) cross-absorbing these interfering antibodies present in the antiserum (Narayanasamy 2005, 2006).

2.2.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Immunodiagnostic assays using both polyclonal antibodies (PABs) and monoclonal antibodies (MABs) have been employed for the detection of bacterial pathogens in seeds, tubers, other propagative materials as well as in whole plants or plant parts. These assays have been found to be particularly useful, when symptoms of infection are not clearly expressed or in the case of latent/quiescent infections

(Narayanasamy 2005, 2006). Among the immunoassays, enzyme-linked immunosorbent assay (ELISA) has been applied more frequently for the detection and differentiation of bacterial pathogens. Several bacterial pathogens such as *Clavibacter michiganensis* subsp. *sepedonicus* (De Boer and Hall 2000), *C. michiganensis* subsp. *insidiosus* (Kokošková et al. 2000), *Erwinia chrysanthemi* (Singh et al. 2000), *Xanthomonas axonopodis* pv. *citri* (Jim et al. 2001), and *X. axonopodis* pv. *dieffenbachiae* (Hseu and Lin 2000) have been detected by using different formats of ELISA. The usefulness of ELISA tests for the detection of other bacterial pathogens has also been highlighted earlier (Narayanasamy 2001).

The presence of *E. chrysanthemi* in potato stems and tubers was detected by using a specific MAB (6A6) in the triple antibody ELISA test. The detection limit of this test was 10^7 CFU/ml (Singh et al. 2000). *Ralstonia solanacearum* (*Rs*) causing potato bacterial wilt and brown rot of tubers is a quarantine organism in many countries. Latent infection of potato tubers which remain unrecognizable is one of the main mode of pathogen spread to other countries. Application of ELISA formats was found to be suitable for large scale certification (Elphinstone et al. 1996, 2000). The sensitivity of ELISA was shown to be enhanced by an enrichment step prior to ELISA on nitrocellulose membrane for the detection of the bacterial pathogen present in seed and tuber (Priou et al. 1999). As this bacterial pathogen can survive in the soil, the population level in the field soil has to be assessed to reduce infection of potato plants. An indirect ELISA was found to be sensitive in detecting as few as 10^4 CFU of *Rs* per gram of soil, when the bacterial suspension was incubated in a modified semi-selective medium prior to ELISA test (Pradhanang et al. 2000). A new semi-selective broth containing a potato tuber infusion was developed by International Potato Center (CIP) for incubation of potato isolates of *Rs* (273) belonging to five different biovars (BV) originating from 33 countries worldwide. By employing specific antibodies in DAS-ELISA format, *Rs* at low population levels could be detected after incubation of soil suspensions for 48 h at 30°C in the newly developed medium. The detection threshold for BV1 and BV2A were 20 and 200 CFU/g of inoculated soil respectively (Priou et al. 2006). The high risks of cross-reactions due to use of PABs could be significantly reduced by using specific MABs. A specific MAB 8B-IVIA reacted positively with 168 typical *R. solanacearum* strains and did not recognize 174 other pathogenic or unidentified bacteria isolated from potato. An initial enrichment step consisting of shaking the samples in modified Wilbrink broth at 29°C for 72 h was followed by application of DAS-ELISA. The test could detect 1–10 CFU of *R. solanacearum* per ml of initial potato extract. Commercial potato lots (233) were analyzed by the procedure and the results were similar to that of conventional methods. Detection of *R. solanacearum* in asymptomatic potato tubers by this enrichment DASI-ELISA may help to prevent the introduction of the pathogen into other countries (Caruso et al. 2002). Infection of cabbage seedlings by *Xanthomonas campestris* pv. *campestris* (*Xcc*) in the seed beds cannot be visually recognized. However, by applying ELISA format, it was possible to detect latent infection of seedlings before production of any visible symptoms of black rot or blight induced by different strains of *Xcc* (Shigaki et al. 2000).

Importation of maize seeds produced in the United States is prohibited by many countries to prevent the introduction of *Erwinia stewartii* (*Pantoea stewartii*) causing Stewart's wilt disease. Maize seeds should be certified as free from *E. stewartii* before export to other countries. An ELISA-based seed health test has been prescribed by the National Seed Health System as the standard method for phytosanitary seed health testing for *E. stewartii.* Among the four ELISA procedures tested to detect *E. stewartii* in pure culture and mixed corn-seed tissue, DAS-ELISA format using PABs for capture and MABs for detection was the most effective. The presence of viable *E. stewartii* in seeds from inoculated plants could be confirmed. There was an absolute positive correlation between recovery of bacteria and ELISA detection in eight seeds (Lamka et al. 1991). Samples of 100-kernel lots are tested for the presence of *E. stewartii*, using a commercially available ELISA reagent set (Agadia, Inc., Elkhart, IN) that is sensitive to detect about 10^5 CFU/ml. Samples are considered positive for *E. stewartii*, if the absorbance values at 490 nm are three times greater than the mean value of the negative control samples. The sensitivity of this ELISA-based seed health test to detect *E. stewartii* in maize seed was not affected due to treatment with fungicides and insecticides (Lamka et al. 1991; Pataky et al. 2004). [Appendix 8].

Development of effective disease management strategies depends on the knowledge of various sources of disease inoculum, especially when the pathogens has a wide host range of which some plant species may be symptomless carriers. One such pathogen is *Xylella fastidiosa* which is a xylem-limited bacterium causing destructive diseases in grapevine (Pierce's disease), citrus (variegated chlorosis) and pear (leaf scorch). By using the PathoScreen Kit (Agadia Inc., Elkhart, IN), over 60 species of plants were tested for the presence of *X. fastidiosa*. In comparison to PCR, ELISA test was found to be easier, less expensive and less time-consuming and the best method for screening the various plant hosts capable of serving as sources of infection of different strains of this bacterial pathogen (Costa et al. 2004). Detection of *X. fastidiosa* prior to symptom expression is critical for effective disease management. The efficiency of ELISA in detecting *X. fastidiosa* in whole tissue samples and xylem fluid samples from grapevine was compared. Collection of xylem fluid samples improved sensitivity of pathogen detection by ELISA (41.0%) compared with whole tissue samples (20.5%) in asymptomatic grapevine. No significant differences could be seen in the frequencies of detection by ELISA or PCR in the case of symptomatic grapevine plants (Bextine and Miller 2004). In another study, the leaves from the most basal nodes of grapevine plants were the most preferable tissues for detection of *X. fastidiosa* by ELISA format (Krell et al. 2006). Almond leaf scorch (ALS) disease caused by *X. fastidiosa* is a serious threat to almond production in San Joaquin Valley, California. ELISA test was as effective as polymerase chain reaction in detecting *X. fastidiosa* in asymptomatic almond trees during the field survey. The isolates of *X. fastidiosa* detected, consisted of mixtures of grape or "G-genotype" and almond or "A-genotype" strains. The immunoassays showed the presence of clusters of ALS-affected trees frequently occurring in the outermost orchard rows (Groves et al. 2005).

Xanthomonas axonopodis pv. *citri* (*Xac*) is known to occur in different strains with varying potential of causing canker disease on citrus. It is desirable to detect and identify the strains of *Xac*. The strain infecting Key/Mexican lime (*Citrus aurantifolia*) and alemow (*C. macrophylla*) trees could be differentiated as *Xac-A* and *Xac*-A ($^{\wedge *}$) strains using ELISA, PCR-based tests, fatty acid analysis pulse-field gel electrophoresis of genomic DNA and host specificity (Sun et al. 2004). *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) causing bacterial spot disease in tomato and pepper accounts for considerable loss in production. Specific monoclonal antibodies (MABs) against bacterial lipopolyaccharides were produced. The sensitivity and specificity of three selected MABs were tested in three ELISA formats. The most sensitive ELISA was a competitive one able to detect populations of *Xcv* as low as 10^3–10^4 CFU/ml. Bacterial proliferation in artificially inoculated tomato and pepper plants was successfully monitored and assays on field-collected samples reconfirmed the utility of the MABs for diagnosis of bacterial infection. Immunogold labeling with MAB 4AD2 showed the uniform distribution of the epitopes on the cell wall of *Xav* (Fig. 2.5) (Tsuchiya and d'Ursel 2004).

2.2.2.2 Immunofluorescence (IF) Technique

Polyclonal (PABs) and monoclonal (MABs) antibodies have been used for the detection of bacterial pathogens by applying direct/indirect immunofluorescene (IF) technique. *Pseudomonas andropogonis* infecting carnations (Li et al. 1993), *Xanthomonas campestris* pv. *undulosa* in wheat seeds (Bragard and Verhoyen 1993) and *Ralstonia solanacearum* in potato tubers (Elphinstone and Stanford 1998; van der Wolf et al. 1998) are some of the bacterial pathogens efficiently detected in the infected tissues. Antisera prepared against some bacterial cell components have

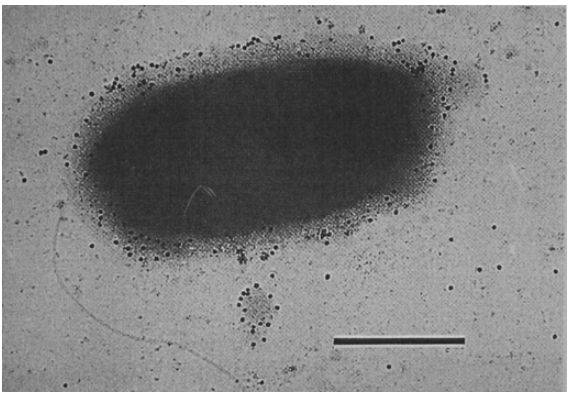

Fig. 2.5 Immunolabeling of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) 265 cells probed with the monoclonal antibody MAB 4AD2
Note the uniform distribution of epitopes on the cell wall of *Xcv.* Bar = 1µm. (Courtesy of Tsuchiya and d'Ursel 2004; The Phytopathological Society of Japan, Tokyo and Springer Verlag, Tokyo)

been found to be useful in detecting respective bacterial pathogens. Monoclonal antibodies specific to lipopolysaccharides of *R. solanacearum* and flagellin presenting the flagella of *Pseudomonas syringae* pathovars could be employed for detection and even differentiation of serotypes (Griep et al. 1998; Malandrin and Samson 1999). In the case of *Erwinia chrysanthemi*, use of MABs resulted in more sensitive detection without any cross-reactions that were seen when PABs were employed (Singh et al. 2000). Likewise, MABs or recombinant antibodies against the bacterial pathogens associated with tomato seeds such as *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* were able to react specifically with the pathogen concerned. This assay has the potential for large scale application, since it is economical, easy to perform and rapid giving the results in 4–5 days as against 30–45 days required for conventional methods (Veena and van Vuurde 2002).

2.2.3 Fungal Pathogens

Fungal pathogens, in most cases, can be isolated in appropriate media favoring their growth and reproduction. The morphological characters of asexual and sexual spores have been used as the basis for their identification and differentiation into species. Sets of differential plant species/cultivars have been used for the recognition of races/biotypes. But these classical methods require several days or even weeks to yield results. Even then, the results remain inconclusive in certain cases. Immunological techniques are able to provide results rapidly and precisely in a cost-effective manner. They can be automated and they may be advantageously applied widely for detection, differentiation and quantification of fungal pathogens. Monoclonal antibody technology has significantly enhanced the sensitivity and specificity of serological tests and helped to avoid cross-reaction with closely related species infecting the same crop/plants. The possibility of detecting the fungal pathogens in seeds and clonal materials such as tubers, bulbs, setts and cuttings which may not exhibit any visible symptoms is another important advantage for preferring immunological assays for disease diagnosis. Domestic and international plant quarantines apply different immunodiagnostic techniques to filter out infected plants and propagative materials to prevent the entry of new fungal pathogens into areas/countries where the pathogen (s) in question is absent or less important. Among the immunoassays, enzyme-linked immunosorbent assay (ELISA) has been employed more frequently. Dot-immuno binding assay (DIBA) and immunofluorescence (IF) assay also have been found to be effective in some pathosystems.

2.2.3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The presence of fungal pathogens in plant materials, soil and water has been detected by employing different ELISA formats to suit the requirements. Diagnostic kits with necessary reactants and instructions of manufacturers are available and the tests can be performed under field conditions also. The fungal pathogens

as well as their charateristic metabolic products such as enzymes and mycotoxins may be detected and quantified rapidly. Many destructive fungal pathogens such as *Aphanomyces euteiches* (Petersen et al. 1996), *Elsinoe fewcetii* (Tan et al. 1996), *Plasmodiophora brassicae* (Wakeham and White 1996), *Magnaporthe grisea* (Gergerich et al. 1996), *Verticillium dahliae* (Plasencia et al. 1996), *Colletotrichum falcatum* (Viswanathan et al. 2000), *Botrytis cinerea* (Meyer and Spotts 2000), *Ustilago scitaminea* (Nallathambi et al. 2001), *Uncinula necator* (Markovic et al. 2002) and *Ustilago nuda* (Eibel et al. 2005a) have been detected efficiently by using PABs or MABs in their respective hosts, some of them carrying the pathogen (s) without showing any visible symptoms.

The antisera may be prepared against antigens present on the surface of mycelium or spores. In some cases, antisera have been prepared against specific protein (s) extracted from the test fungal pathogens and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified mycelial proteins were used to generate PABs against *Verticillium dahliae* and these antibodies reacted positively with 11 of 12 isolates of *V. dahliae* from potato, cotton and soil, but it did react with tomato isolate. The presence of *V. dahilae* and *V. albo-atrum* in potato roots was revealed by DAS-ELISA (Sundaram et al. 1991). The fungal pathogens produce various spore forms at different stages in their life cycle and hence the antigenic nature of the pathogens may also vary. Antisera against the mycelium and ascospores of *Sclerotinia sclerotiorum* present on the petals of rapeseed flowers were tested for their efficiency in detecting the pathogen using DAS-ELISA format. The sensitivity of antimycelium serum (Smy) was greater compared with antiascospore serum (Ssp), indicating the variation in the protein profiles of mycelium and ascospores of *S. sclerotiorum* (Jamaux and Spire 1994, 1999). The results showed that appropriate fungal antigens should be used for efficient detection of fungal pathogens by immunoassays. The effectiveness of early detection of (at 12 h after inoculation of tea) *Pestalotiopsis theae* causing gray blight disease by employing PABs in indirect ELISA format was reported. The ELISA technique was effective in assessing very low level of infection and helped to initiate suitable management strategies (Chakraborty et al. 1996).

The polyclonal antiserum was prepared against the conidia from four genetically distinct isolates of *Uncinula necator* causing grapevine powdery mildew disease. The antibody reacted specifically to antigens present on both conidia and mycelium. Three antigens (MW21, 29 and >250 kDa) present on the conidia reacting with the PABs were recognized. The PAB detected homologous *U. necator* conidial antigens in a plate-trapped antigen (PTA)-ELISA, with linear range of detection extending from 1000 to 9000 conidia/ml at a 1:5000 dilution of antiserum. Monoclonal antibodies (MABs) were produced against the 21 kDa conidial antigen to avoid cross-reactivity with the fungal pathogens associated with grapevine. The MABs were specific to *U. necator* and could be used to group grapes cultivars on the basis of powdery mildew disease levels (Markovic et al. 2002).

Plate-trapped antigen (PTA)-ELISA has been shown to be effective in detecting fungal pathogens. The monoclonal antibodies were characterized by PTA-ELISA. The MABs were captured in wells in the microtiter plates coated with a dilution

of goat-antimouse IgG+IgM (Sigma No. M 8015) in PBST. The subclass of the captured antibodies was determined by probing with goat anti-mouse-peroxidase conjugates specific for the subclasses (IgA, IgG, IgM) (Bossi and Dewey 1992). PTA-ELISA format was employed to determine the sensitivity of MABs against the surface epitopes present on the conidia of *Stagnospora nodorum* causing leaf and glume blotch disease of cereals. By using two monoclonal antibodies, the compositional differences in the stage-specific secretion and development of extracellular mattrices (ECMs) secreted by *S. nodorum* could be studied (Zelinger et al. 2004).

Fungal pathogens elaborate different kinds of metabolites required for infection and breaking down complex materials for their nutrition. These compounds of pathogen/host origin may also be detected by immunoassays which are useful to differentiate strains/isolates of the pathogens based on the production of the specific substances. In grape berries infected by *Botrytis cinerea* causing gray mold disease, the activity of invertase was stimulated. In addition, a new invertase similar to that of *Botrytis* invertase (BIT) was also detected in infected grape berries. The anti-BIT-IgY antibodies generated in chicken were found to be very specific to BIT, indicating the possible use of BIT as a target molecule for immunological detection of *B. cinerea* (Ruiz and Ruffner 2002). Melanins derived from 1,8-dihydroxy naphthalene (DHN) have an important role in the pathogenicity and survival of fungal pathogens like *Alternaria alternata.* Competitive inhibition-ELISA format revealed that the phage-display antibody (scFV) M1 bound specifically to 1,8 DHN that was located in the septa and outer (primary) walls of wild type *A. alternata* conidia. It is possible to detect melanized fungal pathogens in different plant tissues by using M1 antibody (Carzaniga et al. 2002).

Detection of fungal pathogens in seeds and other propagative materials by conventional methods involving their isolation and cultivation in suitable medium has been not only time-consuming, but also difficult because of the fast-growing saprophytes associated with plant materials. ELISA formats allow sensitive and specific detection of several fungal pathogens belonging to different taxonomic groups or species. In addition, quantification of fungal pathogens in host tissue is also possible. The loose smut disease of barley caused by *Ustilago nuda* is internally seedborne. The pathogen is carried passively by the developing plant at the growing point which is transformed into a smutted ear at maturity. A DAS-ELISA with biotinylated detection antibodies, was applied to test naturally infected barley seeds. The results of the assay were comparable with that of conventional seed embryo test. In the case of artificial inoculation experiment, ELISA showed higher level of seed infection compared with embryo test. The results suggest the possibility of application of ELISA for early prediction of the efficacy of seed treating chemicals, elucidation of the pathogen biology and characterization of resistance mechanism operating in barley plants (Eibel et al. 2005a) [Appendix 9].

The fungal pathogens may contain unique proteins that may be used for preparing antisera which can be employed for detecting the pathogens concerned. *Tilletia indica* causing Karnal bunt of wheat has a protein (64 kDa) with antigenic properties. The antibodies reacted specifically with the pathogen cells in a microwell sandwich-ELISA and dipstick immunoassay (Kutilek et al. 2001). *Phytophthora*

fragariae infecting strawberry plants through roots could be detected by using PABs and MABs prepared against specific proteins of *P. fragariae* (Pekárová et al. 2001). Likewise, the MABs specific to the glycoproteins present in the cell walls of *Pythium sulcatum* were applied in indirect competitive ELISA format for efficient detection of *P. sulcatum* in naturally infected carrot tissues and in soil samples from fields where infected carrots were grown (Kageyama et al. 2002).

2.2.3.2 Dot-Immunobinding Assay

Dot-immunobinding assay (DIBA) provides an advantage over ELISA test in minimizing the nonspecific interference. *Phomopsis phaseoli* causes pod and stem blight, while *P. longicolla* is responsible for seed decay of soybean. These pathogens could be detected in asymptomatic tissues by using DIBA technique. Quantification of fungal antigens based on "antigen units" in place of absorption values was possible (Velicheti et al. 1993). Infection of wheat seeds by Karnal bunt disese caused by *Tilletia indica* was detected by the seed immunoblot assay (SIBA) (Anil Kumar et al. 1987), a variant of DIBA developed by Gleason et al. (1987) for the detection of *P. longicolla* in infected soybean seeds. The zoospores of *Phytophthora nicotianae* have a unique glycoprotein with a MW 40 kDa. The MAB generated against this glycoprotein efficiently detected the pathogen (Robold and Hardham 1998).

2.2.3.3 Western Blot Analysis

A polyclonal antiserum (A379) reacting with water soluble proteins from *Phytophthora cinnamoni* mycelium did not allow a clear-cut discrimination among congenerous species. But this antiserum exhibited positive reactions in Western blots against mycelial proteins from nine species of *Phytophthora* and *Pythium* sp. A species-specific protein of 55 kDa was immuno-decorated only in *P. cinnamomi* samples resulting in unambiguous identification of this pathogen. The antibody generated against this protein produced diagnostic bands of 55 and 51 kDa in *P. cinnamomi* only. This antiserum was found to be suitable for the specific detection and identification of *P. cinnamomi* emerging in distilled water from infected tissues of chestnut, blue-berry and azalea (Ferraris et al. 2004).

2.3 Detection of Microbial Plant Pathogens by Nucleic Acid-Based Techniques

Nucleic acid-based techniques are either based on the specificity by which nucleic acids (DNA or RNA) hybridize to form double-stranded molecules or detection of similarities between nucleic acids by using restriction enzymes to cleave DNA into fragments at or near a defined recognition sequence. Closely related organisms are known to share a greater nucleotide similarity than with distantly related ones. Nucleic acid sequences from the target organism hybridize only to the nucleic

acid from the related organism. Specific sequences can be identified, labeled and employed as a probe for hybridization with nucleic acid from target organism. Different hybridization methods such as colony and dot-blot hybridization have been performed for detection and qualitative differentiation to distinguish groups of plant pathogens, when a specific probe is available. By using appropriate restriction enzymes, restriction fragment length polymorphism (RFLPs) and analyes of natural variations in the genomes of different groups or strains of organisms can be assessed. The variations (polymorphisms) in fragment sizes can be generated by loss or gain of restriction endonuclease recognition sites, or by other events that change fragment sizes such as deletions or insertions in the DNA sequences. After digestion of the DNA with restriction enzymes followed by separation of the fragments by electrophoresis in agarose or polyacrylamide gels difference in the sizes of the DNA fragments can be resolved. The number and size of fragments produced after digestion is determined by the distribution of restriction sites in the DNA.

Availability of suitable probes that can discriminate between the DNA of the target and other organisms makes the nucleic acid hybridization assays specific. The probes are frequently derived by cloning double stranded genomic DNA or cDNA (by reverse transcription from mRNA) into plasmid or phage vectors followed by amplification in *Escherichia coli*. By screening the clones from libraries, clones specific for a pathogen can be identified. Nucleic acid probes may be labeled with radioactive (^{32}P or ^{35}S) or non-radioactive (biotin, digoxigenin, fluorescein, enzyme, steroid antigens) markers. The hazards associated with handling radioisotopes, despite the greater sensitivity of probes labeled with radioactive markers, have resulted in wider application of nonradioactive markers. Among the nonradioactive labels, biotin and digoxigenin labeled deoxyuridine triphosphate (Dig-dUTP) are incorporated into the DNA more frequently. Biotin can be readily cross-linked to deoxyribonucleotides and DNA with biotin is able to form stable complexes with avidin. Enzymes such as peroxidase and alkaline phosphatase (ALP) can be attached to avidin. The avidin enzyme conjugate is then used to detect the presence of biotin colorimeterically. The digoxigenin labeled hybrids can be detected by ELISA, by using an anti-digoxigenin antibody-ALP conjugate.

Enzymatic amplification of the signal probe or target nucleic acid sequences enhances the sensitivity and specificity of the diagnostic technique. Amplification of the target of the probe using the polymerase chain reaction (PCR) procedure has dramatically increased the sensitivity and specificity of diagnostic assays. The potential power of PCR lies in the possibility of amplifying a DNA fragment with known end sequences into billions of copies under specified conditions. Three temperature regimes (i) to melt the target DNA into single strands, (ii) to anneal specific oligonucleotide primers and (iii) to extend the primers by using a thermostable DNA polymerase are adopted. Since only a small amount (a few nanograms) of the initial template DNA either in the form of a discrete molecule or as part of a larger DNA for amplification and subsequent of detection is required, PCR has become the most important technique employed in plant pathology for various kinds of studies on plant-pathogen interaction, mechanisms of disease resistance, and disease epidemiology and management.

Nucleic acid-based techniques involving hybridization with and amplification of nucleic acid sequences have been shown to be useful to study various aspects of microbial pathogens and their interaction with host plants, in addition to detection, differentiation and quantification of pathogens and their strains/races/pathotypes: (i) production of disease-free seeds and propagative materials; (ii) prevention of introduction of new pathogens by domestic and international quarantines; (iii) field surveys to determine incidence and distribution in a geographical location/country (iv) detection and resolution of disease complexes; (v) identification of additional hosts to determine the manner of pathogen perpetuation; (vi) studying the nature of pathogen-vector relationships particularly in the case of viruses; (vii) screening to assess the levels of disease resistance in genotypes, cultivars and breeding lines; (viii) studying of the mechanisms of disease resistance of plants following incorporation of genes or induced by inducers of resistance and (ix) functions of transgenes of pathogen, plant and animal origins.

Nucleic acid-based techniques offer many advantages over immunological assays. The fungal and bacterial pathogens are complex antigens, compared to viruses. The nature of antigenic compounds present on the surfaces of pathogen cells may vary depending on the stage of the development, necessitating the requirement of different antisera for the detection of a fungal pathogen. But the pathogen genome is constant at all stages. Hence, the same test can be applied at different stages in the life cycle of the fungal pathogens. In the case of viruses, the viral coat proteins (CPs) represent only about 2.5% of the viral nucleic acids. Hence, the differences in the other segments of viral nucleic acid responsible for other biological characteristics cannot be determined by immuno-diagnositic techniques. Furthermore, the NA-based methods are more sensitive and specific providing results rapidly than immunological tests. For the detection of viroids which do not have protein capsids as the viruses, the NA-based procedures are the ones used for diagnosis exclusively.

2.3.1 Detection of Viral Pathogens

2.3.1.1 Hybridization Techniques

Dot-blot hybridization and microplate hybridization using radioactive and nonradioactive probes have been applied for qualitative detection and quantitative determination of plant viruses with RNA and DNA genomes. A ^{32}P-labeled DNA prepared from the N gene of *Calla lily chlorotic spot virus* (CCSV) was employed to hybridize with the ds-RNAs of CCSV, *Watermelon silver mottle virus* (WSMoV) and *Tomato spotted wilt virus* (TSWV). A weak signal was observed when the probe hybridized with ds-SRNA of WSMoV, whereas the probe did not hybridize with ds SRNA of TSWV indicating the relatedness between the viruses under test (Lin et al. 2005). Due to the hazards associated with the use of radioactive labels, their applicability has been very much limited. The use of digoxigenin (DIG)-labeled cDNA and cRNA probes has substantially enhanced the sensitivity of detection of viruses. DIG-labeled cDNA probes were useful for the detection of *Peanut-chlorotic streak*

virus (PCSV) (Satyanarayana et al. 1997), *Banana bract mosaic virus* (BBSV) (Rodoni et al. 1997), *Cucumber mosaic virus* (CMV) (Kiranmai et al. 1998) and *Pea seed-borne mosaic virus* (PSbMV) (Ali et al. 1998). Dot-blot hybridiation assay was found to be effective for the quantitative assay of geminiviruses.

Tomato yellow leaf curl virus (TYLC) was detected in infected tomato plants and whitefly vector *Bemisia tabaci*, using DIG-labeled probes. The virus was detected after a period of 30 min after acquisition and a single whitefly could acquire about 0.5–1.6 ng of TYLCV-DNA (Zeidan and Czosnek 1991; Caciagli and Bosco 1996, 1997). cDNA from the coat protein (*CP*) gene and the *hsp70* homolog protein gene from *Cucurbit yellow stunting disorder virus* (CYSDV) were prepared by RT-PCR from the viruliferous whiteflies (*Bemisia tabaci*) and cloned into plasmids. DIG-labeled cDNA probes reacted with the extracts of the viruliferous whiteflies applied on nylon membrane. The *hsp70* probe was employed to evaluate natural *B. tabaci* populations in commercial cucumber crops and concentrations of CYSDV per whitefly ranged from 5.6 fg to approximately 2.5 pg of corresponding *hsp*-cDNA (Ruiz et al. 2002).

The DIG-labeled probes could detect, in dot-blots, upto 10 fg of *Cucumber mosaic virus* RNA. It was possible to follow the pattern of accumulation of CMV-RNA in the inoculated leaves of bottle gourd plants (*Lagenaria siceraria*) (Takeshita et al. 1999). *Prunus necrotic ringspot virus* (PNRSV) was detected in peach shoots cultured at 4°C for long periods using cRNA probes (Heuss et al. 1999). The 3′ ends of RNAs of various isolates of *Apricot latent virus* have been sequenced, enabling the development specific riboprobes that can be used for their detection. In dot-blot assays the riboprobe pApr-47 specificially hybridized with the total nucleic acid (TNA) extracts from infected apricot and *Nicotiana occidentalis* (assay host) plants, but not with extracts from healthy controls. Further, there was no cross reaction with TNA extracts from plants infected by *Apple stem pitting virus* (ASPV), *Cherry green ring mottle virus* (CGRMV) or *Cherry necrotic rusty mottle virus* (CNRMV) isolates (Ghanen-Sabanadzovic et al. 2005). Northern hybridization technique was used to confirm the infection of rose geraniums (*Pelargonium* spp) by *Prunus necrotic ringspot virus* detected by ELISA format (Kulshreshtha et al. 2005).

Detection of two or more viruses simultaneously through the nonisotopic molecular hybridization technique was demonstrated by using a cocktail of specific single probes against viruses infecting vegetable crops (Saito et al. 1995) ornamental plants (Sánchez-Navarro et al. 1999) and stone fruit crops (Saade et al. 2000). A new strategy for the simultaneous detection of up to six viruses by molecular hybridization was developed. The sequences of two, four or six viruses were fused in tanden and transcribed to be employed as unique riboprobes named as "polyprobes". "Polyprobe four" (poly 4) could be used for the detection of four ilarviruses affecting stone fruit trees such as *Apple mosaic virus* (ApMV), *Prunus necrotic ringpsot virus* (PNRSV), *Prune dwarf virus* (PDV) and *American plum line pattern virus* (APLPV), whereas *Plum pox virus* (PPV) and *Apple chlorotic leaf spot virus* (ACLSV) were detectable by using "polyprobe two" (poly 2). Detection of any one of these six viruses was possible by employing "polysix" (poly 6) (Herranz et al. 2005). All the six viruses were successfully detected in 46 field samples taken from

infected stone fruit trees by molecular hybridization technique. Poly 2 to detect PPV and ACLSV, poly 4 to detect PNRSV, ApMV, PDV and APLPV and poly 6 to detect all the six viruses were equally effective as the respective individual riboprobe specific for the particular virus (Fig. 2.6) (Herranz et al. 2005).

The tissue-print hybridization, involves the transfer the viral nucleic acid from infected plant tissues directly on to nitrocellulose or nylon membrane followed by hybridization of the printed membrane with radioactive or nonradioactive chemiluminescent DIG-labeled probes. This technique is useful for studying the localization pattern of the virus in specific host plant tissues. The DIG-labeled probes could be employed efficiently for the detection and differentiation of *Citrus tristeza virus* (CTV) isolates from greenhouse and field (Narváez et al. 2000). *Artichoke latent virus* could be reliably detected in Globe artichoke field samples by employing either tissue imprint hybridization or one step RT-PCR technique (Lumia et al. 2003). Tissue-print hybridization technique has been shown to be the most suitable procedure for large scale testing in field surveys to assess the extent of CTV infection. This technique has several advantages: (i) no sample processing is necessary; (ii) imprinted membranes can be sent to far away laboratories for processing; (iii) no plant quarantine risk exists for the imprinted membranes and (iv) membranes can be stored for long periods before processing. The results reported in this study (after 2–3 years) were similar to those obtained with fresh or desiccated tissue (Martin et al. 2004).

2.3.1.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is considered as one of the major scientific development that has provided immense opportunities for studying various aspects of biological entities from primitive viroids to highly developed humans. PCR has been the widely applied technique for plant virus diagnosis, virus/strain differentiation and genome characterization. There are several conditions/factors that may interfere with PCR, resulting in reduction in the sensitivity of tests such as (i) presence of inhibitors in plant extracts, (ii) requirement of highly purified virus preparations. (iii) contamination from extraneous sources giving false positive results and (iv) labor-intensive sample preparation and more expensive compared with immunoassays. But PCR-based methods have been preferred because of their greater sensitivity, specificity and rapidity in providing results.

Detection of plant viruses with single-stranded (ss) DNA and double-stranded (ds)-DNA genome can be carried out directly, while a reverse transcription (RT) step is necessary to generate the complementary (c) DNA prior to PCR amplification (RT-PCR). Specific PCR and RT-PCR procedures have been developed for most of the viruses causing economically important crop diseases. The presence of plant viruses in their respective vector insects, nematodes and fungi has also been revealed by PCR assay (Narayanasamy 2001; Dietzgen 2002). A good quality DNA is the basic requirement for reliable detection of viruses by PCR. Addition of sodium sulphite improved the yield, quality and stability of the genome of *Citrus yellow mosaic virus* (CYMV), a non-enveloped bacilliform DNA virus infecting sweet

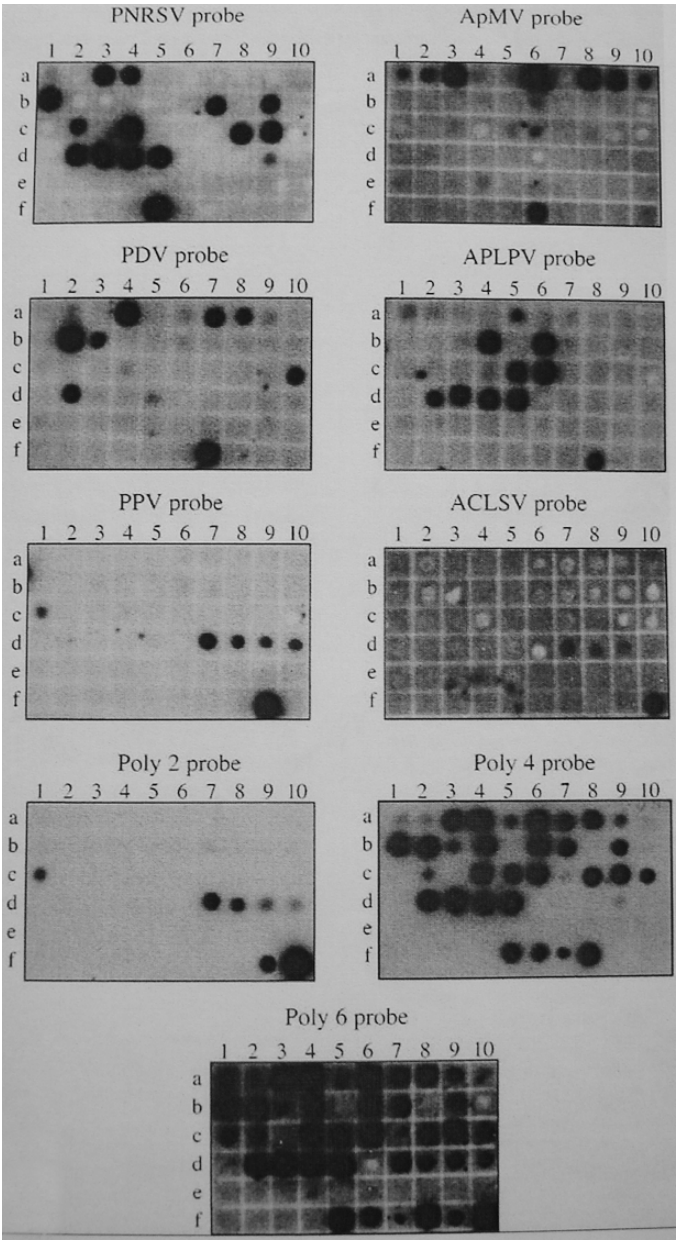

Fig. 2.6 Simultaneous detection of six stone fruit viruses by non-isotopic molecular hybridization, using virus-specific and polyprobes 2, 4 and 6
Viruses detected by specific probes: *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV), *Prunus dwarf virus* (PDV), *American plum line pattern virus* (APLPV), *Plum pox virus* (PPV) and *Apple chlorotic leaf spot virus* (ACLSV). Viruses detected by polyprobes: Poly 2 probe: PPV and ACLSV; Poly 4 probe: PNRSV, ApMV, PDV and APLPV; Poly 6 probe: All the six viruses. (Courtesy of Herranz et al. 2005; Elsevier, Oxford, UK)

oranges in India. Furthermore, the viral DNA extracted with sodium sulphite had greater stabilities at various temperatures compared with those extracted with the commercial kit (Quiagen) DNeasy kit (Baranwal et al. 2003). The specificity of PCR assays may be variable depending on the nature of universal, broad specificity as well as species-, genus-, or family-specific primers. These primers may be designed from conserved nucleotide or amino acid motifs shared by all or several members of the same taxonomic group. The sensitivity and specificity of PCR may be enhanced by using a second pair of primers nested within the original PCR product. With the increasing availability of information on viral nucleotide sequences provision of diagnostic PCR kits for the detection of many viruses rapidly is a distinct possibility. Application of PCR-based assays in the recent years to detect viruses in plants, clonal materials, and vectors that are involved in the natural mode of transmission has been shown to be more effective and advantageous resulting in the prevention of entry of new viruses and restriction of spread of viruses already present in a geographical location or country. *Banana bunchy top virus* (BBTV) in banana and the aphid vectors (Manickam 2000), *Citrus psorosis virus* (CPsV) (Martin et al. 2002, 2004), *Bean golden mosaic virus, Tomato mottle virus, Tomato yellow leafcurl virus* and *Sida golden mosaic virus* (Rampersad and Umaheran 2003; Maruthi et al. 2005), *Citrus tristeza virus* (Huang et al. 2004), *Plum pox virus* (Spiegel et al. 2004), *Grapevine fan leaf virus* in nematode vector *Xiphinema index* (Finetti-Sialer and Ciancio 2005), *Tomato spotted wilt virus, Impatiens necrotic spot virus, Water melon silver mottle virus*, *Melon yellow spot virus Tomato yellow leaf curl virus* (Ueda et al. 2005) and *Iris yellow spot virus* (Lin et al. 2005; Uga and Tsuda 2005) are some of the plant viruses detected by using PCR-based techniques. A rapid and simple PCR-based detection method was developed to distinguish four genetic groups viz., Ng, Sz, Ai and Tosa of *Tomato yellow leaf curl virus* (TYLCV) (Ueda et al. 2005). A variant PVYN-W of *Potato virus* Y (PVY) causes symptomless infection, becoming a concern in seed potato production. By using primers located in Hc-Pro region and NIa region of the viral genome, a PCR protocol was developed for amplication of PVYN-W only, but not other strains of PVY. This technique is rapid and easy-to-use and suitable for large scale application (Glaisa et al. 2005).

Detection of viruses in seeds and propagative materials for eliminating the infected plants/propagative materials, is a mandatory requirement of quarantine and certification programs. Certification for freedom from viruses like *Strawberry vein banding virus* (Mráz et al. 1997), *Narcissus yellow stripe potyvirus, Narcissus silver streak potyvirus* and *Narcissus late season yellows potyvirus* (Langeveld et al. 1997), *Apple stem pitting virus* (Nemchinov et al. 1998) and *Little cherry clostero virus* (Jelkmann et al. 1998) is essential to permit the plant materials. The presence of *Cowpea aphidborne mosaic virus* (CAMV) in peanut seeds could be more efficiently detected, compared with immunoassays. Samples consisting of one infected among 100 leaves gave positive reaction in RT-PCR tests (Gillapspie Jr. et al. 2001). Isolates of *Lettuce mosaic virus* (LMV)-Most are seedborne in lettuce cultivars with *mo1* gene. RT-PCR assay using primers designed to amplify a central region of the genome provided sensitive detection of LMV-Most in situations of single as well as mixed infections (Peypelut et al. 2004). RT-PCR tests with *Wheat streak*

mosaic virus (WSMV) – specific primers proved the seed transmission of WSMV in eight different wheat genotypes at rates 0.5–1.5% unequivocally (Jones et al. 2005). *Banana bunchy top virus* (BBTV) could be detected in banana plants as well as in aphid vectors by using PCR assay which was 1000 times more sensitive than dot-blot hybridization and ELISA tests (Xie and Hu 1995; Manickam 2000). *Plum pox virus* (PPV) was detected in both seed coat and cotyledons of apricots by RT-PCR assay. On the other hand, ELISA test could detect PPV only in seed coat, indicating the higher sensitivity of RT-PCR in detecting PPV in plant tissues with low virus concentration (Pasquini et al. 1998). A rapid reliable RT-PCR format was developed for the detection of *Onion yellow dwarf virus* (OYDV) infecting onion and garlic. Primers were designed from conserved RNA-dependent RNA polymerase and 3′-UTR region for detection of OYDV in garlic and onion. The leaf samples from different states in India were tested. The presence of approximately 1.1 kb fragment indicating the OYDV infection was observed. The immunoassay could not detect the OYDV in onion using antisera raised against garlic isolates. The RT-PCR assay was more sensitive than ELISA test for OYDV detection (Meenakshi Arya et al. 2006).

It is possible to detect plant viruses in crude plant extracts by applying RT-PCR technique, eliminating the need for having purified virus preparation. *Cucumber mosaic virus* (CMV) in crude extracts was detected by employing primers complementary to conserved sequences of CMV-RNA3 for broad spectrum detection of isolates belonging to subgroups I and II from different geographical locations (Blas et al. 1994). A modified RT-PCR technique was successfully applied for the detection of *Prunus necrotic ringspot virus* (PNRSV) in dormant peach trees. Furthermore, this technique could be used for screening imported budwood materials in post-entry quarantine (PEQ) programs and also for generating virus-free planting materials (Spiegel et al. 1996). The PCR assay was applied for the detection of begomoviruses in extracts of tomato, *Sida acuta*, *S. rhombifolia, Calapogonium mucunoides* and *Rhynchosia minima* known hosts of geminiviruses (Rampersad and Umaheran 2003). The RT-PCR assay targeting the coat protein (CP) gene in RNA3 of *Potato mop top virus* (PMTV) was highly effective in detecting the virus in potato seed tuber lots and ware potato during the surveillance in United States and Canada (Xu et al. 2004). The PCR assay based on degenerate primers SPG1/SPG G were employed to detect nine uncharacterized isolates of geminiviruses infecting sweet potato including *Sweet potato leaf curl virus* (SPLCV-Taiwan). The assay detected the viruses in highly diluted samples ($10^{\wedge -9}$) proving the PCR to be very sensitive and specific (Li et al. 2004). The presence of viruses causing symptom resembling *Beet curly top virus* (BCTV) in pepper plants was detected by using primers designed to detect a portion of CP gene and primers to detect a portion of replication-associated protein (rep) gene. Field isolates exhibiting homology to *Beet mild curly top virus (BMCTV)* and *Beet severe curly top virus* (BSCTV) were identified (Creamer et al. 2005). Seoh et al. (1998) demonstrated that by using a single pair of PCR primers two unrelated viruses *Cymbidium mosaic potex virus* (CyMV) and *Odontoglossum ringspot tobamovirus* (ORSV) infecting orchids could be effectively detected simultaneously. Primers based on specific virus sequences or components of viral nucleic acid can be used for virus detection. By employing

sequence-specific primers in RT-PCR technique, the pathotypes P1 and P4 of *Pea seedborne mosaic virus* (PSbMV) were detected (Kohnen et al. 1995).

Specific detection of molecular variants of Grapevine virus A (GVA) was reported by application of RT-PCR assay. GVA from various grapevines and isolates recovered from grapevines in *Nicotiana benthamiana* were detected and identified by RT-PCR (Goszczynski and Jooste 2003). The DNA of *Tomato leaf curl virus* was amplified from tomato plants showing mild and severe symptoms by PCR. An isolate of the bipartite *Tomato leafcurl New Delhi Virus – Severe* (ToLCNDV-Svr) was found to be associated with induction of severe symptoms, whereas a monopartite virus, *Tomato leaf curl* Joydebpur virus-Mild (ToLCJV-Mld) was present in plants with mild symptoms (Maruthi et al. 2005). Identification of *Soybean mosaic virus* (SMV) strains by symptom phenotypes was well correlated with RT-PCR/RFLP analysis. A primer pair that amplified a 1385-bp fragment of the cylindrical inclusion (CI) coding region of SMV was employed for the identification of five strains G2, G5, G5H, G7 and G7H, as well as the seedborne SMV isolates from soybean cultivars. The sensitivity of the RT-PCR enabled detection of SMV from plants with necrotic symptoms in which the virus titre was too low to be detected by ELISA, indicating the higher level of sensitivity of PCR assay (Kim et al. 2004). RT-PCR was more sensitive than ELISA in detecting *Prune necrotic ring spot virus* (PNRSV) and *Prune dwarf virus* (PDV) with the additional advantage of being able to detect the viruses at any time throughout the growing season (Mekuria et al. 2003). RT-PCR using the CP-specific primers detected *Rupestris stem pitting virus* (RSPaV) in all samples including those from symptomless plants. In contrast a pair of primers designed from the replicase gene detected RSPaV only in symptomatic plants (Habili et al. 2006). The efficacy of RT-PCR ELISA and DTBIA for the detection of non-decline inducing and decline inducing isolates of *Citrus tristeza virus* (CTV) in sweet orange and grapefruit plants was assessed. RT-PCR assay was not only able to detect the CTV isolates, but also to differentiate the decline-inducing and non-decline inducing isolates of CTV. Both isolates could be detected in a single field-infected sweet orange or grapefruit tree. The results showed that RT-PCR had a greater sensitivity than immunoassays (Huang et al. 2004). *Plum pox virus* (PPV) was detected in wild apricot and cultivated plum in germplasm entries in Kazakhstan by RT-PCR assay using primers that amplified a 243-bp fragment in the C-terminus of coat protein (CP) coding region. The isolates from wild apricot and plum cultivars were identified as D strain (Spiegel et al. 2004) [Appendix 10].

The RT-PCR assay has found wide applicability for the detection of a wide range of viruses in the recent years. In the case of the *High Plains virus* (HPV) causing a potentially serious economic disease of cereals, a procedure for inspecting plants and testing cereal seedlings in quarantine and testing cereal seedlings in quarantine using RT-PCR was developed (Lebas et al. 2005b). With the availability of nucleotide sequence data for many viruses infecting fruit trees, the applicability of RT-PCR assay as a routine tool for disease diagnosis has become feasible. Cellular location of *Prune dwarf virus* (PDV) in young leaves and flower buds could be visualized by in situ RT-PCR technique. The CP gene of PDV was used as target to produce a cDNA copy that was amplified by PCR and visualized using a

direct detection method using digoxigenin-labeled nucleotides (Silva et al. 2003). A simplified single tube RT-PCR protocol for the detection of *Little cherry virus* 1 and 2 (LChV-1, LCHV-2) had reduced unspecific amplification that resulted in false positive results. This protocol could be used for reliable detection of both viruses in different types of leaves of cherry (Rybak et al. 2004). As the RT-PCR was effective in detecting several plant viruses, attempts were made to develop simple and rapid methods such as direct binding (DB)-RT-PCR (Rowhani et al. 1995) and tube capture (TC)-RT-PCR procedures (James 1999). These procedures are easy and useful for efficient detection of viruses infecting woody plants. However, long time (several hours) was required to extract viral RNAs from infected tissues. An easy and rapid procedure designated simple-direct-tube (SDT) method was developed for preparing viral RNA for cDNA synthesis. This method may be completed in approximately 15 min and does not require the use of antiserum, filtering or centrifugation. This method involves grinding of plant tissues in phosphate-buffered saline containing Tween-20 (PBST) and placing the extract in a microfuge tube for a few min and allowing absorption of virus particles to the tube wall. *Turnip mosaic virus, Cucumber mosaic virus* and *Cucumber green mottle mosaic virus* were readily detected using SDT protocol (Suehiro et al. 2005) Chickpea chlorotic stunt virus was detected and identified using degenerate primers in RT-PCR for amplification of the CP coding region (Abraham et al. 2006).

In the nested PCR technique, two PCRs are performed. In the first reaction, the amount of template for the second reaction is increased, making the detection effective particularly for the virus occurring in low titres or when inhibitors of DNA polymerase are present in the host tissue extract. In this technique, a combination of degenerate deoxyinosine (dI)- substituted primers amplified part of the RNA-dependent RNA polymerase (RdRP) domain, followed by a semi-nested PR amplification that increased the sensitivity of virus detection is employed. If viral genomic dsRNA or viral RNA is not available RNA extracts from infected plants can be used for testing. *Potato black ringspot virus* (PBRSV) and *Cherry leaf roll virus* (CLRV) were detected efficiently (Maliogka et al. 2004). Viruses belonging to *Vitivirus* and *Foveavirus* species infecting grapevines were detected by the protocol developed by Dovas and Katis (2003). *Artichoke yellow ringspot virus* (AYRSV), a member of the family *Comoviridae* was detected in onion crops by using degenerate primers specific for *Comoviridae* in RT-PCR assay. Based on the RNA dependent sequence analysis and comparison with AYRSV isolates from *Cyanara scolymus* (AYRSV-AtG) and *Vicia faba* (AYRSV-F), the virus isolate infecting onion was identified as a nepovirus infecting onion under field conditions (Maliogka et al. 2006).

A nested RT-PCR protocol was developed for the detection and identification of *Comoviridae* species. A polyvalent nested RT-PCR assay using degenerate primers containing inosine was developed for the detection of filamentous virus belonging to genera *Trichovirus, Capilovirus* and *Foveavirus*. The 362-bp product was amplified from nucleic acid extracts from *Prunus* and *Malus* leaf samples. All targeted viruses were detected by this technique (Foissac et al. 2005). The detection of *Bean leaf roll virus* (BLRV) was optimized by using selective precipitation of BLRV-RNA by LiCl from small amounts of infected plant tissues followed RT-PCR procedure.

Required quantity and quality of extracted viral nucleic acids seem to be a critical factor for successful detection of vectors. *Aphis fabae* was earlier considered to be a nonvector of BLRV. However, it was found that *A. fabae* could acquire BLRV from infected plants using this procedure (Ortiz et al. 2005). The presence of *Sugarcane yellow leaf virus* (SCYLV) was detected using RT-PCR assay in the leaves, shoots and roots of all sugarcane cultivars tested. The cv. R575 was most severely infected by SCYLV reaching a mean of 98% infected stalks. Furthermore, SCYLV was also detected by RT-PCR in the aphid vector *Melanaphis sacchari* (Rassaby et al. 2004). By employing primers designed based on the highly conserved RNA-1 segment of the bipartite genome of *Tobacco rattle virus* (TRV) in RT-PCR procedure, TRV was detected in individual *Trichodorus* spp., the nematode vectors of TRV (Boutsika et al. 2004). The possibility of the aphid vectors *Myzus persicae* and *Aphis spiraecola* transmitting *Plum pox virus* (PPV) after feeding on PPV-infected peach fruit to peach seedlings was indicated by RT-PCR assay. Primers based on the sequences of the CP gene of PPV amplified the viral fragments from the inoculated seedlings confirming the positive transmission of PPV from infected fruits which may serve as sources of PPV (Gildow et al. 2004).

Detection of *Potato yellow vein virus* (PYVV) a quarantine pathogen in the European and Mediterranean Plant Protection Organization (EPPO) area has to be rapid and reliable to prevent its introduction or restrict its further spread. A sensitive, high throughput, real-time reverse transcription (RT)-PCR assay based on TaqMan® chemistry was developed for efficient detection of PYVV in tubers, in addition to the conventional RT-PCR protocol. Although real-time RT-PCR technique was more sensitive (1000-folds) compared with conventional PCR assay, the latter method may be preferred as an alternative to the real-time technique in some laboratories due to nonavailabilty of sophisticated equipment. These two methods may assist in enforcing quarantine regulation by reliable detection of PYVV and in routine indexing of PYVV for production of virus-free seed potatoes in areas of South America where incidence of PYVV is quite high (López et al. 2006).

A *Peach mosaic virus* (PcMV)-specific RT-PCR was developed by employing the PcMV-dervied primers PM16AFF and PM16AFR for screening a range of virus isolates representing different genera within the family *Flexiviridae*. These primers targeted the 3′ terminus of the coding region of the replication protein (ORF1) and amplified a fragment of 419-bp. Since there were cross reactions with certain *Apple chlorotic leaf spot virus* (ACLSV) isolates in the conventional RT-PCR procedure, a semi-nested RT-PCR using cDNA generated by PM16AFR for amplification with the PM-AF1 and PM-AFR primers was formulated. The PcMV-specific fragments were amplified making this protocol to be very useful for specific detection of this virus (Fig. 2.7) (James et al. 2006).

A simple and sensitive diagnostic procedure involving RT-PCR in combination with restriction analysis of the amplification products with *HindIII* and *PvuII* endonucleases was developed. The *Sweet potato virus 2* (SPV2; synonymous with *Sweet potato virus Y* and *Ipomea vein mosaic virus*) a tentative member of the genus *Potyvirus* and the *Sweet potato feathery mottle virus* (SPFMV) were detected and differentiated by employing oligoT (25) primer for reverse transcription and

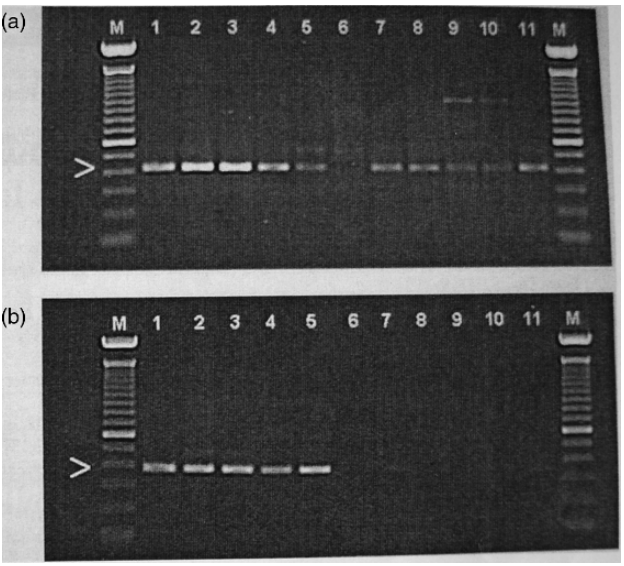

Fig. 2.7 Detection of *Peach mosaic virus* (PcMV) by RT-PCR analysis using primer sets (**a**) (PM16AFF/PM16AFR) for amplification of a 419-bp fragment and (**b**) (PMAF1/PMAFR) for amplification of a 383-bp fragment. Lane M: 100 bp DNA ladder; Lane 1: PcMV CA1 in peach; Lane 2: Another accession of PcMV CA1 in peach; Lane 3: PcMV CA2 in peach; Lane 4: PcMV CA3 in peach; Lane 5: PcMV CL2 in peach; Lane 6: Healthy peach; Lane 7: *Apple chlorotic spot virus* (ACLSV); Lane 8: ACLSV isolate 996-1A in apple; Lane 9: ACLSV isolate 982-11P5 in apple; Lane 10: Another sample of ACLSV isolate 982-11P5 in apple; Lane 11: Another sample of ACLSV isolate 1288-4 in peach. Arrow heads indicate the amplified fragment in the infected samples. (Courtesy of James et al. 2006; The American Phytopathological Society, St. Paul, MN, USA)

combination of degenerate primers for PCR amplification (Tairo et al. 2006). Specific primer pairs for the detection of four genotype BRA-(Brazil), CUB-(Cuba), PER-(Peru) and REU-C (Réunion Island) of *Sugarcane yellow leaf virus* (SCYLV) were used in RT-PCR assay. The presence of these genotypes of SCYLV was detected in 245 leaf samples collected from 18 different sugarcane growing locations in the world. Most of the samples were found to be infected by one of the three genotypes, but some samples showed mixed infections by more than one genotype (Ahmad and Royer 2006).

Several modifications of standard PR protocol have been made to suit the needs of host-virus combination to be examined. The spot-PCR was developed to detect *Grapevine A virus* (GAV), *Grapevine B Virus* (GBV) and *Grapevine leaf roll- associated virus 3* (GLRaV3). The genomic fragments were specifically amplified by employing 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 been earlier spotted. A heat treatment at 95°C for 10 min enhanced the ease of release of viral template from the spot on nylon membrane. The spot-PCR had similar sensitivity as standard PCR assay in addition to the additional advantage of

storing the blots for about 1 month after spotting (Notte et al. 1997). Detection of immobilized amplified product in a one-phase system (DIAPOPs), variant of PCR assay was developed for the detection of *Potato virus* Y (PVY) and its strains in dormant potato tubers (Nielsen et al. 1998). An RT step was included prior to DIAPOPS for enhancement of sensitivity. The sensitivity of RT-DIAPOPS was 0.5 pg, when virions were added directly to the RT reaction. All isolates of PVY, representing all taxonomic groups of PVY could be detected by this technique. The distinct advantage of RT-DIAPOPS is that there is no need for transfer of PCR products to gels for detection, thus avoiding the risk of contamination (Nicolaisen et al. 2001) [Appendix 11].

Amplification of two or more specific DNA fragments simultaneously can be done by using a combination of several primers in the same PCR assay. Such multiplex RT-PCR methods have been applied to detect multiple species of a virus in a single plant as in the case of detection of *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* in orchids (Seoh et al. 1998), *Soilborne wheat mosaic virus* and *Wheat spindle streak mosaic virus* (Gitton et al. 1999), three ilarviruses affecting stone fruit trees (Saade et al. 2000), six citrus viroids and *Apple stem grooving virus* from citrus (Ito et al. 2002) and Criniviruses causing yellowing disease in tomatoes in Greece (Dovas et al. 2002). A multiplex RT-PCR, using a cocktail of nine primers was developed for the detection of five seedborne legume viruses by Bariana et al. (1994). Similarly detection of many viruses infecting banana (Sharman et al. 2000) and peanut (Dietzgen 2002) was possible by employing multiplex PCR formats. This approach further enlarged by dovetailing the GPRIME, a computer program for enabling the identification of the best regions of aligned genes to target in nucleic acid hybridization assays. The redundant primers for RT-PCR assay were designed by using GPRIME for the detection of *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* (ORSV) and *Ceratobium mosaic virus* (CcMV) infecting orchids (Gibbs et al. 1998) In a study to assess the comparative efficacy of conventional RT-PCR and multiplex RT-PCR techniques, both tests gave similar results for the detection of *Prunus necrotic ringspot virus* and *Plum pox virus* (PPV). Multiplex RT-PCR could be used for testing a limited number of samples to verify the health status of plant materials of especially when ELISA tests could not provide reliable results (Kölber et al. 1998). The multiplex PCR provides certain advantages over standard PCR assay such as simultaneous detection and differentiation of several viruses/strains in the same sample and saving of time and reagents. The critical factors such as suitable relative concentrations of the primers and concentration of the PCR buffer have to be provided. These methods need the treatment of amplified PCR products with restriction endonucleases to identify the virus species.

An improved multiplex one-step RT-PCR was developed for the simultaneous detection and identification of different viruses infecting the same host plant species. A single universal degenerate primer (actually a mixture of primers in which the nucleotides at one or more defined positions vary by design to represent a consensus sequence) corresponding to the 3′- noncoding region conserved among tospoviruses was used with five species-specific primers against viral S RNAs. The distinct

advantage of this one-step RT-PCR system is its capacity to detect and discriminate individual tospoviruses, *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INS), *Watermelon silver mottle virus* (WSMoV), *Iris yellow spot virus* (IYSV) and *Melon yellow spot virus* (MYSV) and coinfections without the need for restriction endonuclease treatment or serological methods. Furthermore, the limit of sensitivity when detecting a plural viral RNA infection was comparable to that of a single tospovirus detection using single gene-specific primer pairs, resulting in saving of substantial time that will be required for the detection of viruses one after another. This technique, appears to be the first of its kind, was successfully applied for screening 18 infected cultivated plants of six different species that were harvested in crop fields against the five tospoviruses which could be simultaneously detected (Uga and Tsuda 2005). A multiplex RT-PCR technique was developed for the simultaneous detection of *Strawberry crinkle virus* (SCV), *Strawberry mild yellow edge virus* (SMYEV), *Strawberry mottle virus* (SMoV) and *Strawberry vein banding virus* (SVBV). All combinations of 18 isolates of the viruses could be detected. The upper detection limit of all the four viruses was at an extract dilution of 1/200 (Thompson et al. 2003). *Beet necrotic yellow vein virus* types A and B were detected and differentiated by a multiplex RT-PCR protocol that could be performed in a single PCR tube (Ratti et al. 2005).

Seven viruses infecting citrus viz., *Citrus leaf rugose virus* (CLRV), *Citrus psorosis virus* (CPsV), *Citrus tatter leaf virus* (CTLV), *Citrus tristeza virus* (CTV), *Citrus variegation virus* (CVV), *Citrus yellow mosaic virus* (CYM) and *Indian citrus ringspot virus* (ICRSV) belonging to six different virus genera were detected simultaneously by the multiplex PCR (mPCR) assay. Degenerate primers were designed based on the sequences of respective virus isolates. The cDNA fragments (245–942 bp) specific to the viruses were simultaneously amplified using mPCR and they were identified on the basis of their molecular sizes. The diagnostic technique reduces the risk of contamination, saves time and reduces the cost as compared to other conventional methods employed for citrus virus detection. Furthermore, the mPCR provides a useful rapid method for detecting multiple virus infections in citrus plants that may be required for production of virus- free citrus plants for certification programs (Roy et al. 2005).

A one-step RT-PCR technique was developed to detect and differentiate eight important viruses infecting stone fruit trees viz., *Apple mosaic virus* (ApMV), *Prunus necrotic rinspot virus* (PNRSV), *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* (PBNSPaV). A large number of virus combinations was detected and up to three different viruses were observed in five samples. The sensitivity of the detection by this assay was reduced, when the primer cocktail contained more than five different pairs of primers. However, the test involving the eight virus primers pair was more sensitive than the ELISA and molecular hybridization methods (Sánchez-Navarro et al. 2005). Likewise, a one-step RT-PCR was employed to detect and identify eight viruses infecting olive trees commonly, such as *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Cucumber mosaic virus* (CMV), *Olive leaf*

yellowing-associated virus (OLYaV), *Olive latent ring spot virus* (OLRSV), *Olive latent virus-1* (OLV-1), *Olive latent virus-2* (OLV-2) and *Strawberry latent ring spot virus* (SLRSV). Among the eight viruses assayed OLYaV was the most predominantly occurring virus in Southern Italy, while SLRSV was detected more frequently in the central Italy (Faggioli et al. 2005). *Bean yellow mosaic virus* was detected by using one step RT-PCR from crude sap of infected dwarf gentian plants (*Gentina scabra*) at dilutions upto 10^6-fold indicating that this technique was 100 times more sensitive than indirect ELISA format, and equally sensitive as IC-RT-PCR protocol (Uga 2005).

Melon necrotic spot virus (MNSV) is water and soil-borne, infecting members of Cucurbitaceae. Water samples from a water source pool of a hydroponic culture or from the recirculating nutrient solution were concentrated by ultracentrifugation or precipitation by polyethylene glycol (PEG) followed by RT-PCR analsysis. Non-isotopic riboprobes specific to MNSV were employed to detect MNSV in roots, stems, cotyledons and young leaves of mechanically inoculated melon plants. The virus concentration was maximum in roots. In addition, MNSV was detected in the water samples, making this report to be the first to detect this virus in water (Gosalvez et al. 2003). Mealybug wilt of pineapple (MWP) is reported to have the involvement of an *Ampelovirus* species and mealybug feeding. Two ampeloviruses, *Pineapple mealybug associated virus-1* (PMWaV-1) and PMWaV-2 have been detected in the affected plants (Hu et al. 1996; Sether et al. 2001; Melzer et al. 2001). By using degenerate oligonucleotide based on conserved sequences of related viruses in RT-PCR, the putative PMWaV-3 was detected in pineapple. Sequence analysis of the C-terminal portion of the RdRp, the complete ORF for a small hydrophobic protein and the N-terminal portion of the HSP-70-like ORF suggest that putative PMWaV-3 was a distinct virus and not a strain of PMWaV-1. The specific RT-PCR protocol efficiently detected the putative PMWaV-3 (Sether et al. 2005).

A multiplex RT-PCR procedure for simultaneous detection of five potato viruses using 18S RNA as an internal control was able to detect *Potato virus A, PVX, PVY, Potato leafroll virus* and *Potato virus S* individually and in different combinations in potato tubers. This assay was more sensitive (100-fold) for detection of PVX than the commercial DAS-ELISA format. In addition, this multiplex RT-PCR technique detected viruses in some samples that were DAS-ELISA negative (Du et al. 2006).

Grapevine (*Vitis* spp.) are known to be infected by several viruses. The most important strategy for controlling virus diseases is the use of virus-free propagation materials for which a reliable and specific detection technique providing results rapidly is necessary. The immunoassays were unsatisfactory, because virus titre in plant tissues was low. Multiplex RT-PCR (mRT-PCR) protocol for detecting 2–5 plant viruses has been adopted for virus detection (Lopez et al. 2003). A protocol for simultaneous detection of nine grapevine-infecting viruses, *Arabis mosaic virus* (ArMV), *Grapevine fan leaf virus*, *Grapevine fleck virus*, *Grapevine leafroll-associated virus-1* (GLRaV-1), GLRaV-2 and GLV-3 was developed in combination with a plant RNA internal control used as an indicator of the effectiveness of RNA extraction and RT-PCR. Primers were designed from conserved regions of

each virus and their specificity was confirmed by sequencing PCR products. One to nine fragments specific for viruses were simultaneously amplified from infected grapevine samples and identified by their specific molecular sizes in agarose gel electrophoresis. In the two-step mRT-PCR, the detection limits were 10^{-3} to 10^{-4} extract dilutions, depending on the virus (Gambino and Gribaudo 2006).

In a later investigation the differential and simultaneous detection of nepoviruses of subgroups A, B and C was performed using degenerate and species-specific primers. Three sets of degenerate primers one for each of three subgroups of the genus (A, B and C) were designed based on the nucleotide sequence homology of RNA-1 and RNA-2 of nepoviruses isolated from grapevines. Primers designed specifically for detecting subgroup A species amplified a fragment of 255-bp from samples infected by GFLV, ArMV, *Tobacco ringspot virus* (TRSV) and *Grapevine deformation virus* (GDefV), but not from samples infected by other nepovirus species. Likewise, primers for detection of subgroup B nepoviruses amplified a 390-bp product from samples infected by *Grapevine chrome mosaic virus* (GCMV), *Tomato black ring virus* (TBRV), *Grapevine Anatolian ringspot virus* (GARSV) and *Artichoke Italian latent virus* (AILV). The third set of primers amplified a 640-bp fragment, only from samples infected by subgroup (nepoviruses viz., *Tomato ringspot virus, Grapevine Bulgarian latent virus* (GBLV) and *Grapevine Tunisian ringspot virus* (GTRSV)). Multiplex-PCR detection of subgroup A and B nepoviruses could be achieved by using a specific primer (sense for subgroup A and antisense for subgroup B) for each of the species of the same subgroup in combination with the degenerate subgroup-specific primers. It was possible to detect four different viral species in single samples containing mixtures of viruses of the same sub-group using sense and antisense-specific primers, as indicated for subgroups A and B. Amplicons for viruses of subgroup A (TRSV, GFLV, ArMV and GDefV) were respectively 190, 259, 301 and 371-bp, whereas 190, 278, 425 and 485-bp fragments were amplified respectively from viruses of subgroup (GCMV, AILV, GARSV and TBRV) (Digiaro et al. 2007). The potential of this one-step RT-PCR system for use in field-based epidemiological studies can be exploited.

New strains of *Potato virus Y* (PVY) emerge now and then posing a serious problem for the seed potato industry, as rejection of seed lots submitted for certification with increased virus incidence is possible. Although several RT-PCR protocols have been earlier used, they could detect only certain combinations of mixed strain infection making a more efficient diagnostic method absolutely necessary. A single multiplex RT-PCR assay was developed for assigning PVY strain type as well as for detecting mixed infections with respect to the major strain types. This new procedure was validated by testing 119 archieved PVY isolates that had been earlier characterized by serology and bioassay and/or previously published RT-PCR assays. Results obtained with tests for single-strain isolates compared well with previous reports in most cases. It is of significance that 16 mixed infections that could not be detected earlier, were differentiated effectively. This new protocol has the potential for use by seed potato industry widely (Lorenzen et al. 2006). RT-PCR technique was demonstrated to be effective for the simultaneous cDNA synthesis of viral and viroid RNAs in plant extracts. *Grapevine leafroll-associated virus-1,2*

and 3, *Grapevine virus A and B, Grapevine rupestris stem pitting-associated virus, Grapevine fleck virus and Grapevine fan leaf virus* were reliably detected up to a 10^{-3}-fold or higher dilution of the original plant extract. Further *Hop stunt viroid* and *Grapevine yellow speckle viroid* were amplified to the same level as the viruses. As the viruses and viroids can be detected simultaneously, these procedures can contribute to cost-effective diagnosis of a large number of samples throughout the year (Nakaune and Nakano 2006).

In the PCR-microplate hybridization procedure, hybridization of PCR products to oligonucleotides probes is carried out and immobilized on the microplate wells followed by colorimetric detection. A cDNA fragment from the CP coding region of PVY RNA genome amplified by reverse transcription followed by PCR was directly adsorbed onto polystryrene microplate wells after heat denaturation. DIG-labeled cDNA probe was employed for hybridization with the adsorbed cDNA. Alkaline phosphatase-conjugated anti-DIG antibody was allowed to react with the hybrid of adsorbed cDNA and DIG-labeled probe. The enzyme activity was then detected by hydrolysis of a substrate and the absorbance values were determined using a microplate reader. This nucleic acid-based ELISA-like highly sensitive diagnostic method has the potential for detection of plant viruses and viroids (Hataya et al. 1994).

In PCR-ELISA format, multiple aligments of CP gene sequences of the viruses to be detected are made to select PCR primers in the regions conserved between viruses. Oligonucleotides specific to viruses are used as capture probes. By selecting suitable primers *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) were detected simultaneously and the sensitivity of detection was significantly enhanced by the protocol developed by Candresse et al. (1998). PCR-ELISA was found to be useful for the detection of *Plum pox virus* (PPV), *Cherry leaf roll virus*, CTV, PNRSV and *Tomato ringspot virus* (ToRSV) (Olmos et al. 1997; Rowhani et al. 1998). Attempts to simplify the RT-PCR technique resulted in the development of the print or spot-capture (PC)-PCR for the detection of plant viruses. The PC-PCR-analogous to tissue blot immunoassay (TBIA) was successfully applied for the detection of PPV (Olmos et al. 1996), *Apple chlorotic leaf spot virus* (ACLSV), PNRSV and ApMV (Cambra et al. 1998). *Tomato yellow leaf curl virus* (TYLCV) isolates, TYLCV-Sv and TYLCV-Is were detected and differentiated by applying PC-PCR technique (Navas-Castillo et al. 1998). Likewise, *Cucumber vein yellowing virus* (CVYV) was detected in cucumber, zucchini or melon plants which showed irregular distribution of CVYV (Rubio et al. 2003). *Artichoke latent virus* (ArLV) was detected by using a denatured DIG-labeled ArLV DNA probe in the tissue imprint hybridization technique, in addition to a one-step RT-PCR protocol. Both diagnostic method were effective in detecting ArLV in late globe artichoke plant samples (Lumia et al. 2003).

A combination of RT-PCR and restriction fragment length polymorphism (RFLP) has been shown to be effective for detection and differentiation of plant viruses. The serologically related *Tobamovirus* spp. could be detected and discriminated by using RT-PCR/RFLP protocol (Letschert et al. 2002). For the detection and identification of *Soybean mosaic virus* (SMV) strains, a primer pair amplifying

a 1385-bp fragment of cylindrical inclusion (CI) coding region was designed. Following RT-PCR, the RFLP profiles of RT-PCR products were compared after restriction digestion with *RsaI, EcoRI* or *AccI* restriction endonucleases. The five strains, in addition of seedborne SMV isolates from soybean cultivars were differentiated by RT-PCR/RFLP analysis. The results of this experiment correlated well with strain identification by symptom phenotypes produced on differential cultivars inoculated with strains and isolates (Kim et al. 2004). The primers amplifying a 605-bp fragment containing a part of the coat protein (CP) of *Grapevine fanleaf virus* (GFLV) were employed for the detection of 20 isolates of GLV in Tunisia and sequence variation among isolates was characterized by RFLP analsysis and confirmed by sequencing (Fattouch et al. 2005). An RT-PCR-RFLP protocol was employed to detect and discriminate new severe strains of *Melon necrotic spot virus* (MNSV) (Kubo et al. 2005). A combination of RT-PCR and reverse dot blot hybridization for detection and identification of potyviruses was developed based on three degenerate primers located at the NIb and CP region. The cDNA fragments (1.0–1.2 kb) of the viruses were amplified from infected plant tissues. For further precise identification, sequences located between the 3' end of the NIb gene and the 5' end of the CP gene were used to design species-specific probes which hybridized with DIG-labeled RT-PCR products amplified by potyvirus degenerate primers (Hsu et al. 2005).

Plant viruses depend on specific vectors for their spread under natural conditions. Estimation of population of viruliferous vectors and their efficiency is of great epidemiological importance, in addition to helping the formulation of effective disease management systems. RT-PCR assay has been successfully applied for the detection and quantification of virus content in the vectors. The presence of *Grapevine fanleaf virus* (GFLV) in the nematode vectors (Esmenjand et al. 1994), *Tomato spotted wilt virus* (TSWV) in thrips vectors (Tsuda et al. 1994). *Potato leafroll virus* (PLRV) in three aphid vector species (Singh et al. 1997) and *Citrus tristeza virus* (CTV) in two aphid vector species (Mehta et al. 1997) was detected efficiently by using RT-PCR technique. Rice stripe virus (RSV) was detected in the vector, small brown planthopper (*Laodelphax striatellus*) by applying RT-PCR technique. Primers matching the viral RNA-dependent RNA polymerase gene in RNA1 were employed for the amplification of a specific 445-bp product in viruliferous insects (Lijun et al. 2003).

Grapevine fan leaf virus (GFLV) contains a bipartite single-strand positive RNA genome consisting of RNA-1 and RNA-2 each one coding for a polyprotein. The coat protein (CP) gene located in RNA-2 and the contiguous nine C-terminal residues are involved in the transmission by the nematode vector *Xiphinema index* (Belin et al. 2001; Wetzel et al. 2001). The potential of two types of molecular probes to detect and identify GFLV in plant and nematode tissues was assessed. Amplification of the CP gene of GFLV using RT-PCR technique was possible when the extract of 30 nematodes (*X. index*) was tested. There was no amplification in the case of healthy grapevine, virus-free *X. index* and other nonvector nematode species (Finetti-Sialer and Ciancio 2005) [Appendix 12]. The presence of *Strawberry latent ringspot virus* (SLRSV) and *Arabis mosaic virus* (ArMV) in their nematode vectors *Xiphinema diversicaudatum* and *Longidorus macrosoma* was detected by applying

RT-PCR technique. From the total-RNA extract of nematodes from soil around rose cultivars RNA later™ generated amplicon of 520 bp for ArMV and 200 bp for SLRSV, whereas the nematodes from soil around lily showed the presence of SLRSV (Kulshreshtha et al. 2005).

The combination of an immunological reaction with RT-PCR enhances the sensitivity of virus detection dramatically. The immuno-capture (IC)-RT-PCR has been found to be 100–1000 times more sensitive compared to ELISA technique. The virus particles are trapped on the wall of tubes or wells in microplates using specific antiserum followed by removal of inhibitory substances of plant origin. Using the viral RNA as template, the cDNA was synthesized by employing reverse transcriptase and amplification of the cDNA was carried out with virus-specific primers. The IC-RT-PCR technique was reported to be 100 times more sensitive for the detection of *Yam mosaic virus* and *Yam mild mosaic virus* (Mumford and Seal 1997). While this test was 1000 times more sensitive in detecting *Lettuce mosaic virus* than ELISA (Vlugt et al. 1997). *Peanut stripe virus* (PStV) and *Peanut mottle virus* (PeMV) were efficiently detected in extracts of small slices taken from peanut seeds distal to the radicle by applying IC-RT-PCR test. This technique was more sensitive than ELISA and has the potential for large scale testing of peanut germplasm (Gillaspie et al. 2000).

Immunocapture of *Potato leafroll virus* (PLRV) coupled to one-tube RT-PCR format using *Thermus thermophilus* (*Tth*) instead of *Taq* DNA polymerase was shown to be effective in detecting PLRV in potato tubers. Inspection time of seed potatoes for PLRV infection was reduced to 1 day from 5 weeks required for conventional testing for certification (Leone and Schoen 1997). The immunocapture (IC)-RT-PCR format to combine the simplicity of ELISA and sensitivity of PCR was more efficient in detecting *Citrus tristeza virus* (CTV) strains in 20% of samples that were ELISA-negative (Nolasco et al. 1997). Likewise, *Plum Pox virus* (PPV) was detected in 31% of ELISA-negative leaf samples and in 23% of ELISA doubtful trees indicating the usefulness of IC-RT-PCR for field surveys to assess the incidence of virus diseases precisely (Varveri and Boutsika 1998). The higher levels of sensitivity of IC-RT-PCR compared to ELISA were demonstrated in the case of *Prunus necrotic ringspot virus* (PNRSV) (Rosner et al. 1998), PPV in root samples (Adams et al. 1999) and PVY (Varveri 2000).

Detection of viruses by IC-RT-PCR can be performed in microplate wells also. The virus particles are captured by antibodies coated in the microplate wells for enrichment followed by lysis of virus particles and reverse transcription and implication of viral RNA genome. *Raspberry bushy dwarf idaeovirus* (RDBV) RNA3 was efficiently detected by this procedure using combinations of four primers (Kokko et al. 1996). Different protocols of IC-PCR, RT-PCR with plant extracts and RT-PCR with total RNA were found to be more sensitive (>100-folds) than DAS-ELISA for the detection of *Onion yellow dwarf virus* (OYDV), *Leek yellow stripe virus* (LYSV) and allexiviruses infecting *Allium* spp. A one-step RT-PCR suitable for large scale application was also developed for detection of viruses in leaf extracts (Dovas et al. 2001). A one-step IC-RT-PCR using degenerate primers was developed for the detection and differentiation of *Leek yellow stripe virus* (LYSV) and *Onion*

yellow dwarf virus (OYDV) in single and mixed infections in several *Allium* spp. IC-RT-nested-PCR was conducted directly in microtitre plates as well as in microcentrifuge tubes. This detection technique was 10^4 times more sensitive than the DAS-ELISA technique (Lunello et al. 2005).

Rapid detection and precise identification of new viruses by applying IC-RT-PCR format have been useful to plan proper disease management strategies. The involvement of a nepovirus in the development of the black currant reversion disease was indicated by IC-RT-PCR technique and the virus named *Black currant reversion-associated virus* (BRAV) (Lemmetty et al. 1998). The tuber necrotic strain of *Potato virus Y* was shown to cause the potato tuber necrotic ringspot disease by IC-RT-PCR procedure (Tomassoli et al. 1998). By employing specific primers, *Grapevine leafroll –associated viruses 1* and *3* (GLRaV-1 and GLRaV-3) were detected using IC-RT-PCR method in plants as well as in the mealy bug vector, *Planococcus ficus* (Acheche et al. 1999; Sefc et al. 2000). A single pair of degenerate primers designed from the sequences coding for movement proteins of *Arabis mosaic virus* (ArMV) and *Grapevine fan leaf virus* (GFLV) was used for the detection of ArMV and GFLV simultaneously by applying IC-RT-PCR procedure. This technique was ten times more sensitive than ELISA tests (Wetzel et al. 2002). *Plum pox virus* (PPV) was detected by using IC-RT-PCR technique in nine wild apricot accessions, including eight ELISA-negative and one ELISA-positive, indicating higher sensitivity level of this technique (Spiegel et al. 2004). The IC-RT-PCR assay using the MABs and specific primers in the region of the coat protein (CP) gene was applied for the detection of CMV in field samples. The S-I isolates of CMV showed one specific band about 500 nucleotides in length, whereas S-II isolates gave a single band containing about 600 nucleotides indicating the differences among the two subgroup isolates (Yu et al. 2005).

2.3.1.3 Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was developed to overcome the limitations of standard PCR procedure that requires post-PCR manipulations and processing of the reaction with slabgel. The real-time PCR consists of the fluorigenic 5′-nuclease assay designated TaqMan and spectrofluorimetric thermal cycler. TaqMan technology exploits the 5′–3′ nuclease activity of *Taq* polymerase. A fluorescence reasonance energy transfer (FRET) probe consisting of a green fluorescent "reporter" dye at the 5′ end and an orange "quencher" dye at the 3′ end is employed. During the PCR, the probe anneals to a complementary strand of an amplified product, whereas *Taq* polymerase cleaves the probe during extension of one of the primers and the dye molecules are displaced and separated. After the separation, the electronically excited reporter cannot be suppressed by the quencher dye. Hence, variation occurs in the green emission intensity concentration of PCR amplicons in the reaction. The number of PCR cycle at which the fluorescent signal (emission) exceeds a certain background fluorescence level is called as the threshold cycle (Ct), is directly proportional to the amount of the target DNA present in the sample. The advanced nucleic acid analyzer (ANAA) with silicon chip-based spectrofluorimetric

thermocyclers has been developed for field use (Belgrader et al. 1999). Real-time PCR technique has been more frequently applied for the detection of bacterial and fungal pathogens (Volume 1, Sections 2.3.3 and 2.3.5).

Real-time RT-PCR procedure was employed for the detection of a wide range of isolates of *Tomato spotted wilt virus* (TSWV) in infected plants (Roberts et al. 2000) and in individual thrips (Boonham et al. 2002). The real-time RT-PCR assay based on TaqMan™ chemistry reliably detected TSWV in as little as 500 fg total RNA. This technique was more sensitive (10-folds) than the conventional PCR and detected reproducibly 1000 molecules of the target transcript (Roberts et al. 2000). *Barley yellow mosaic virus* (BaYMV) and *Barley yellow and mild mosaic virus* (BaMMV) could be more reliably detected especially in late-season and mixed infection samples compared with ELISA. Rapid automation of extraction procedure was an added advantage for routine disease diagnosis (Mumford et al. 2004). The potential for large-scale application of a sensitive real-time RT-PCR was assessed for the detection of TSWV in single and bulked leaf samples and its sensitivity was compared with the standard DAS-ELISA. Real-time RT-PCR was effective in detecting TSWV in leaf tissues of all 22 plant species tested at a wide range of concentrations. This technique generally detected one infected sample present along with 1000 uninfected ones. DAS-ELISA format was less sensitive and less reliable than real-time RT-PCR, when the virus concentration was low (Dietzgen et al. 2005). Real-time RT-PCR assay was developed for the detection of *Cucumber vein yellowing virus* (CVYV) using specific primers designed from a nucleotide sequence of the RNA polymerase gene (NIb) conserved among all the available CVYV strains. This technique reproducibly detected titres as low as 10^3 molecules of the target CVYV DNA and also quantified CVYV concentration in young leaves following mechanical inoculation (Picó et al. 2005).

Based on the sequence information, *Dahlia mosaic virus* (DMV)–specific primers were used in an adapted real-time PCR assay for the detection of DMV in dahlias which are often severely affected by viral diseases. This procedure is expected to facilitate not only production of virus-free dahlias, but also elimination of virus-infected material from breeding and propagating stocks (Pappu et al. 2005). Real-time RT-PCR tests were shown to be effective in detecting *Grapevine fan leaf virus* (GFLV) in the nematode vector *Xiphinema index* collected from the rhizosphere of GFLV-infected grapevine plants in Italy. A 1157-bp fragment of the GFLV RNA-2 coat protein (CP) gene was amplified. A fluorescent scorpion probe based on the highly conserved CP region was used for the detection of GFLV. This procedure could be used as a diagnostic tool or for studies on GFLV in acquisition, rentention and transmission experiments (Finetti-Sialer and Ciancio 2005). In a later study, a real-time RT-PCR (TaqMan®) assay was developed for the specific detection of isolates of *Grapevine leafroll-associated viruses 1-5* and *9* (GLRaV1-5 and -9) from South Africa, Europe, Australia, Asia, Latin America and United States. TaqMan® primers and probes were designed targeting the regions with 100% sequence identity. The real-time TaqMan® and conventional RT-PCR assays were compared for detection of viruses using purified total RNA as well as crude extract. TaqMan® RT-PCR was found to be more sensitive than the conventional one-step RT-PCR

for testing different isolates of GLRaV1-5 and -9 either using RNA or crude tissue extracts (Osman et al. 2007).

A real-time RT-PCR protocol was developed for the specific detection of *Beet necrotic yellow vein virus* (BNYVV). Two assays, one detecting RNA2 of all types and the other detecting types containing RNA5 were performed. The real-time assays were 10,000 more sensitive in detecting BNYVV compared to the conventional RT-PCR (Harju et al. 2005). For the detection and differentiation of *Plum pox virus* (PPV), a real-time multiplex PCR procedure with SYBR Green I was developed. This technique using inexpensive dye SYBR Green I is simple and provides more reliable results. Further it eliminates the need for electrophoretic analysis of amplicons or RFLP patterns using ethidium bromide (Vargo and James 2005). An RT-PCR assay using primers targeting isolates of the *Wheat spindle streak mosaic virus* (WSSM) from Canada, France, Germany, Italy and the United Kingdom and a real-time PCR with SYBR-Green for quantification of WSSMV were developed. These assays allowed a more sensitive detection of WSSMV than ELISA format. The virus was also detected in soil samples in addition to samples from infected wheat plants. There was no amplification of other viruses infecting wheat, indicating the specificity of the RT-PCR protocol (Vaïanopoulos et al. 2006).

A real-time PCR protocol was developed for the detection and relative quantification of potyviruses, *Sweet potato feathery mottle virus* (SPFMV). *Sweet-potato virus G* (SVPG) and *Ipomoea vein mosaic virus* (IVMV) and *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato leaf curl virus* (SPLCV) in singleplex reactions directly from infected sweet potato plants. There was no discernible adverse effects due to the presence of potential PCR inhibitors. The titres of SPFMV, IVMV and SPVG, as determined by real-time PCR were lower in singly-infected sweet potato plants compared with singly-infected *Ipomoea setosa*- cv. Brazilian Morning-glory and *I. nil* cv. Scarlet O'Hara plants (Kokkinos and Clark 2006). A *Potato virus Y* (PVY) single nucleotide polymorphism (A/G_{2213}), identified as a molecular determinant of the tobacco leaf necrosis symptom induced by PVY^N isolates was used as a target to develop two PVY group-specific (PVY^N and PVY^O) fluorescent (Taq-Man-based) real-time RT-PCR assays. Detection, characterization and quantitation of a wide range of PVY isolates in samples containing 10^3–10^8 viral transcripts have been performed by using these protocols. High specificity of these two techniques is useful for simultaneous detection and reliable quantitation of PVY^N and PVY^O isolates in mixed solutions regardless of the Y^N/Y^O ratio (Balme-Sinibaldi et al. 2006).

The presence of fungal and bacterial pathogens, outside of their natural host plant species, in soil, water and air has been demonstrated by various techniques and the assessment of their populations in the environment is important to study epidemiology of the diseases caused by them. Very few investigations have been taken up to detect the plant viruses in a free state in soil and water, probably because of the belief that the viruses may be inactivated rapidly when the infected plants dry up under field conditions. A quantitative real-time RT-PCR method was developed for the detection of *Tomato mosaic virus* (ToMV) in irrigation waters in order to monitor health status of environmental waters. A concentrating procedure using convective Interaction Media® chromatographic column was adopted prior to

real-time RT-PCR technique. ToMV was detected in water samples from the rivers KrKa and Vipava in Slovenia. The detection limit of the technique was as low as 12 viral particles per ml of sample. The results were confirmed by infectivity tests, DAS-ELISA and electron microscopy. This report demonstrated that the technique developed was simple, rapid, efficient and sensitive for monitoring of irrigation waters for the presence of plant, animal and human viruses (Boben et al. 2007).

2.3.1.4 Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Technique

An improvement in the sensitivity of virus detection could be achieved by using immunocapture-reverse transcription loop-mediated isothermal amplification (IC-RT-LAMP) for the detection of TSWV in infected chrysanthemum plants. TSWV genomic RNA could be amplified under isothermal (65°C) conditions within 1 h. The resulting amplicons were detected by estimating the intensity of turbidity of reaction mixture. IC-RT-LAMP was shown to be 100 times more sensitive than IC-RT-PCR assay (Fukuta et al. 2004). A reverse transcription loop-mediated isothermal amplification of DNA (RT-LAMP) method was developed for the detection of *Potato virus Y* (PVY) and its sensitivity of detection was compared with that of RT-PCR. A set of four primers matching a total of six sequences of CP gene of PVY were designed in such a way that a loop could be formed and elongated during DNA amplification. Complementary DNA clones of PVY-CP were used as templates. The LAMP reaction was optimized by adjusting the concentration of $MgSO_4$ dNTPs and *Bst* DNA polymerase. The positive reaction of LAMP was indicated by the turbidity formed due to precipitation of magnesium pyrophosphate which was measured by a spectrophotometer. The results of one-step RT-LAMP-turbidity method and the two step RT-PCR assay were comprable. Both methods detected PVY in 234 out of a total 240 samples infected by PVY (Nie 2005) [Appendix 13].

A new approach for the detection of pathogenic microbes using molecular beacons was attempted by Tyagi and Kramer (1996). This novel fluorescence based nucleic acid detection involves the use of molecular beacon (the probe) consisting of a single-stranded DNA with a stem-loop structure. The loop portion contains a probe sequence that is complementary to a target sequence, whereas 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 and a quenching moiety are attached to the 5′ arm terminus and 3′ arm respectively at opposite ends. The presence of the target nucleic acid in the test solution is detected by adding the molecular beacon and by heating the mixture to 80°C followed by cooling to 20°C. The intensity of fluorescence emitted by the beacon is continuously monitored during the entire period. In the presence of the target with the complementary sequence, the probe forms a hybrid within the loop region resulting in the displacement of the fluorescent moiety from the quenching moiety leading to the emission of fluorescence. In the case of negative reaction due to the absence of the complementary sequence in the target nucleic acid tested, 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. Fluorescent signals will be emitted only when the molecular beacons hybridize with their complementary nucleic acids. Hence, removal of unhybridized molecular beacons from the mixture is not required as they do not fluoresce (Fig. 2.8). By tagging fluorescent moieties that have different emission wavelengths, it is possible to employ multiple molecular beacons for the detection of two or more plant viruses or pathogens.

Molecular beacons (4) were designed specific to the RNA-dependent RNA polymerase (RdRP) and coat protein (CP) genes of two viruses, *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV). The molecular beacons detected up to 0.5 ng of both CymMV and ORSV purified RNA. Only tubes containing total RNA isolated from CymMV-and ORSV-coinfected *Oncidium* leaves exhibited significant increases in fluorescence intensities following addition of both sets of molecular beacons specific for CymMV and ORSV (Eun and Wong 2000). A fluorescent scorpion probe was designed based on the highly conserved CP region of the genome of *Grapevine fanleaf virus* (GFLV). This probe allowed quantitative estimation of GLFV RNA2 in single nematode vector *Xiphinema index* collected from the rhizosphere of GFLV-infected grapevine plants. This diagnostic technique was as effective as real-time RT-PCR procedure for the detection of GFLV in the nematodes (Finetti-Sialer and Ciancio 2005).

Molecular beacon technology offers several advantages over other nucleic acid-based diagnostic tests. The high specificity of molecular beacons can be useful to discriminate even with one nucleotide mismatch, because of the presence of the stem-loop structure as probes. There is no necessity of removing the unhybridized molecular beacons which do not fluoresce. Quantitative estimation is possible immediately, since this technique does not require any post-analysis steps such as gel electrophoresis or spectrophotometry. Further, the molecular beacon technology provides 96-well simultaneous analysis that is comparable to both RT-PCR and ELISA.

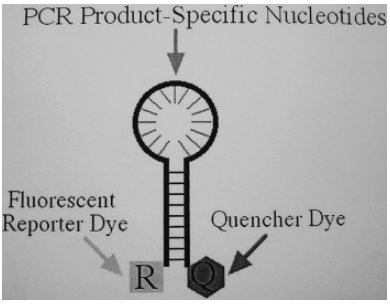

Fig. 2.8 Detection of plant viruses using molecular beacons
A reporter fluorescent dye (R) is attached to the 5′end and a quencher (Q) is attached to the 3′ end; molecular beacons that bind to the PCR product, remove the ability of the quencher to block the fluorescence from the reporter dye; as the PCR product accumulates, a linear increase in the fluorescence is seen

2.3.1.5 Heteroduplex Mobility Analysis (HMA)

Detection of microbial pathogens by the heteroduplex mobility analysis (HMA) is based on the delay in the rate of migration of a DNA heteroduplex in comparison with a DNA homoduplex to identify mismatches or deletions in DNA sequences. The variability of *Human immuno-deficiency virus* (HIV) Type 1 was first determined by employing HMA technique (Delwart et al. 1994). *Grapevine leafroll-associated virus 2* (GLRaV-2) was detected in samples obtained from several grapevine accessions of different varieties from Italian, Greek, French and Brazilian Vineyards during survey in 2001–2002. The HMA technique detected the differences in the sequences in ORF coding the coat protein (CP) of GLRaV-2 (Angelini et al. 2004).

2.3.1.6 DNA Array Technology

Plant pathogen detection by employing DNA array technology aims to miniaturize traditional bioanalytical detection system so that hundreds or even thousands of biomolecules with unique identity can be detected simultaneously in one single experiment by using a very small amount of test samples. DNA microarray provides a medium for hybridization of known with unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. Common assay systems such as microplates or standard blotting membranes may be used in the experiments based on microarrays. Depending on the size of the deposited sample spots, the DNA arrays may be designated as macoarrays (>300 microns in diameter) or microarrays (<200 microns in diameter). The microarrays generally contain thousands of spots and require specialized robotics and imaging equipment.

DNA arrays were earlier developed for the detection of human pathogens such as *Escherichia coli* 0.57: H7 (Schena et al. 1996; Call et al. 2001). DNA array systems require amplified and labeled samples of DNA to act as probes in an array with specific oligonucleotides anchored to a solid support such as nylon membranes (macroarrays) or glass slides (microarrays). The results of the experiments may be captured on X-ray film. A positive reaction is indicated by the presence of a light gray to black dot, whereas white color is seen in the case of negative reaction. Fluorescent DNA label can be scanned directly. The hybridization intensity is converted into grayscale values to indicate reaction strength for quantification of the results.

DNA microarray technology has two major applications – identification/detection of sequences (genes or gene mutations) and determination of expression levels or abundance of genes. Probes are the tethered nucleic acids with known identity or sequence and they are applied to detect the targets which are free nucleic acid samples whose identity is to be established or abundance is to be quantified. Probes are arrayed on the microarray substrate to capture the targets of complementary nature. Two major types of probes have been distinguished. Clones of cDNA (generally 500–5000 base pairs in length) and oligonucleotides (generally 25- to 80-mer) have been employed as probes. The target total RNA or mRNA has to be isolated from cells or tissue samples as rapidly as possible to avoid any potential

changes in transcript profiles during the procedure. Commerical kits (eg. Qiagen kit) are available giving suitable instructions. Various terminologies such as biochip, DNA chip, gene chips and gene array have been used by different researchers (Shi et al. 2003).

Microarray technology has been primarily developed to allow highly parallel examination of gene expression (Schena et al. 2006). Later the possibility of exploitation of microarray methodology was examined for its potential in viral diagnostics. In medical virology, microacrray technology was employed for detection and characterization of single virus such as *Hepatitis C virus* (Park et al. 2001) and characterization of *Poliovirus* vaccine (Proudhikov et al. 2000). Microarray technology can be applied for the detection of several viruses in a single generic assay. DNA probes (upto 30,000) may be arrayed onto a single glass microscope slide which forms the microarray. The DNA probes arrayed are gene sequences from each of the viruses that are to be detected in a single assay. The microarray is then exposed to fluorescently labeled cDNA from the sample to be tested and finally scanned using a microarray scanner to detect the presence of any of the target in the sample (s) under investigation.

A microarray protocol was developed to detect four different potato viruses viz., *Potato virus X* (PVX), *Potato virus Y* (PVY), *Potato virus A* (PVA) and *Potato Virus S* (PVS) either individually or in mixtures in infected plants. This technique was able to detect closely related viruses and strains and also to discriminate sequences with less than 80% sequence identity. It could select sequence variants with greater than 90% sequences identity. The technique was comparable to ELISA in the sensitivity of detection (Boonham et al. 2003). In another study, detection of potato viruses, using microarray technology was attempted. In this investigation, short synthetic single-stranded oligomers (40 nt) were employed as capture probes, instead of PCR products. A microchip detecting potato viruses, PVA, PVS, PVM, PVX, PVY and PLRV in both single and mixed infections was developed. The main strains of PVY and PVS could be detected and differentiated by this oligonucleotide-based microarray technique (Bystricka et al. 2005). By employing a system of microarrays, *Cucumer mosaic virus* (CMV) serogroups and subgroups could be detected and differentiated. The coat protein (CP) genes of 14 different isolates were amplified using cy3-labeled generic- and species-specific primers. These amplicons were hybridized against a set of five different serotype and subgroup-specific 24-mer oligonucleotides bound to an aldehyde-coated glass slide via an aminolinker (Deyonga et al. 2005).

2.3.2 Detection of Viroids

Among the plant pathogens, viroids are the simplest in structure and they are capable of independent replication reaching sufficient concentration, when introduced into cells/tissues of susceptible plants so as to produce characteristic symptoms. Viroids have only nucleic acids composed of a few hundred nucleotides without a protein component as in the case of viruses. As such the viroid nucleic acids do not posses

any messenger activity resulting in the absence of any viroid-specific protein in the infected plants. But enhanced levels of host-specific proteins have been reported in the infected plants following infection by viroids. Hence, the application of serodiagnostic techniques for the detection of viroids has not been possible. Different nucleic acid-based assays have been successfully employed for the detection and differentiation of viroids.

2.3.2.1 Nucleic Acid Hybridization Assays

A technique based on hybridization of highly radioactive recombinant DNA to viroid RNA that is attached to a solid support (nitrocellulose membrane) was developed for the detection of *Potato spindle tuber viroid* (PSTVd) in potato tubers. The amount of PSTVd that could be detected in the potato tuber sprouts was equivalent to a concentration of 0.04–0.125 μg of PSTVd per gram of tuber sprouts tissue (Owens and Diener 1981). The disadvantages and potential health hazards associated with the use of radioactive probes led to the development of non-radioactive labeling techniques for viroid detection. *Potato spindle tuber viroid* (PSTVd) was detected by employing digoxigenin (DIG)-labeled probes using dot-blot hybridization protocol. The assay procedure was as sensitive as the tests using radioactive probes. PSTVd was detected in composite samples taken for mass indexing programs (Welnicki and Hiruki 1992). The effectiveness of detection of *Citrus exocortis viroid* by performing PCR-microplate protocol was reported by Saito et al. (1995). Dot-blot hybridization procedure using DIG-labeled viroid-specific probes was employed to detect *Peach latent mosaic viroid* (PMLVd) and *Hop stunt viroid* (HSVd) infection in peach trees. High incidence of PMLVd (77%) and HSVd (69%) as single or mixed infection in samples from orchards in Czech Republic was indicated by the diagnostic tests (Hassan and Ryšánek 2004a). Northern blot hybridization technique was applied using viroid-specific probes for the detection of *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid (CBLVd), Hop stunt viroid* (HSVd), *Citrus viroid IV* (Cvd-III) and *Citrus viroid III* (Cvd-IV) in inoculated Etrog citron (Barbosa et al. 2005).

2.3.2.2 Polymerase Chain Reaction (PCR)

By using DNA primers for cDNA synthesis, a full-length viroid product was amplified by PCR from extracts of peach tissue infected by *Peach latent mosaic viroid* (PLMVd). The amplified viroid cDNA hybridized to ^{32}P-labeled PLMVd cRNA probe. The viroid was detected in different tissues such as fruits, leaf and bark of infected peach trees. PLMVd was shown to be wide spread in the peach germplasm in different countries (Shamloul et al. 1995). Digoxigenin-labeled probes prepared by PCR have been reported to be useful for the detection of several viroids. The total nucleic acid extracted from leaves infected by *Citrus exocortis viroids* (CEVd) was amplified by RT-PCR technique. CEVd-specific probes labeled with digoxigenin were successfully employed for detecting CEVd in citrus samples that were found to be CEVd-negative by biological indexing on indicator plants (Saito et al. 1995).

Potato spindle tuber viroid (Shamloul et al. 1997; Lebas et al. 2005) and *Avocado sunblotch viroid* (ASBVd) (Mathews et al. 1997; Schnell et al. 1997), *Coconut tinangaja viroid* (CTiVd) (Hodgson et al. 1998), *Hop stunt viroid* (HSVd) (Nakahara et al. 1999) and *Citrus viroid-OS* (Ito et al. 2001, 2002) were efficiently detected by application of RT-PCR technique. With suitable modification for the extraction of RNA and careful selection of DNA primers for optimization for viroids in low copy number, five viroids infecting grapevines could be detected by a highly sensitive RT-PCR protocol (Wah and Symons 1997). Viroids, *Citrus exocortis viroid, Citrus bent leaf viroid, Hopstunt viroid, Citrus viroids III* and *IV* were detected using DIG-labeled specific probes in RT-PCR format (Barbosa et al. 2005). *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) belonging to Avsun viroidae and Popsiviroidae respectively commonly infect stone fruit trees. Using RNeasy extraction kit (Qiagen) total RNA was extracted from different tissues (leaves, petioles or bark). RT-PCR technique was applied using viroid specific primers for the detection of PLMVd and HSVd in the extracts. The protocol developed has the potential for use in certification programs for freedom from viroid infection (Hassan et al. 2004a, b).

The RT-PCR technique has been reported to be effective for the detection of five viroids infecting grapevines (Wah and Symons 1997). A single-tube RT-PCR format was developed for amplification of nucleic acids of *Citrus exocortis viroid* (CEVd) and *Citrus cachexia viroid* (CaCaVd) (Turturo et al. 1998). The tissue printing method involving immobilization of plant extract onto nitrocellulose membrane or filter paper followed by application of RT-PCR protocol, has been shown to be efficient for the detection of *Potato spindle tuber viroid* (PStVd) in primarily and secondarily infected potato plants (Weidemann and Buchta 1998).

Fluorescence RT-PCR using TaqMan^{TM} technology was employed to detect *Potato spindle tuber viroid* (PSTVd), a quarantine pathogen in Europe. This procedure was 1000 times more sensitive compared with chemiluminescent assay (Boonham et al. 2004). A microtissue direct RT-PCR method was developed for the detection of *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CCLMVd) in very small chrysanthemum plants in order to identify viroid-free plants. In this method, tissue samples are taken at a depth of 0.1–0.2 mm using a razor or syringe and the sample is directly transferred to the RT mixtures. Both viroids could be detected in plants with high and low viroid concentrations. The procedure has the potential for application for viroid detection in microtissues such as shoot apical meristem (Hosokawa et al. 2006).

The high specificity and sensitivity of RT-PCR technique has been responsible for its wide applicability. Furthermore, requirement of only small amounts of tissue samples or crude extract, but not necessarily purified preparations is the additional advantage offered by this technique. A simple protocol for the preparation of nucleic acids for RT-PCR detection of viroids from small quantities of plant tissue has been developed. This procedure involves preparation of crude extracts in a solution containing NaOH and EDTA and testing the supernatant solution for the presence of the viroid in question, after an incubation period of 15 min at room temperature (Singh et al. 2005). The RT-PCR assay was applied for the detection of PLMVd

in peach and pear trees, HSVd in pear, peach and almond trees and *Pear blister canker viroid* (PBCVd) in pear trees. Natural mixed infections by PBCVd-HSVd and PLMVd-HSVd were observed in pear trees. The identity of the different viroids was confirmed by comparing their sequences with characterized isolates. This protocol could be applied for the detection of viroids in crude extracts of leaves or bark tissues and in total RNA preparations (Fig. 2.9). This procedure has the potential for use in certification programs (Hassen et al. 2006) [Appendix 14].

2.3.3 Detection of Bacterial Pathogens

Bacterial pathogens invade different plant organs such as stems leaves, flowers, fruits, seeds and propagative plant materials such as tubers, corms and setts. Infected seeds and propagative plant materials form primary sources of infection carrying the pathogens to different countries or parts of the same country and through different seasons facilitating the perpetuation of the bacterial pathogens. The seeds and planting materials exhibiting no visual symptoms are more dangerous sources of infection. Furthermore, majority of bacterial diseases are spread through contaminated seeds or propagative materials. Hence, it is of paramount importance that the bacterial pathogens have to be detected rapidly and identified precisely to restrict the incidence and spread of the bacterial diseases and to sustain international trade.

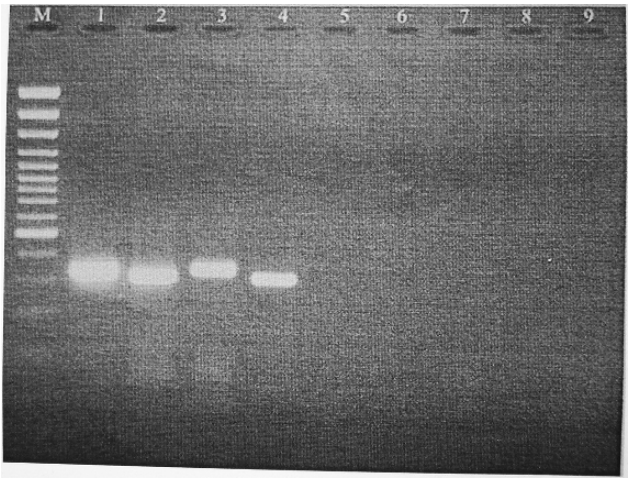

Fig. 2.9 Detection of viroids infecting fruit trees using agarose electrophoretic analysis of RT-PCR products amplified from total RNA preparations of viroid-infected plant tissues
Lane M: 100-bp DNA ladder; Lane 1: *Apple scar skin viroid* (ASSVd) in infected apple; Lane 2: *Pear blister canker viroid* (PBCVd) in infected pear; Lane 3: *Peach latent mosaic viroid* (PLMVd) in infected peach; Lane 4: *Hop stunt viroid* (HSVd) in infected cucumber; Lanes 5 to 9: Negative controls consisting of water and total RNA extracts of healthy apple, pear, peach and cucumber respectively. (Courtesy of Hassen et al. 2006; Blackwell Verlag, Berlin, Germany)

2.3.3.1 Nucleic Acid Hybridization Techniques

With the development of diagnostic DNA probes specific for the pathogen(s), it has been possible to detect, differentiate and quantify various phytopathogenic bacteria. Fluorescent in situ hybridization (FISH) method was effective in detecting *Ralstonia solanacearum* race 3 biovar2 causing potato brown rot disease (Wullings et al. 1998). A tissue blot hybridization protocol was developed based on the probe (560- bp) amplified from the intergenic region of the 16S/23S rDNA of *Clavibacter xyli* subsp. *xyli* causing sugarcane ratoon stunting disease to identify infected sugarcane plants (Pan et al. 1998). The Southern hybridization technique using DNA probes derived from plasmid-borne genes *CelA* (encoding an endocellulose) and *pat-1* (involved in bacterial pathogenicity) was shown to be useful for the detection of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) inducing tomato wilt and canker disease. This technique could be employed not only for the detection of *Cmm,* but also for the differentiation of virulent and avirulent strains of *Cmm* and for specific identification of subspecies *Cmm,* when it was present along with *C. michiganensis* subsp. *sepedonicus* causing potato ringrot disease (Dreier et al. 1995).

The colony hybridization test can be applied to detect the bacterial pathogen in the suspension prepared by macerating infected tissue in liquid. The suspension is then spread onto the culture medium, to permit its growth and covered with a nylon or nitrocellulose membrane. The bacteria in soil extracts and seed-soak washes may also be tested in a similar manner. After incubation for required period, appropriate DNA probe may be employed for the detection of the pathogen in question (Cuppels et al. 1990; Ward and De Boer 1990). Dot blot hybridization has been shown to be effective for the detection of pathogens such as *Pseudomonas syringae* pv. *phaseolicola* causing halo blight disease of beans and *Candidatus Liberibacter* causing citrus greening disease. The sensitivity of detection appears to be significantly affected by the nature of probe, bacterial species and the minimum number of bacterial cells required [varying widely from 200 to 10^6 colony forming units (CFU)]. In the case of *P. campestris* pv. *phaseolicola* a semi-selective medium that allows rapid multiplication is used, followed by concentration of bacteria before spotting on the nitrocellulose membrane (Schaad et al. 1989). By employing the pathogen-specific DNA fragment (0.24 kb) labeled with biotinylated nucleotides, the citrus greening pathogen was detected in various citrus hosts including mandarins, tangerins, oranges and pummelos (Hung et al. 1999).

2.3.3.2 Restriction Fragment Length Polymorphism

Digestion of bacterial DNA with specific restriction endonucleases results in restriction fragment length polymorphism (RFLP) patterns characteristic of the bacterial species under investigation. Detection and differentiation of *Xanthomonas axonopodis* pv. *citri* (*Xav*) and *X. oryzae* pv. *oryzae* (*Xoo*), causing citrus canker and rice bacterial leaf blight diseases and their strains has been possible by RFLP analysis. The cosmid clone PXCF 13–38 isolated from *X. axonopodis* pv. *citri*, covering the entire *hrp* gene cluster, was employed as a probe for RFLP analysis. This

probe was useful for the identification of various xanthomonads (Kanamori et al. 1999). The RFLP patterns recognized following digestion with *Pst*1 enzyme were specific for a group of strains of *Xoo* within a single race. The strains of race 2 frequently occurring in the Philippines were distinguishable by the RFLP patterns (Leach and White 1991).

The usefulness of RFLP technique to identify strains of *X. campestris* causing bacterial leaf streak disease in cereals has been demonstrated (Alizadeh et al. 1997). Specific sequences of bacterial genomes may be amplified by PCR and the amplicons may subjected to RFLP analysis as in *Burkholderia* spp. infecting rice. This combination of PCR-RFLP was applied for the identification of *B. glumae, B. gladioli, B. plantarii* and *B. vandii*. The ITS region (5S plus ITS1 and ITS2) of rDNA of respective units was amplified by PCR and digested with *Hha I* and *Sau* 3AI restriction enzymes. RFLP patterns could be used for the detection and differentiation of bacterial species present in naturally infected rice plant tissues (Ura et al. 1998).

2.3.3.3 Polymerase Chain Reaction

Among the nucleic acid-based diagnostic techniques employed for the detection of bacterial pathogens, polymerase chain reaction (PCR)-based methods have been shown to be suitable for the detection of more numerous bacterial pathogens. Amplification of desired sequences that are unique in the bacterial genome is performed by PCR, so that the test bacterial species is detected and identified. The nucleotide sequences coding genes involved in virulence, enzyme production or toxin production may be targeted by designing appropriate primer pairs and amplified by PCR, leading to the identification of the bacterial pathogen concerned (Table 2.1).

Development of PCR tests to detect pathogenic strains has been very useful to predict the extent of disease incidence and to identify the bacterial infection in asymptomatic seeds and propagative plant materials. A wide variety of pathogenic strains of *Agrobacterium tumefaciens* could be detected by using two PCR primers based on the sequences of *vir* D2 and *ipt* genes. The *ipt* gene coding for the cytokinin synthesis present only in *A. tumefaciens* differentiated this pathogen from *A. rhizogenes* (Haas et al. 1995). Primers based on the sequences of *tms 2* gene present in T-DNA required for the pathogenicity of *A. tumefaciens* detected T-DNA in infected plants and also in infected soils (Sachadyn and Kur 1975). The primers derived from the *pat-1* region of the plasmid (involved in pathogenicity) were shown to be efficient in detecting virulent strains of *Clavibacter michiganensis* subsp. *michiganensis* in the extracts of infected tomato plants and contaminated seeds (Dreier et al. 1995).

The PCR has been applied to detect bacterial pathogens in different organs and tissues of infected plants. The presence of *Xylella fastidiosa* (*Xf*), a xyelm-limited bacterium causing citrus variegated chlorosis (CVC) disease was detected in all main sweet orange fruit vascular bundles, in addition to seeds and seed parts, though no visual changes in the seeds could be discerned (Li et al. 2003). By using species-specific primers, the potato scab pathogens *Streptomyces scabies* and *S. turgidiscabies* were detected in the field-grown potato tubers cvs. Matilda and Sabina by

Table 2.1 Target sequences amplified by PCR for detection and identification of bacterial pathogens

Bacterial pathogen	Target sequences/primers	References
Acidovorax avenae subsp. *citrulli*	16S rRNA	Walcott and Gitaitis (2000)
Agrobacterium tumefaciens	T-DNA	Sachadyn and Kur (1997); Cubero et al. (1999)
Burkholderia glumae, B. plantarii and B. vandii	ITS1 and 2 region of rDNA	Ura et al. (1998)
Candidatus Liberibacter	16S rDNA	Coletta-Filho et al. (2005)
Clavibacter xyli subsp. *xyli*	Intergenic spacer region of 16S–23S rDNA sequences	Pan et al. (1998)
Erwinia amylovora	16S–23S rDNA sequences	Bereswill and Geider (1996)
	23S rDNA sequences	Maes et al. (1996)
	50-mer oligonucleotides	Merighi et al. 2000
Erwinia carotovora subsp. *atroseptica*	PEL (pectate lyase) encoding gene sequence	Helias et al. (1998)
Erwinia carotovora subsp. *carotovora*	16S–23S rDNA sequences	Toth et al. (1999)
E. herbicola pv. *gysophilae*	Cytokinins or IAA bio-synthetic gene sequences	Manulis et al. (1998)
Pseudomonas savastanoi (*syringae*) pv. *phaseolicola*	Phaseolotoxin-insensitive ornithy carbamoyl transferase (*arg K*) gene sequences	Mosqueda-Cano and Herrera-Estrella (1997)
	phaseoloxin (*tox*) gene	Rico et al. (2003)
P. syringae pv. *atropurpurea*	Gene sequences associated with pathogenicity and coronatine synthesis	Takahaski et al. (1996)
Ralstonia (Pseudomonas) solanacearum	16S rDNA sequence	Seal et al. (1999)
		Van der Wolf et al. (2004)
Streptomyces scabies	16S rDNA sequences	Lehtonen et al. (2004)
Xanthomonas axonopodis (*campestris*) pv. *citri* (*Xac*)	Intergenic region between 16S and 23S rDNA	Miyoshi et al. (1998); Taylor et al. (2002)
	468 bp fragment of plasmid DNA specific to *Xac*	Sun et al. (2004)
	rpf gene cluster specific to *Xac*	Coletta-Filho et al. (2006)
Xanthomonas axonopodis pv. *glycines*	DNA sequences encoding glycinecin	Oh et al. (1999)
Xanthomonas campestris	*hrp* gene	Berg et al. (2005)
X. campestris pv. *carotae*	350 bp of pathogen DNA	Meng et al. (2004)
Xylella fastidosa	16S–23S rRNA spacer region	Costa et al. (2004)

the PCR format that was found to be rapid and cost-effective (Lehtonen et al. 2004). A PCR-based lesion assays were employed for the detection of bacterial pathogenic strains causing bacterial speck and bacterial spot lesions in tomatoes. By using crude DNA extracts and primer sets COR 1/2 (bacterial speck) and BSX 1/2 (bacterial spot) amplification of a 689-bp fragment with COR 1/2 was obtained in the case of all 29 pathogenic strains of *P. syringae* pv. *tomato* (*Pst*) tested. On the other

hand, 28 of 37 strains causing bacterial spot generated the 579-bp amplicon with BSX 1/2 primer. The detection limit with plant extracts was determined as 30–50 CFU/reaction (Cuppels et al. 2006).

Detection Based on Specific Genes

Studies on genomic sequencing have provided information on array of genes predicted to control the processes like adhesion, production of phytotoxins, resistance to oxidative stress, plant cell wall degradation, secretory systems and interference and/or suppression of host defenses (Puhler et al. 2004). Bacterial pathogens are known to produce enzymes and toxins that have vital role in their pathogenicity/virulence. A PCR fragment (420 bp) generated from pure cultures that were selected based on the immunofluroescence colony-staining (IFC) assay was amplified by the primers for the detection of *Erwinia chrysanthemi* (Van der Wolf et al. 1995). The usefulness of the amplification of a segment of the necrotizing (*nec1*) gene which is involved in the pathogenicity for the detection of *Streptomyces scabies, S. acidiscabies* and *S. turgidiscabies* was demonstrated by Bukhalid et al. (1998) and Joshi et al. (2007). The sequences of gene segments associated with virulence factors such as ethylene-forming enzyme gene *efe* and cyclic lipodepsinonapeptide have been used for designing primers for the amplification of genomes of phytobacterial pathogens (Sato et al. 1997; Sorensen et al. 1998).

Primers based on sequences of genes coding for bacterial toxins have been used for detection of the pathogenic bacteria producing them. By using nested pairs of primers required to amplify the *tox* (phaseolotoxin) gene region of *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) the pathogen causing halo blight disease in bean could be detected (Schaad et al. 1995). From the phaseolotoxin gene cluster of *Psp*, G + C-rich oligonucleotide primers were designed. The HB14 primers specifically amplified a 1.4 kb fragment from DNA of 19 *Psp* isolates. Likewise, G + C-rich X4 primers were specific for the amplification of *X. campestris* pv. *phaseoli* causing bean common blight disease. These primers (HB14 and X4) in combination could be employed successfully for the detection of both individual and mixed infections of bean common and halo blight infections of bean seeds. Distinctive DNA fragments were produced from seed lots containing as few as 1 infected seed in 10,000 seeds (Audy et al. 1996). A majority of the strains of *Psp* (95 out of 138) did not produce phaseolotoxin in vitro, though they produced typical water-soaked lesions on bean pods. These strains did not produce the expected amplicons after PCR using the primers specific for ORF6 of the phaseolotoxin (*tox*) gene cluster and did not contain DNA homologous to ORF6 as revealed by Southern hybridization experiments. Hence, it may not be possible to detect the Tox^{-} isolates by using current PCR or serological techniques (Rico et al. 2003).

Coronatine-producing genes are located in a large transmissible plasmid in *P. syringae* pathovars and the sequences of the gene cluster have been used for designing primers for their amplification in PCR tests employed for detection of this bacterial pathogen. However, some pathogenic strains lacking the coronatine gene cluster are capable of producing coronatine and such strains would

remain undetectable (Bereswill et al. 1994). Later primers for specific and sensitive detection of pCOR1 plasmid gene segments associated with coronatine production and pathogenicity of *Pseudomonas syringae* pv. *atroseptica* (*Psa*) were designed. This test was able to detect 0.1–1.0 CFU from serially diluted pure cultures, in addition to direct detection of *Psa* in infected tissues of Italian ryegrass showing symptoms of halo blight disease (Takahashi et al. 1996). By amplifying the *Cfl* gene from the gene cluster encoding coronatine it was possible to detect and differentiate coronatine-producing strains of *P. syringae* pv. *maculicola* infecting leafy crucifers in Oklahoma (Zhao et al. 2002).

The *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) gene cluster in bacteria is vital for interaction with host plants. These *hrp* gene clusters are generally conserved among phytopathogenic bacteria, encoding Type III secretion systems that deliver pathogenicity factors, elicitors and avirulence proteins to plant cells. By designing primers based on DNA sequences related to *hrp* genes and amplifying them in PCR, the possibility of detection and identification of *X. campestris* pv. *vesicatoria* was reported by Leite et al. (1994). The *hrpF* gene from *X. campestris* was detected by employing the primer pair DLH 120 and DLH 125 that amplified the 3′ end of *hrpF* gene. The primers specifically amplified a 619 bp fragment of the *hrpF* gene and no amplification products could be detected from other *Xanthomonas* spp. By including primers targeting a 360 bp section of the ITS region from *Brassica* spp. in a multiplex PCR, seed borne *X. campestris* pv. *campestris* (*Xcc*) could be detected even if one seed in a lot of 10,000, was infected by the bacterial pathogen (Berg et al. 2005). Primers were designed based on the *hrpZPst* gene of *Pseudomonas syringae* pv. *tomato* (*Pst*) which maps on a pathogenicity-associated operon of the *hrp/hrc* pathogenicity island. A 532 bp product corresponding to the internal fragment of the *hrpZPst* was amplified from 50 isoaltes of *Pst*. This PCR assay was effectively used to detect *Pst* in leaf and fruit spots from naturally infected tomato plants and asymptomatic seedlings and artificially contaminated seeds (Zaccardelli et al. 2005). The *rpf* genes regulate the expression of pathogenicity factors in *Xcc.* A *X. axonopodis* pv. *citri* (*Xac*)-specific genomic region was identified inside the *rpf* gene cluster between *rpfB* and *recJ* of strain JAPAR 306 (da Silva et al. 2002). The *rpf* cluster in *Xac* was shown to be different from the *rpf* cluster of *Xcc* by computer analysis. Two primers (Xaco1 and Xaco2) directed the amplification of a 581 bp fragment from DNA strains of *Xac* only. This PCR protocol detected as few as 100 bacterial cells and also *Xac* in extracts of both fresh and dried canker lesions and from washes of inoculated, but asymptomatic leaf surfaces (Coletta-Filho et al. 2006).

Detection Using Nonnuclear Nucleic Acids as Targets

Primers for PCR amplification have been designed from different DNAs of pathogenic bacterial species to be detected, identified and differentiated. Several studies have been carried out to assess the effectiveness of plasmid DNA as target. Specific detection of *Calvibacter michiganensis* subsp. *sepedonicus* (*Cms*) was possible by using primers to a promoter-like sequence from plasmid pCS1. However, plasmid-less strains of *Cms* are unlikely to be detected by this PCR assay (Schneider

et al. 1993). On the other hand, a plasmid-derived *Cms*-specific primer set could be successfully employed for the detection of all strains tested, including the one which was presumably with a plasmid (Rademaker and Janse 1994). *Xanthomonas axonopodis* pv. *citri* (*Xac*) strains (pathotype A) could be reliably and consistently detected by employing plasmid-derived specific primers (Hartung et al. 1996). The worldwide strains of *Xac* were detected and identified by using sets of primers based on sequence differences in the ITS and on a sequence from the plasmid gene *pthA* involved in virulence of *Xac* under specific conditions. Pathotypes of *Xac* could be differentiated. In addition, subgroups of pathotypes were identified (Cubero and Graham 2002). A new *Xac* strain causing canker on Key/Mexican lime (*Citrus aurantifolia*), designated *Xac-A*^{W} could be differentiated by using plasmid-derived primers specific for *Xac* (Sun et al. 2004). The primers deisnged from a plasmid fragment associated with pathogenicity of *X. axonopodis* pv.*manihotis* (*Xam*) infecting cassava were employed in the PCR assay. The primers amplified a specific 898-bp fragment from 107 pathogenic strains of *Xam,* whereas there was no amplification of the fragment in five nonpathogenic strains, indicating the specificity of the PCR assay (Verdier et al. 1998). The long term reliability of PCR tests using plasmid-derived primers has to be assessed based on the nature and persistence of the target plasmid DNA.

Three functionally and evolutionarily conserved genes, viz., the small subunit 16S (rRNA) gene (rrs), the large subunit 23S rRNA gene and 5S rRNA gene interspersed with variable spacer regions (intergenic transcribed spacer) constitute the ribosomal DNA operon (Schmidt 1994). A primer set was designed by combining a universal 16S rDNA with a reverse primer specific to *Xanthomonas*. This PCR test allowed detection of *Xanthomonas* DNA effectively in wheat seed extracts (Maes et al. 1996b). The PCR primers specific to the 23S rRNA gene of *Erwinia amylovora* were successfully employed for the detection of *E. amylovora* strains in asymptomatic tissues (Maes et al. 1996a). The oligonucleotide primers derived from the intergenic region between 16S and 23S rRNA genes amplified the sequences specific to *Clavibacter xyli* subsp. *xyli*, causing sugarcane ratoon stunting disease. No amplicons were generated by these primers from closely related subsp. *cyanodontis,* indicating the specificity of the PCR test (Fegan et al. 1998).

The three 16S rDNA subgroups of *Ralstonia solanacearum* were detected by employing primers designed based on the sequences of intergenic region between 16S and 23S rDNA genes. Selective PCR amplification of DNA sequences of biovars 3, 4 and 5 (in Division I) and biovars 1 and 2 (in Division II) was possible by using different combination of forward and reverse primers (Seal et al. 1999). Primers within the ITS region between the 16S and 23S rRNA genes were designed to allow amplification of sequences for specific detection of *Burkholderia plantarii* and *B. glumae* associated with seedling disease of rice (Takeuchi et al. 1997). The synthetic oligonucleotide primers designed based on the 16S rRNA gene of a known strain of *Acidovorax avenae* subsp. *citrulli* (*Aac*), amplified DNA from all *Aac* strains tested. However, amplicons from several closely related bacteria were also generated by these primers. The sensitivity of the assay could be enhanced

substantially (100-folds) by introducing an immunomagnetic separation (IMS) of *Aac* step prior to PCR (Walcott and Gitaitis 2000).

Amplification of the 16S–23S rRNA intergenic spacer regions of *Xylella fastidiosa* causing Pierce's disease of grapevine, using primers G1 and L1 was performed to detect infection of different plant species such as almond, oleander, Spanish broom (*Spartium junceum*) and wild mustard (*Brasica* spp.). This PCR protocol was useful to identify potential inoclum sources for infection of grapevines of California vineyards (Costa et al. 2004). During the survey in Brazil for the detection of *Candidatus* Liberibacter (a phloem-limited nonculturable bacterium) infection in citrus, the primers OI1 and OI2c based on the sequences of 16S rDNA were employed for PCR assay. But the PCR assay using these primers could detect the pathogen in 28% of the samples analyzed (Coletta-Filho et al. 2004). In a later study, the primers LSg 2f and LSg 2r were developed based on consensus sequences of 16S rDNA fragments. The 38 samples that did not amplify with OI1 and OI2c showed positive amplification with the LSg2f and LSg2r primers. The Asian "*Ca*. Liberibacter" was also detected in 6 of the 53 samples tested (Coletta-Filho et al. 2005).

Conserved primers to the 16S and 23S ribosomal genes are used to amplify the internally transcribed spacer (ITS) region. Several tRNA genes and noncoding regions exhibiting more variability than 16S and 23S rRNA genes themselves are included in the ITS regions. Identification of bacteria based on the ITS sequences, when a universal primer set is used, depends on the number and length of PCR-amplified products (Normand et al. 1996). The rice seed-associated pathogens belonging to genus *Pseudomonas, Xanthomonas* and *Erwinia* were detected and identified based entirely on size polymorphisms of the primary and secondary ITS-PCR products generated by universal primers to the 16S (R16-1) and 23S (R23-2R) rRNA genes (Kim and Song 1996). Based on the DNA sequence of the spacer region between the 16S and 23S rRNA genes for five different *Calvibacter* subspecies, a single pair of primers was designed. A 215-bp fragment was amplified from *C. michiganesis* subsp. s*epedonicus* (*Cms*) by the primer pair in the PCR protocol developed by Li and De Boer (1995). This PCR assay was more sensitive in detecting the bacterial pathogens in naturally infected potato tissues than the standard ELISA and immunofluorescence tests. Based on the DNA sequence analysis of the 16S–23S ITS regions of *Clavibacter xyli* subsp. x*yli* (*Cxx*), primers were designed for the specific detection of the pathogen in vascular sap from sugarcane infected by ratoon stunting disease. A multiplex PCR assay was also developed to enhance the level or reliability of pathogen detection (Fegan et al. 1998).

Polymerase chain reaction (PCR) has been widely used to detect, identify and differentiate bacterial plant pathogens in infected seeds, plant tissues, in soil and water sources, in heterogeneous mixtures along with other pathogens or saprophytes. Different forms of PCR or coupled with one or more techniques have been applied to amplify pathogen-specific genes or-segments of DNA. The PCR assay, due to higher level of sensitivity, specificity and reliability has been frequently employed for detection of bacterial pathogens in asymptomatic plants and propagative materials (Manulis et al. 2002; Koh and Nou 2002; Stöger et al. 2006).

Variants of PCR for Detection

Among the variants of PCR, the repetitive sequence-based (rep)-PCR genomic fingerprinting technique has been applied more frequently. This procedure is based on PCR-mediated amplification of DNA sequences located between specific interspersed repeated sequences in prokaryotic genomes. These repeated sequences are variously named as BOX, REP and ERIC elements. There is no need for DNA extraction from the test plants, since the technique can be applied directly to cell suspensions prepared from infected plants. The field isolates of *Xanthomonas fragariae* collected from the nurseries in California were detected and differentiated by the rep-PCR fingerprints of isolates. The results of rep-PCR were in good agreement with that of pathogenicity tests that need a long time. In comparison to indirect ELISA, rep-PCR fingerprint technique was more sensitive and provided results more rapidly and precisely (Opgenorth et al. 1996). The strains (60) of *Pseudomonas avellanae*, causing hazelnut decline disease could be accurately detected and identified by rep-PCR using ERIC primers very rapidly and accurately, while the conventional methods required more than 6 months (Scortichini et al. 2000). Establishing the identity of a new pathogen rapidly is essentially required to plan effective strategies to contain the spread of the new pathogen (s) and to prevent its introduction in new locations. A new bacterial blight disease was noted on leek (*Allium porrum*) in California. By applying rep-PCR technique, the pathogen was identified as *Pseudomonas syringae* pv. *porri* (*Psp*) based on the DNA fingerprints of leek isolate of *Psp.* The rep-PCR analysis provided precise identification unambiguously whereas results of fatty acid analysis were inconclusive in nature (Koike et al. 1999). The isolates of *Acidovorax avenae* subsp. *citrulli,* causing bacterial fruit blotch in watermelon and melon were detected by rep-PCR and pulsed field gel electrophoresis (PFGE). Rep-PCR has the advantage of being more accessible, less expensive and faster than PFGE. However, rep-PCR technique provided levels of reproducibility of results compared with PFGE (Burdman et al. 2005).

Ralstonia solanacearum (*Rs*) causing bacterial wilt disease is distributed in tropical and sub-tropical countries inducing diseases in many crops. Based on the differences in host ranges, isolates of *R. solanacearum* have been grouped into five races. The race 4 infects ginger, mioga and curcuma. Two primer sets were desinged based on the sequence of polymorphic bands that were derived from repetitive sequences-based PCR (rep-PCR) fingerprinting and specifically detected *Rs* race 4 strains. One primer set (AKIF-AKIR) amplified a single band (165-bp) from genomic DNA obtained from all mioga and curcuma and some ginger isolates, while another set (21F-21R) amplified one band (125-bp) from other ginger isolates. Both the primer sets did not amplify the bands from genomic DNA of other *Rs* strains or of other related bacterial species. The sensitivity of PCR detection by the rep-PCR was 2×10^2 CFU. The detection limit for *Rs* inoculated into soil artificially was 3×10^7 CFU/g of soil (Horita et al. 2004). Rep-PCR genomic fingerprint profiles from 33 isolates of *Xanthomonas translucens* pathogenic to asparagus, in addition to 61 *X. translucens* reference strains pathogenic to cereals and grasses were generated. Amplified ribosomal restriction analysis profiles were prepared for most of

these strains and they were compared with those in a large *Xanthomonas* database using computer-assisted analysis. The isolates from ornamental asparagus (tree fern; *Asparagus virgatus*) were identified as *X. translucens* pv. *undulosa*. A unique amplified small subunit ribosomal gene *Msp I/Alu* I restriction profile was found to be specific for all *X. translucens* strains tested, including those strains pathogenic to asparagus, allowing discrimination from other species of *Xanthomonas.* All hosts of *X. translucens* pathovars are known to belong to *Gramineae* and *Poaceae*. On the other hand, the novel asparagus isolates infect host plant species belonging to the phylogenetically distant Liliaceae (Rademaker et al. 2006). Rep-DNA-PCR-based fingerprinting technique was applied to determine the genetic diversity of the isolates of *Xanthomonas* causing leaf spot disease of *Brassicas* and closely related pathovars. The leaf spot isolates were clustered separately from *X. campestris* pv. *campestris* isolates. Based on the results, it was proposed that *X. campestris* that induce non-vascular leaf spot disease should be identified as pv. *raphani*, but not pv. *armoraciae* (Vicente et al. 2006).

The usefulness of nested-PCR for the detection of certain bacterial pathogens has been reported. For the detection of *Erwinia amylovora* (*Ea*) causing fireblight disease, a unique DNA fragment of plasmid pEA 29 was the basis for designing two oligonucleotides primers that were employed in nested-PCR assay. This assay was more sensitive (1000-folds) compared with a single-round PCR and even single cells of *Ea* in pure cultures could be detected by this nested-PCR protocol. The presence of *Ea* in leaves, axillary bud and mature fruit calyx samples was detected successfully (McManus and Jones 1996). Three primer pairs were designed based on the sequences of the cytokinins (*etz*) or IAA-biosynthetic genes of *E. herbicola* pv. *gysophilae* infecting *Gysophila paniculata* plants. When nested PCR assay employing *etz* primers was applied, it was possible to detect single bacterial cell in pure culture, indicating the higher level of sensitivity of nested-PCR format compared with standard PCR procedure (Manulis et al. 1998). *Erwinia amylovora* causing fire blight disease of apple, could be detected in asymptomatic plant material by performing nested-PCR in a single closed tube, using two consective PCRs. A higher annealing temperature that permitted amplification of only an external primer pair was maintained for the first PCR. This was followed by the standard PCR with the internal primer pair. Amplification of a specific DNA fragment from plasmid pEA29 was directed by the second PCR. Both endophytic and epiphytic populations of *E. amylovora* were detected by this nested-PCR protocol which showed promise for the routine use in quarantine programs due to its higher level of sensitivity, specificity, simplicity and rapidity compared with the standard PCR assay (Llop et al. 2000). By using a specific primer set, generation of an amplicon of 500 bp in samples of citrus leaf and citrus xylem extract was observed following the PCR, indicating the presence of *Xylella fastidiosa (Xf).* Detection of *Xf* within sharpshooter heads was possible with nested-PCR. Insoluble acid-washed polyvinyl pyrrolidone (PVPP) was added prior to DNA extraction from the insect tissues for enhancing the sensitivity of nested-PCR format which detected up to two bacteria per reaction (Ciapina et al. 2004).

Huang long bing (HLB), also called citrus greening disease that reduces citrus production heavily, is usally diagnosed by symptoms. The PCR assay and dot- hybridization (Jagoueix et al. 1994; Hung et al. 2000) did not provide consistent results for detection of the pathogen. However, a conventional PCR protocol using a non-chloroform (sodium sulfite method) for DNA extraction, a primer set to amplify a 451-bp amplicon and Klen *Taq* polymerase (a mixture of two polymerses) was demonstrated to be very efficient in detecting HLB in citrus trees. The sequence of cloned amplicon from 16S rRNA gene had 89%–100% sequence identity with corresponding *Candidatus* Liberibacter asiaticus from China, Brazil, Japan and India, *Ca*. Liberibacter americanus from Brazil and *Ca*. Liberibacter africanus from Africa (Gouda et al. 2006). The competitive PCR for detection and quantification is based on competitive amplification of two template DNA and competitor DNA, during progression of PCR program. The number of cycles for the PCR program was increased to 45 for higher sensitivity. The amplified DNAs were evaluated by image analysis of the products in an electrophoretic gel. The leaves from two citrus cultivars grown in southern Vietnam were tested for the presence HLB by using the competitive PCR followed by image-analyzing by software developed in this study. The pathogen-related DNA was found to be less in the citrus cultivar tolerant to HLB compared with susceptible plants. This system was found to be better than real-time PCR assay (Kawabe et al. 2006).

Multiplex PCR assay is of paramount importance, when it is essential to detect and identify several pathogens simultaneously. Infection of a plant species by many bacterial pathogens simultaneously is not common as in the case of plant viruses. Multiplex PCR procedure has been found to be successful in detecting bacterial pathogens infecting simultaneously as in the case of bean pathogens *X. axonopodis* pv. *phaseoli* and pv. *phaseoli* var. *fuscans* (Audy et al. 1994, 1996), *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria* in peppers and tomatoes (Kuflu and Cuppels 1997), potato soft rot pathogens *Erwinia carotovora* subsp. *atroseptica* and *E. chrysathemi* (Smid et al. 1995) and *Clavibacter xyli* subsp. *xyli* and *C. xyli* subsp. *cyanodontis* (Fegan et al. 1998). Multiplex PCR assay was demonstrated to be effective in the detection of four RNA viruses and the bacterial pathogen *Pseudomonas savastanoi* pv. *savastanoi* infecting olive trees (Bertolini et al. 2003). A multiplex PCR protocol using primers based on the sequences of *hrpF* gene rapidly detected pathovars of *Xanthomonas campestris* involved in black rot of crucifers. The limit of detection was one infected seed among 10,000 healthy seeds (Berg et al. 2005).

Integration of PCR with Other Detection Techniques

Integration of PCR with other diagnostic method has resulted in marked improvement in the sensitivity and specificity of detection of bacterial pathogens. The subspecies of *Erwinia carotovora* (*Ec*) could be detected by the PCR-random fragment length polymorphism (RFLP) assay based on a pectate lyase-encoding gene, since pectate lyases have important role in the development of soft rot diseases caused by *Ec*. Wide molecular diversity among isolates of *E. carotovora* subsp. *atroseptica*

(*Eca*) infecting potato was revealed by RFLP analysis. By coupling PCR with a 48-hr enrichment step in a polypectate-rich medium, the sensitivity of PCR-RFLP could be substantially enhanced. The presence of *Eca* in wash water, leaves, stem and tuber peel extracts was detected reliably (Helias et al. 1998). *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), transmitted through infected plant material and contaminated seed, has world wide distribution and hence this pathogen is brought under quarantine regulations to restrict its spread and prevent its introduction into new areas/countries. Based on RFLP analysis, a specific 898-bp PCR fragment was generated from 107 pathogenic strains of *Xam*. However, no amplification occurred when five nonpathogenic strains of *Xam* or several closely related xanthomonads or cassava associated saprophytes were tested, indicating the suitability of this technique for the detection of the *Xam* strains in plant materials (Verdier et al. 1998).

BIO-PCR procedure developed by Schaad et al. (1995) involves a combination of biological and enzymatic amplification of PCR targets. The infection of bean seeds by *P. syringae* pv. *phaseolicola* (*Psp*) was detected by plating the seed soak solution onto a general agar medium and incubation for 45–58 h. The bacterial cells collected by washing with water were subjected to two consecutive cycles of PCR, using nested pairs of primers for amplication of *tox* gene sequences of *Psp*. A modified BIO-PCR, allowing *Psp* present in the bean seed extract to multiply in a semisolid medium for 18 h prior to PCR amplification, showed improvement in the sensitivity of detection (Mosqueda-Cano and Herrera-Estrella 1997). BIO-PCR was also useful for the detection of *Erwinia herbicola* pv. *gysophilae* in the program to establish disease-free nuclear stock of mother plants of gysophila (Manulis et al. 1998). *Clavibacter michiganensis* subsp. *michiganensis* in naturally infected and artificially contaminated tomato seeds was detected only by BIO-PCR format, the limit of detection being one infected seed in lots of 10,000 seeds (Hadas et al. 2005). Absence of false positive results due to the presence of dead bacterial cells and false-negative results due to the presence of PCR inhibitors in the seeds and non-requirement of DNA extraction are the distinct advantages of BIO-PCR technique.

A combined agar-absorption and BIO-PCR assay was developed to overcome the problems associated with slow growing pathogens like *Xylella fastidiosa* (*Xf*). Inhibition of PCR by inhibitors may be avoided by this procedure. *Xf* requires 10–14 days to produce visible colonies. The petioles of grapes and citrus leaves with symptoms were spotted onto agar media, followed by washing after various time intervals and assaying by realtime-PCR. The presence of *Xf* was detected in 97% and 100% of spots after 2 d and 4 h of absorption in agar, respectively in grapes and citrus. The field samples of grapevine were tested and 93% of the samples tested positive after 5 days using agar-absorption PCR. In contrast, all samples tested by direct PCR did not give positive reaction. The agar-absorption-based PCR has the potential for use in detecting of *Xf* and other slow-growing bacterial pathogens in the presence of PCR inhibitors (Fatmi et al. 2005).

The sensitivity of PCR may be considerably increased by immunomagnetic separation (IMS) – PCR or enrichment-involved BIO-PCR. The BIO-PCR is better placed because of the recovery and use of viable culture of the target bacteria. In

order to further increase the sensitivity and reduce the labor required for BIO-PCR a high throughput 96-well membrane BIO-PCR technique was developed for ultra-sensitive detection of *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) in washings of seeds and leaves of bean (*Phaseolus vulgaris*) using available conventional PCR primers and newly designed real-time primers and probe. The primers and probe, designed from a *tox-arg K* chromosomal cluster of the *Psp*-specific phaseolotoxin gene were shown to be specific to *Psp*. Comparison of conventional PCR and the newly developed high throughput membrane BIO-PCR techniques revealed that in the spiked seed washings, conventional PCR was unable to detect *Psp* at mean concentration of 40 CFU/ml. BIO-PCR detected *Psp* in five out of six samples at 40 CFU/ml concentration, but none at mean concentrations of 4.2 and 0.4 mean CFU/ml. On the other hand, membrane BIO-PCR could detect *Psp* in all the six samples containing as few as 0.4 mean CFU/ml. The sensitivity of detection of leaf washings was lower but the results were similar. Classical and BIO-PCR were negative for all three levels of inoculum, while membrane BIO-PCR detected *Psp* in all three samples with a mean concentration of 80 CFU/ml and one out of three at 40 CFU/ml. The sensitivity of membrane BIO-PCR was enhanced by 500-folds over BIO-PCR (without membrane) and real-time PCR (Schaad et al. 2007) [Appendix 15].

The combination of techniques functioning on immunological properties and PCR amplification has been found to be very effective for the detection of bacterial pathogens. Sensitivity and rapidity of detection of bacterial pathogens can be significantly increased by immuno-enzymatic detection of amplified products as in PCR-ELISA procedure, circumventing the requirements of electrophoresis, image capture and other associated steps. A sensitive method of detection of *Erwinia amylovora* (*Ea*) was developed by labeling the PCR amplicons with 11-digoxigenin (DIG) dUTP during the PCR amplification. Then the labeled amplicons were captured by hybridization to a biotinylated oligonucleotide in streptavidin-coated ELISA microplates, followed by detection using anti-DIG-Fab′-peroxidase conjugated antibodies. Strains of *Ea* from different host plant species and geographical origin, could be detected and identified precisely. This PCR-ELISA coupled with chemiluminescent detection could detect as few as 4×10^2 CFU/g of artificially infected pear twigs and also the pathogen in naturally infected plant parts (Merighi et al. 2000).

Immunomagnetic separation (IMS) prior to PCR assay was demonstrated to be more sensitive (100-fold) than direct PCR for the detection of *Acidovorax avenae* subsp. *citrulli* (*Aac*) in water melon seeds. Super-paramagnetic beads pre-coated with sheep anti-rabbit antibodies are coated with purified IgG fractions of anti-*Aac* as per the instructions of the manufacturers (Dynabead M280 sheep anti-rabbit Dynal, Oslo, Norway). The IMS-PCR was not affected by the PCR inhibitors present in the seeds, as in the case of direct PCR assay. The presence of *Aac* in watermelon seed washes containing different levels of infected seeds was not detected by both indirect-ELISA and PCR format. IMS-PCR could detect *Aac* in 100% of seed lots with 10% infestation, whereas seed lots with 5.1 and 0.1% infestation showed positive reaction in 80% of tests. Furthermore, it was possible to detect *Aac*

also in seeds treated with Thiram by employing IMS-PCR technique, suggesting the seed treatment with the fungicide did not influence pathogen detection (Walcott and Gitaitis 2000). Immunocapture (IC)–PCR technique involves the use of antibody-coated magnetic immunocapture beads for capturing cells of *Xylella fastidiosa* (*Xf*). infecting grapevines, followed by PCR assay. IC-PCR technique did not detect *Xf* in more number of naturally infected grapevine samples, compared with standard ELISA format. Hence, it was considered that ELISA technique was preferable, since it was easier, less expensive and less time-consuming than IC-PCR technique for the detection of *Xf* in grapevines (Costa et al. 2004). *Xanthomonas axonopodis* pv. *dieffenbachiae* (*Xad*) causing the destructive bacterial blight of *Anthurium* was efficiently detected by employing a genus-specific monoclonal antibody for capturing *Xad* followed by PCR amplification with specific primers. *Xad* was reliably detected in latently infected plants also (Khoodoo et al. 2005). The IC-PCR technique for detection of *Xad* was more sensitive than both standard PCR and indirect ELISA format.

Suppression subtractive hybridization (SSH) is a PCR-based technique that can be employed to detect differences between prokaryotic genomes with differing phenotypes, including those of pathogenic and nonpathogenic strains of the same pathogen species and between different, closely related species (Brown and Beacham 2000; Miyazaki et al. 2002). The SSH procedure was used to isolate sequences from *Erwinia amylovora* (*Ea*) strain Ea 110, infecting apples and pears. Six subtractive libraries were generated for comparing the genomes of *Ea* strains infecting fruit trees with those of *E. pyrifoliae* (occurring on Japan) and a *Rubus*-infecting strain of *E. amylovora*. The sequences recovered, included Type III secretion components, hypothetical membrane proteins and ATP-binding proteins. An Ea 110-specific sequence showing homology to a Type III secretion component of the insect endosymbiont *Sodalis glossinidius* and also an Ep1/96-specific sequence with homology to the *Yersinia pestis* effector protein tyrosine phosphatase YopH were identified. The sequences that have been identified can be candidates for analyses of their roles in host range differentiation and virulence (Triplett et al. 2006).

2.3.3.4 Real-time PCR

The available conventional methods, although quite sensitive, are unable to comprehensively detect all strains of some bacterial phytopathogens like *Xanthomonas axonopodis* pv. *citri* (*Xac*) and its phylogenetically distinct strains. In addition, gel visualization and other post-PCR amplification steps are also required extending the time required for pathogen detection. Furthermore, to assess the extent of disease incidence and intensity during field surveys, the assay procedure has to (i) be portable, (ii) require mimimal sample handling and (iii) work with suboptimal (old, partially degraded or stored) samples. Real-time PCR technology provides sensitive and reliable detection and identification of phytopathogens rapidly. Real-time PCR is simpler to perform, less labor intensive and faster compared with conventional PCR procedure. Results can be obtained within 1 h, if proper sampling method is followed.

The nonavailability of a practical and efficient method of detecting *Burkholderia glumae* causing panicle blight disease in rice grains necessitated the development of real-time PCR method. The specific primers designed based on the 16S–23S rDNA ITS sequence of several representative isolates from the US and Japan were employed to detect *B. glumae* in seed lots and in whole plants. The real-time PCR protocol developed in this investigation was highly sensitive, rapid and reliable and has great potential for analyzing large number of samples without the requirement of DNA extraction in agarose gel electrophoresis steps (Sayler et al. 2006).

Citrus bacterial canker (CBC) is caused by at least two groups of phylogenetically distinct *X. axonopodis* pv. *citri* (*Xac*) being responsible for Type A citrus canker, whereas *X. axonopodis* pv. *aurantifolii* causes Type B and C canker (Schubert et al. 2001). Citrus bacterial spot (CBS) is due to a diverse group of strains classified as *X. axonopodis* pv. *citrumelo* (*Xacm*) (Graham and Gottwald 1990). A fast sensitive and reliable real-time PCR protocol was developed employing a portable, field hardened mobile RAPID 72,000 system (Idaho Technology, Salt Lake City, UT) and primers designed to detect all canker-causing strains. Single-lesion sampling methods requiring minimal handing were followed and the complete real-time diagnosis was completed in a total time of 4 h with an apparent sensitivity of less than 10 CFU of target cells from diseased lesions. It was possible to detect *Xac* in a herbarium sample from a 1912 canker outbreak by this real-time PCR technique for the first time (Mavrodieva et al. 2004).

A quantitative real-time (QRT) PCR assay was developed for specific detection and quantification of strains by using three sets of primers based on the pathogenicity gene (*pth*) in *Xac*, a ribosomal gene in *Xacm* and the leucine-responsive regulatory protein (*lrp*) in both pathovars. These primers were combined with TaqMan probes and applied for developing calibration curves for bacterial abundance in plant samples. The sensitivity for quantification of *Xac* was higher than for *Xacm*, due to a greater affinity of TaqMan probes to the target sequence. TaqMan technology does not require the post-PCR evaluation, since the fluorescence recorded is due to the amplification of the target sequence and not from a nonspecific product, as may be noted, when SYBR green protocols are employed (Mavrodieva et al. 2004). The QRT-PCR reduced the risk of false positives and enhanced the reliability of detection. In addition to quantification, differentiation of *Xanthomonas* strains infecting citrus tissues was made by allelic discrimination (Cubero and Graham 2005).

Leifsonia xyli subsp. *xyli* (*Lxx*) causing ratoon stunting disease of sugarcane was efficiently detected in the youngest, fully expanded leaf of three cultivars collected at biweekly interval from the field nurseries, by applying real-time PCR assay. The presence of the bacterium even in 1 month old plant could detected. Real-time PCR assay was more effective than conventional PCR for detection of *Lxx* in leaf tissue. Quantification of *Lxx* using real-time PCR formed the basis for ranking the cultivars to indicate the levels of resistance to the disease. The results were in close agreement with those of tissue-blot immunoassay performed on tissues from 7- to 9-month old stalks. The real-time PCR assay could be applied for determining the resistance levels of cultivars using 3 or 4 month old plants providing the advantage of using younger plants for testing. Thus results may be available much earlier, if

real-time PCR technique is used for detection and quantification of *Lxx* in sugarcane plants (Grisham et al. 2007). A combination of real-time PCR and BIO-PCR assay approaches resulted in highly sensitive detection of strains of race 3 biovar (bv) 2 of *Ralstonia solanacearum* (*Rs*) in asymptomatic potato tubers. Real-time PCR primers and probes of real-time PCR detected all 17 strains of bv2 including 12 from potato and 5 from geranium. Other strains of *Rs* and 13 bacterial species associated with potato were not detected by this procedure, indicating its high level of specificity of detection of the target pathogen only. On the other hand, the standard real-time PCR failed to detect *Rs* and its strains, confirming the greater sensitivity of the combination of the two techniques (Ozakman and Schaad 2004).

Detection of *Xylella fastidiosa* (*Xf*) a xylem-limited pathogen has been found to be difficult due to low concentrations of the bacteria in asymptomatic plant tissues and in vector insects in addition to its irregular distribution in infected plants. Earlier investigations based on the use of conserved sequences of the 16S rRNA and 16S–23S ITS and primers based on the target sequence identified by RAPD have been successful in the detection of *Xf* in citrus infected by variegated chlorosis (Pooler and Hartung 1995; Schaad et al. 2002). By using SYBR Green, *Xf* was detected in the vector glassy-winged sharp-shooter (GWSS), *Homalodisca coagulata*. But this technique was less specific and reliable than the TaqMan system (Giulietti et al. 2001; Bextine et al. 2005). The transmissibility of CVC strain of *X. fastidiosa* by GWSS was tested by PCR assay and membrane entrapment immunofluroescene (MEIF) test after inoculating Madam Vinous sweet orange plants. Six of the 16 inoculated plants were positive for PCR and MEIF analysis. Scanning electron micrographs of xylem vessels from the six CVC-infected sweet orange seedlings and cibaria of 27 sharp shooters that had fed on infected source plants showed the presence of bacterial populations abundantly (Fig. 2.10) (Damsteegt et al. 2006).

Xylella fastidiosa (Xb) causing almond leaf scorch disease (ALSD) and grapevine Pierce's disease (PD) were considered to be the same pathogenic strain of *Xf.* The strains of ALSD sampled from two locations in California were found to be two genotypically distinct types of *X. fastidiosa* strains. Single nucleotide polymorphisms (SNPs) in the (16S rRNA gene (16SrDNA) of *Xf* were identified to characterize the population in infected trees. However, when genotype-specific-SNPs were employed to design primers for multiplex PCR assays of early passage cultures, two genotypically distinct types of *Xf* strains, G type and A type were found to coexist in the same infected almond orchard. The RFLP analysis of a different genetic locus RST 31-RST33 confirmed the findings of multiplex PCR assays (Fig. 2.11). This report on the mixed genotype infection of *Xf* in the same location under natural conditions appears to be the first indicating the epidemiological importance of this finding (Chen et al. 2005b).

In a later investigation a dual purpose conventional PCR and quantitative PCR (Taq Man^{TM}) system was developed for the generic detection of *Xf* strains. The primers HL5 and HL6 were designed to amplify a unique region common to the sequenced genomes of four *Xf* strains. A 221-bp fragment from strains associated with grapes Pierce's disease, almond leaf scorch and oleander leaf scorch disease

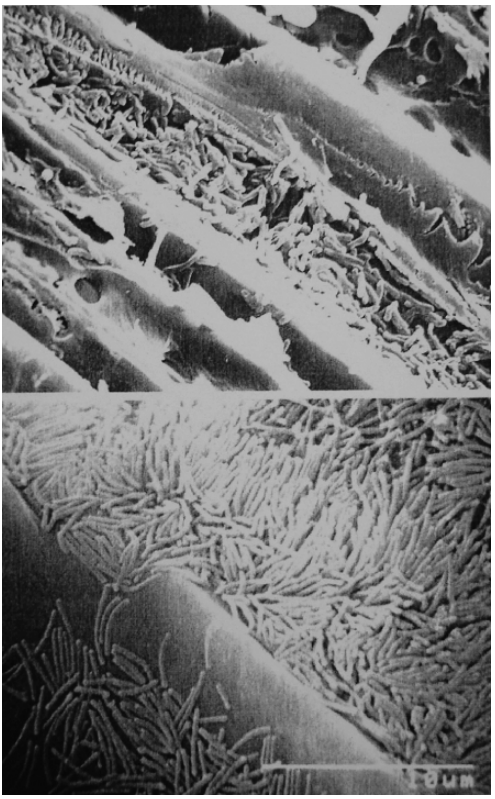

Fig. 2.10 Detection of *Xylella fastidiosa* (*Xf*) in plant host and vector tissues using scanning electron microscopy
Top: *Xf* in xylem vessels from infected sweet orange seedlings; *Bottom*: Cibarium of *Homalodisca coagulata* (glassy-winged sharpshooters) after feeding on sweet orange plant infected by citrus variegated chlorosis disease. (Courtesy of Damsteegt et al. 2006; The American Phytopathological Society, St. Paul, MN, USA)

and also citrus variegated chlorosis was amplified by these primers. The standard curves based on known dilution of *Xf* in water, and grape extracts and insect cells were prepared. The regression curves were similar with high correlation coefficients ($r^2 > 0.97$). In quantitative PCR, Ct values ranged between 20 and 36 cycles for $5–10^5$ bacterial cells per reaction. The protocol developed in the study, provided a reliable detection of *Xf* in grapes, almonds and insect vectors with a high degree of sensitivity and specificity (Francis et al. 2006).

Xylophilus ampelinus (*Xa*) causing grapevine bacterial blight disease is transmitted mainly through infected plant propagation material and sometimes via contaminated tools. Detection of *Xa* in plant materials and elimination of such infected ones form the practicable disease mangement method. Nucleic acid-based procedures have to encounter the problem of inhibitory substances present in plant extracts interfering with DNA amplification. Nested PCR procedure targeting the 16S–23S

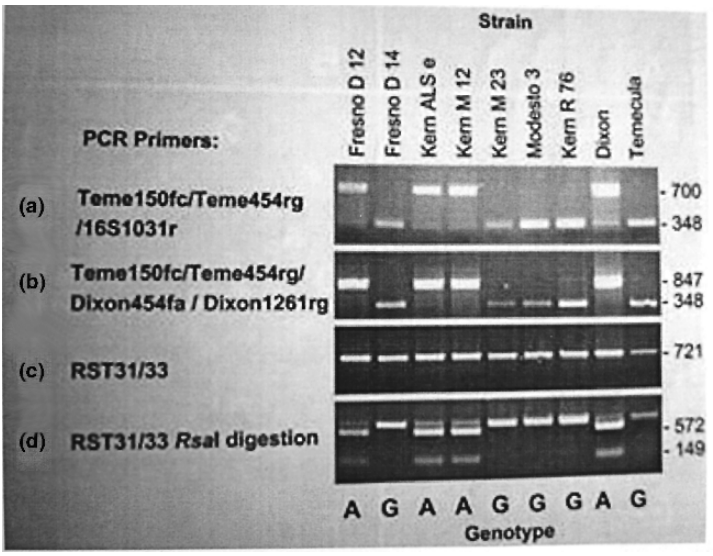

Fig. 2.11 Detection of different strains of *Xylella fastidiosa* (*Xf*) causing almond leaf scorch disease using different primer sets in PCR assay
(**a**) Three-primer format; (**b**) Four-primer format; (**c**) RST31/RST33 primer set; (**d**) *Rsa* I digestion of RST31/RST33 amplicons. (Courtesy of Chen et al. 2005b; The American Phytopathological Society, St. Paul, MN, USA)

rDNA intergenic spacer region was followed to overcome the effects of inhibitory substances in grapevine plants (Botha et al. 2001). A PCR protocol combining with an ELISA-based signal amplification was developed by Manceau et al. (2005). Improvement of detection of *Xa* even in grapevine tissues containing low numbers of bacteria was achieved by using an agar absorption based technique prior to BIO-PCR (enrichment) procedure (Fatmi et al. 2005). However a high contamination risk from handling enriched samples or PCR products between reaction limits its applicability considerably. A real-time method based on a 5′-nuclease and minor groove binding (MGB) probe was developed to overcome the shortcoming of the methods followed earlier. This protocol was able to provide a fast, sensitive and reliable detection of *Xa* in plant tissues and to identify isolated bacteria. Used in combination with DNeasy plant minikit, the sensitivity of *Xa* detection was approximately 100 cells from tissue extract, proving to be more sensitive than the nested-PCR technique at least 10-fold. The real-time PCR assay amplified all isolates of *Xa* from different geographical locations. The target sequence seemed to be conserved and specific in *Xa* isolates, since no specific signal was obtained with other bacterial pathogens of grapevine. In field samples, a high correlation between real-time PCR cycle threshold (Ct) values obtained and *Xa* isolation on artificial media. The high sensitivity of real-time PCR assay could allow detection of *Xa* in low number in tissues, facilitating detection of latent infection in planting materials which form the principal method of *Xa* dissemination to distant locations (Dreo et al. 2007).

2.3.3.5 Loop-Mediated Isothermal Amplification Technique

The loop-mediated isothermal amplification (LAMP) is a new DNA amplification procedure based on autocycling strand displacement DNA synthesis by a DNA polymerase which has high strand displacement activity and a set of specially designed inner and outer primers (Notomi et al. 2000). This is a simple method of detection of target phytopathogens, not requiring thermal cycler and other expensive equipments as in the case of PCR and real-time PCR techniques. Amplification may be completed within 30 min using a simple waterbath that can maintain a constant temperature of 65°C. LAMP procedure, suitable for under-equipped laboratories of extension centers and quarantine facilities, was successfully employed for the detection of citrus greening disease caused by *Candidatus* Liberibacter. Primers were designed based on the sequence of conserved region of the *nus* G-*rpl* KAJL-*rpoB* gene cluster. The LAMP product was rapidly detected on nylon membranes by staining with AzurB. The LAMP-based assay could detect as low as 300 copies of the *nus* G-rplKAJL-rpoB fragment of the Japanese and Indonesian isolates of *Ca.* Liberibacter. This detection technique is expected to facilitate elimination of infected citrus trees at early stages of infection which exhibit non-specific nature of foliar symptoms (Okuda et al. 2005).

2.3.3.6 DNA Array Technology

The DNA array technology, being essentially a reverse dot-blot technique, is useful for identification of DNA fragments. It is applicable for rapid detection and identification of pathogens in infected plants and in the environment. An array of species-specific oligonucleotide probes representing different pathogens infecting one crop/plant species, is built on a solid support such as nylon membrane or microscope slide. The probes are then probed readily with labeled PCR products amplified from samples of test plant tissues. Conserved primers are used to amplify common bacterial genome fragments from extracts of plant tissues that may contain the bacterial pathogens to be detected. The presence of DNA sequences indicative of pathogens would be revealed by hybridization to species specific-oligonucleotides probes within the array.

For the detection and identification of bacterial pathogen of potato viz. *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), *Ralstonia solanacearum* (*Rs*) *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *E. catorovora* subsp. *carotovora* (*Ecc*) and *E. chrysanthemi* (*Ec*), oligonucleotides in the 16S and –23S intergenic spacer (IGS) region of the ribosomal gene clusters that are specific for each pathogen were designed and formated into an array by pinspotting on nylon membranes. By employing conserved ribosomal primers, the specific genomic DNA from bacterial cultures were amplified and labeled simultaneously with digoxigenin-dUTP. Distinct hybridization patterns for each species and subspecies tested were recognized, following hybridization of amplicons to the array and subsequent serological detection of digoxigenin label. Hybridization patterns were recorded as separate gray values for each hybridized spots and revealed a consistent pattern for multiple strains of each species or subspecies isolated from diverse geographical locations. The bacte-

rial pathogens could be detected and identified precisely from both mixed cultures and inoculated potato tissues (Fessehaie et al. 2003).

A DNA array was formated to detect and identity one bacterial pathogen (*Erwinia amylovora*) and four fungal pathogens (*Botrytis cinerea*, *Penicillium expansum, Podosphaera leucotricha* and *Venturia inaequalis*) causing economically important apple diseases. The oligonucleotides or probes were spotted on nylon membrane by an amine modified linker arm and arranged in a precise pattern to form an array. The DNA array identified all pathogens tested correctly. The probe sequences for EA-H1 and EA-H4C (designed from the ITS region of *Erwinia amylovora* (*Ea*) were specific to two of the three *Ea* isolates and did not react with the third isolate (G-5) from pear. However, the isolate G-5 positively reacted with the probe EA-H3d that was designed for the detection of both *Ea* and *E. pyrifoliae* (a pathogen of Asian pear closely related to *Ea*) (Scholberg et al. 2005) [Appendix 16].

2.3.3.7 Nucleic Acid Sequence-Based Amplification Assay

PCR-based assays have been shown to be sensitive and specific for the detection of plant pathogens. However, PCR-amplification procedures, using primers detected against specific rDNA sequences are unsuitable for determining live pathogenic cells or units as required in studies on pathogen population dynamics. This limitation of PCR assays is due to the fact that DNA from lysed target cells can persist for long time in the natural environment or in plant tissues. Hence, PCR assays cannot differentiate living cells from dead ones. In this context, a method capable of detecting RNA would be helpful to determine the viability of target pathogen, since RNA is degraded rapidly after the collapse of cells (van der Vliet et al. 1994). A technique designated nucleic acid sequence-based amplification assay (NASBA) was developed to amplify isothermally a specific RNA sequence of human immuno-deficiency virus (HIV) (Kievitis et al. 1991). Later, NASBA was successfully applied for the detection and identification of bacterial human pathogen (*Mycobacterium smegmatis* (van der Vliet et al. 1994) and *Mycoplasma pneumoniae* (Ovyn et al. 1996).

The suitability of NASBA for the detection of bacterial pathogen *Ralstonia solanacearum* (*Rs*) in potato tissues was demonstrated by Bentsink et al. (2002). This technique, using oligonucleotide probes targeting *R. solanacearum* (*Rs*) allowed amplification and detection of an amount of nucleic acids corresponding to about 10^4 molecules. This amount is equivalent to what is present in one viable bacterial cell. The probes employed were able to distinguish *Rs* from the phylogenetically related species *R. syzigii* and *R. picketii*. The detection level of NASBA for *Rs* added to potato tuber extract was at 10^4 CFU/ml of extract, equivalent to 100 CFU per reaction. This technique will be particularly valuable for the detection of *Rs* in the ecological studies that need determination of viable cells of target pathogens (Bentsink et al. 2002). In a further investigation, the AmpliDet RNA technique based on NASBA of RNA sequences and homogeneous real-time detection of NASBA amplicons with a molecular beacon was developed for the sensitive and specific detection of *Rs*. As the AmpliDet RNA protocol was carried out in sealed tubes, the risks of carry-over contamination were minimized. Ampli Det RNA provided

reliable detection of specific 16S rRNA sequences of *Rs* in total RNA extracts from potato tuber samples in 90 min at a level of 10 cells per reaction, equivalent to about 10^4 cells/ml of sample. All potato tuber samples (18) contaminated with *Rs* were consistently positive in all tests. The presence of *Rs* in surface water was also detected by AmpliDet RNA, after concentrating the bacterial cells in the water sample (Van der Wolf et al. 2004).

2.3.3.8 Multilocus Sequence Type (MLST) System

Multilocus sequence typing (MLST) is a recently developed procedure to identify bacterial strains entirely based on nucleotide sequence differences in a small number of genes. In this system each allele of a gene is given a number, and each strain characterized (for *n* loci) is represented by a set of *n* numbers defining the alleles at each locus. The sequence type (ST) is characterized by these numbers. By convention, a seven-locus MLST data set is commonly used and such a set provides high level of discrimination. A single-nucleotide difference always produces a new allele in an MLST data set which distinguishes this method from other methods such as multilocus enzyme electrophoresis (MLBE) and pulsed field gel electrophoresis (PFGE) requiring greater number of substitution for discrimination (Peacock et al. 2002). The MLST method was applied to *Xylella fastidiosa* using an initial set of sequences of 10 loci (9.3 kb) for detecting and differentiating 25 strains from different host plants, grapevine (PD strains), oleander (OLS strains), oak (OAK strains), almond (ALS strains) and peach (PP strains). The allelic profiles of the 25 strains studied produced 19 different STs (ST1 to ST19). The MLST methodology grouped the *X. fastidiosa* PD, OLS, ALS, OAK and PP plant host strains into six clonal complexes. The simplicity of MLST compared to a phylogenetic approach is a distinct advantage in providing information, when the spread of the strains has to be tracked and to rapidly recognize the incidence of an unusual or new isolate. Further, the diversity within a bacterial species may be efficiently catalogued by applying MLST procedure (Scally et al. 2005).

2.3.4 Detection of Phytoplasmal Pathogens

The phytoplasmas, except three helical spiroplasmas, have not been characterized due to lack of information on cultural and other biological properties. They have not been cultured on cell-free aritifical media and hence considerable problems arise to obtain purified genomic DNA of phytoplasmas. Different nucleic acid-based techniques have been applied for the detection of phytoplasmas in various plant hosts and the insect vectors that are involved in the spread of the diseases caused by them.

2.3.4.1 Dot-Blot Hybridization Assay

DNA probes designed based on the sequences of chromosomal or plasmid DNA of the phytoplasmal pathogen, labeled either with radio-active ^{32}P or nonradioactive

biotin have been used. The nonradioactive digoxigenin is used more frequently for the detection of phytoplasmas. ^{32}P-labeled single-stranded riboprobe (RNA) with plasmid vector pS64 was employed for the detection of Western X phytoplasma and aster yellows (AY) (Lee and Davis 1988; Davis et al. 1990). Biotinylated cloned DNA probes were used for detection of AY phytoplasma in infected plants and the leaf- hopper vector *Macrosteles fascifrons* (Davis et al. 1990; Davis et al. 1992). The sweet potato witches'-broom phytoplasma was detected in sweet potato and periwinkle plants by employing digoxigenin-labeled DNA probes (Ko and Lin 1994). The phytoplasma causing decline syndrome in coconuts was detected by dot-blot hybridization by using two probes from palm lethal yellowing (PLY) phytoplasma in Florida (Tymon et al. 1997).

2.3.4.2 Restriction Fragment Polymorphism Analysis

The yellow leaf crinkle (PYC) and mosaic (PM) disease of papaya occurring in Australia were shown to be caused by phytoplasmas that are identical by restriction fragment polymorphism. But the phytoplasma causing die back (PDB) symptoms showed distinctly different RFLP pattern (Gibb et al. 1996). *Candidatus* phytoplasma australiense as the cause of pumpkin yellow leaf curl disease in Queensland was established by examining the amplification products using the fP1 and rP7 primers followed by digestion with restriction enzymes *Alu*I and *Rsa*I. The RFLP patterns of the diseased pumpkin samples were distinguishable from the tomato big bud phytoplasma (Streten et al. 2005a). The Argentinean alfalfa witches'-broom (ArAWB) phytoplasma was detected by the RFLP analysis of partial 16S rRNA gene of two ArAWB isolates, digested with 16 restriction enzymes. Differences between the ArAWB and the reference strain (Ash Y1^{T}) in six enzymes patterns were discernible. Restriction patterns unique for the group and an exclusive *Hinf1* restriction site were found in the ArAWB phytoplasma DNA (Conci et al. 2005). Infection of peach trees by aster yellows (AY) was detected by subjecting PCR products to RFLP analysis after digestion with endonucleases *AluI, HpaII, KpnI* and *RsaI*. The restriction profiles of all samples tested, were identical with those of American aster yellows (16SrI) phytoplasma strain (Anfoka and Fattash 2004). Tomato big bud phytoplasma belonging to group 16SrIII occurring in Brazil was detected by RFLP analysis using endonucleases *HhaI* and *RsaI* (Mello et al. 2006).

2.3.4.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) provides a sensitive, specific and fast detection system for phytoplasmas. General and specific primers located in the 16S rDNA intergenic spacer (IGS) and the 23S rDNA region of the phytoplasma genome have been used frequently. The PCR assay has been particularly useful, when the phytoplasmas are irregularly distributed and occur in low concentrations. A cloned fragment of a plasmid from the phytoplasma is sequenced to identify oligonucleotide primers for PCR. Amplified DNA fragments of the expected size are then detected in the DNA extracted from plants or insect vectors containing the

phytoplasma under investigation. In contrast, there will be no amplification in the extracts of healthy plants or nonvector insects.

In most cases, detection of phytoplasma by PCR assay has been reported to be more sensitive, specific and rapid compared with hybridization assay or immunodetection techniques. In the case of aster yellows (AY) phytoplasma, the PCR assay was found to be 500 times more sensitive than the hybridization procedure (Goodwin et al. 1994). PCR detection of the pear decline phytoplasma with primer pairs (fU5/rU3 and f01/r01) from ribosomal DNA sequences was significantly more sensitive than the microscopic detection using the 4′-6-diamidino-2-phenylindole fluorescence procedure (Lorenz et al. 1995). The PCR was shown to be effective for the detection of phytoplasmas causing pear decline, Western X-disease, peach yellow leaf roll, peach rosette, apple proliferation, Australian grapevine yellows and Vaccinium witches' broom diseases (Green et al. 1999). By amplifying a 237-bp DNA frgment from total DNA extracts derived from over 300 stone fruit samples, the infection by European stone fruit yellows phytoplasma was recognized. A high correlation (97%) between the results obtained with specific and universal primers was observed (Jarausch et al. 1998).

New primers using rDNA sequence information from an Australian isolate of European stone fruit yellows (ESFY) phytoplasma were deisgned and they were able operate at high annealing temperatures. Hence, the specificity of detection increased in addition to lowering of the risk of false positive. The primers could reliably detect the apple proliferation (AP), pear decline (PD) and ESFY phytoplasmas. In addition, the primers could be employed for identification of strains by direct PCR followed by RFLP analysis as demonstrated with micro-propagated fruit tree material (Heinrich et al. 2001).

The sensitivity of detection may be enhanced by employing nested-PCR assays using the universal primer pair R16 F 2/R2 and a group specific primer pair (Lee et al. 1994). Grapevine plants showing "Bois noir" disease symptoms in Italy and the weeds in the vineyard were tested for the association of phytoplasma. Nested-PCR assays using primers specific for the phytoplasma 16S rDNA gene revealed the infection of the weeds *Calendula arvensis, Solanum nigrum* and *Chenopodium* spp. by a phytoplasma of the 16SrII-E subgroup. However, the weed species and leafhopper collected in the vineyard tested negative by PCR assays for the stolbur phytoplasma, causing "Bois noir" disease of grapevine (Tolu et al. 2006). A nested-PCR protocol using phytoplasma specific rRNA operon primers was developed for the detection of sugarcane grassy shoot (SCGS) phytoplasma in infected sugarcane plants and SCGS-exposed nymphs of *Deltocephalus vulgaris*. Both plant and insect tissues, following nested-PCR yielded SCGS-exclusive DNA bands. This technique has the potential for assessing the *D. vulgaris* population carrying the phytoplasma (Srivastava et al. 2006).

Candidatus phytoplasma australiense (*C*a Pa) was reported to be associated with strawberry lethal yellows (SLY), strawberry green petal (SGP), papaya dieback (PDB), Australian grapevine yellows (AGY) and Phormium yellow leaf (PYL; New Zealand). By using specific primers, the presence of *Ca* Pa was detected in 18 plant species (Streten et al. 2005b). The phytoplasma universal rRNA primer

pair P1/P7, nested LY-group-specific rRNA primer pair 503f/LY 16Sr or LY phytoplasma-specific nonribosomal primer pair LYF1/R1 were employed to detect lethal yellowing (LY) phytoplasma in the embryos from fruits of diseased Atlantic coconut palms by PCR. The distribution LY phytoplasma in sectioned tissues from PCR-positive embryos was investigated by in situ PCR and digoxigenin-11-deoxy-UTP (Dig) labeling of amplification products. The Dig-labeled DNA products were detected by colorimetric assay (Cordova et al. 2003).

A simplified DNA preparation for PCR amplification for the detection of phytoplasmas in herbaceous and woody plants was developed. Thin free-hand cross-sections were prepared and stored in acetone. Treatment of tissue sections by grinding or boiling in NaOH, sonicating in water, microwaving in water or placing directly in PCR tube provided phytoplasmal template. Then PCR amplification was performed with a universal phytoplasma-specific primer pair in a reaction buffer containing 0.5% (V/V) Triton X −100, 1.5 mM magnesium chloride and 10 mM Tris–HCl. In the case of woody plants (green ash-*Fraxinus pennsylvanica*), grinding, boiling or microwaving procedures resulted in positive amplification, whereas all procedures tested for release of templates were successful for herbaceous plants such as periwinkle, carrot and maize. These procedures are simple, labor-saving and need only small amounts of tissues for testing and indexing of plant materials for freedom from infection (Guo et al. 2003).

Alfalfa plants infected by witches' broom phytoplasma in Sultanate of Oman, showed the specific amplification of 16S–23S rRNA gene of the pathogen in PCR using the phytoplasma-specific universal primer pairs. Restriction fragment length polymorphism (RFLP) profiles P1/P7 primer pair identified the alfalfa phytoplasma belonging to peanut witches'-broom group (16SrII or faba bean phyllody). The restriction enzyme profiles indicated that the alfalfa phytoplasma enzyme profiles indicated that the alfalfa phytoplasma was different from all others included in subgroup 16SrII, except tomato big bud phytoplasma from Australia and hence, it was classified in subgroup 16Sr II-D. The PCR product of P1/P7 primer pair amplification of DNA of alfalfa phytoplasma (AlfWB) was sequenced and it showed 99% similarity with papaya yellow crinkle (Papaya YC) phytoplasma from New Zealand. The results show that AlfWB phytoplasma as a new phytoplasma species with closest relationships to papaya YC phytoplasmas from New Zealand and Chinese pigeonpea witches' broom phytoplasmas from Taiwan (Khan et al. 2002). Carrot crops showing symptoms suggestive of infection by Mollicutes (phytoplasmas and spiroplasmas) were observed in south central Washington. To establish the nature of the causal agents, PCR assays were carried out using primers specific to phytoplasmas and primers specific to plant pathogenic spiroplasma. The PCR amplicons of 16SrDNA sequence were subjected to RFLP analysis. About 81% of affected plants with dark purple or yellow purple leaf symptoms tested positive for *Spiroplasma citri*. Other carrot plants exhibiting mild purple discoloration of leaves showed the infection of clover proliferation group (16SrV1), subgroup 16SrVI-A and aster yellows group (16SrI), subgroup 16SrI-A alone or in combination with *S. citri*. This report appears to be the first in recording spiroplasma infection in carrots in the United States (Lee et al. 2006).

The corn stunt disease caused by *Spiroplasma kunkelii* is a helical, motile, cell wall-less bacterium belonging to the class *Mollicutes.* It is transmitted by phloem-feeding leafhopper *Dalbulus maidis.* It is important to detect the infection of corn by *S. kunkelii*, for disease forecasting to avoid disease spread and reduce crop losses, since foliar symptoms of this disease can be seen only closer to flowering time. The gene encoding a novel adhesin-like protein was identified in the pathogen genome. Adhesins of the sarpin family including SARP1 from *S. citri* and SkARP1 from *S. kunkelii* are considered to have role in the adhesion of spiroplasma cells to gut cells of insect vectors during early stages of infection (Berg et al. 2001; Davis et al. 2005). A field-depolyable real-time PCR protocol was developed for rapid, specific and sensitive detection of *S. kunkelii* using the primers designed based on the sequences of adhesin-like gene and fluorigenic probe. The presence of *S. kunkelii* DNA as low as 5 fg in the plant and vector tissue could be detected by real-time PCR, the sensitivity of assay being 100-folds greater than the conventional PCR format. The conventional PCR detected *S. kunkelii* DNA at a dilution of 50×10^{-5} ng/µl (500 fg template DNA) regardless of whether primer set CSSF1/CSSR1 or primer set sk104F/sk 104R was used for amplification (Fig. 2.12). No fluorescence signal was detected from DNA samples isolated from in vitro cultured *S. citri*, from healthy corn or from aster yellows phytoplasma (*Candidatus* phytoplasma asteris-related strain)-infected plant materials (Wei et al. 2006).

The immunoenzymatic determination of PCR amplified products in the liquid phase simplifies the analysis of the results with an ELISA reader eliminating the need for cumbersome electrophoresis and staining procedures. Apricot chlorotic leaf roll (ACLR), plum leptonecrosis (PLN) and pear decline (PD) phytoplasmas were detected by PCR-ELISA procedure more efficiently. Detection of PCR products by PCR-ELISA was more sensitive (5–15 times) than electrophoresis. The PCR-ELISA technique is simple to use and the determination of amplified products can be adapted to the size and the simple instruments needed by ELISA format,

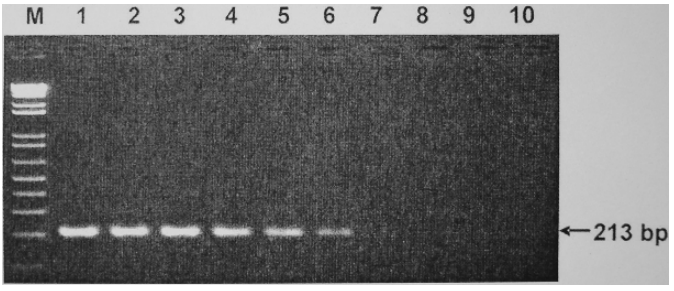

Fig. 2.12 Detection of *Spiroplasma kunkelii* by PCR amplification of targeted gene sequences of the pathogen
Amplicons (213-bp) generated with primer set CSSF1/CSSR1 resolved by agarose gel electrophoresis. Lanes 1–10: DNA template from different dilutions from 50×10 to 50×10^{-9} ng Lane M: DNA ladder (standards). (Courtesy of Wei et al. 2006; The American Phytopathological Society, St. Paul, MN, USA)

indicating the suitability of PCR-ELISA for large scale use (Pollini et al. 1997). Immunocapture (IC) of phytoplasmas with appropriate monoclonal antibody against apple proliferation (AP) phytoplasma was performed to increase the concentration, prior to PCR assay. The primer PA2 F/R was employed for PCR amplification and the amplification product, after dilution was used for nested PCR with NPA2F/R primers. This protocol was found to be very sensitive and detect Ap in vitro infected plant tissues extracts up to dilution of 1:1600. The IC-PCR proved to be more sensitive and reliable compared to ELISA. It may be a valuable alternative for large scale testing of apple trees (Heinrich et al. 2001).

Real-time PCR assays for the specific diagnosis of flavescence dorée (FD), bois noir (BN) and apple proliferation (AP) phytoplasmas and a universal one for the detection of phytoplasmas belonging to groups 16Sr-V, 16Sr-X and 16Sr-XII were developed. The phytoplasmas in field collected samples from grapevines infected with FD and BN phytoplasmas and the insects *Scaphoideus titanus, Hyalesthes obsoletus* and *Cacopsylla melanoneura* were detected by employing the primers designed. The group-specific assays provided highly efficient detection comparable to nested-PCR (Galetto et al. 2005). A quantitative, real-time PCR was applied for detection and quantification of a group 16Sr-VI phytoplasma in DNA extracts prepared from infected tomatoes, potatoes and beet leafhoppers (*Circulifer tenellus*). Primers and probes were prepared from the sequences of 16S rRNA gene of the Columbia Basin potato purple top phytoplasma which is closely related to the beet leafhopper-transmitted virescence agent. The limit of detection of phytoplasma in tomato was determined to be approximately 50 pg DNA. The phytoplasma could be detected in extracts from single or groups of five beet leafhoppers. Aster yellows (group 16Sr-I) and pigeonpea witches' broom (group 16Sr-IX) were also detected by employing the protocol developed in this study. This assay was as sensitive as the more labor-intensive nested-PCR for the detection of the phytoplasma (Crossolin et al. 2006).

Infection of carrot purple leaf disease occurring in Washington State under natural conditions was investigated by using PCR assay employing primers specific for spiroplasmas as well as for phytoplasmas. RFLP analysis of PCR-amplified 16S rDNA sequences revealed that about 8.1% of affected plants tested positive for *S. citri.* The carrot purple leaf disease-causing agent was proved to be *Spiroplasma citri.* Incidence of aster yellows and beet leaf hopper-transmitted virescence agent (BLTVA) yellows phytoplasmal diseases was also confirmed by applying PCR assays (Lee et al. 2006).

2.3.5 Detection of Fungal Pathogens

Nucleic acid-based techniques for the detection of fungal pathogens have distinct advantages over serological techniques. The fungal antigens are complex and variable depending on the growth stages. The production of spore bearing structures and rate of development are significantly influenced by cultural and environmental conditions. On the other hand, nucleic acid-based methods require small quantities

of fungal cells containing DNA. By using specific DNA probes, fungal pathogens causing nonspecific generalized rotting and death of plants may be rapidly detected, precisely identified and distinctively differentiated.

2.3.5.1 Dot-Blot Hybridization Assay

Rhizoctonia solani AG8 soilborne pathogen causing root rot and damping off diseases in several crops, could be detected in soil samples by employing a specific DNA probe pRAG12. A high copy number of AG-8 probe and its specificity constitute a reliable basis for a sensitive detection of *R. solani* in infested soil samples (Whisson et al. 1995). The pathogenic isolates of *Gaeumannomyces graminis* var. *tritici* were detected and differentiated by using a specific DNA probe pG158 both in the soil and roots of wheat in which the pathogen causes a devastating "take all" disease (Harvey and Ophel-Keller 1996). A sensitive, species-specific DNA probe isolated from a library of genomic DNA was employed in dot and slot-blot assays for the detection of *Phytophthora cinnamomi* infecting avocado roots. The probes detected as little as 5 pg of *P. cinnamomi* DNA in the assays. Quantitative assessment indicated that the extent of colonization deduced from the measurement of the relative amounts of pathogen and host plant DNA, increased over time and with increasing amounts of inoculum (Judelson and Messenger-Routh 1996). Identification of *Pythium* spp. is often difficult by using morphological characters. Species-specific probes could be obtained by amplification of the 5S rRNA intergenic spacer for species. The amplicons were probed using DIG- labeled probes (Klassen et al. 1996).

2.3.5.2 Restriction Fragment Length Polymorphism

Diagnosis of fungal diseases by employing restriction fragment length polymorphism (RFLP) analysis has been successful. Natural variations in the genomes of different groups of strains of organisms are revealed by RFLP analyses. A set of restriction enzymes that cleave the genome of the test fungal pathogen(s) at specific restriction (recognition) sites is used to obtain fragments of different sizes. The size and number of fragments formed after digestion depend on the distribution of restriction sites in the pathogen DNA. A specific set of DNA fragments that are considered as fingerprint for the test organism is formed, following digestion with different restriction enzymes. The fragments are separated by electrophroesis in agarose or polyacrylamide gel electrophoresis (PAGE) and they can be visualized after staining the gels with ethidium bromide under ultraviolet light. The DNA fragments have to be transferred to a nitrocellulose or nylon membrane and hybridized with an appropriate probe.

Detection of fungal pathogens may be done by employing appropriate probes as in the case of *Pseudocercosporella herpotrichoides.* Infection of rye seedlings by R type of *P. herpotrichoides* was detected by hybridization of a 6.7 kb DNA fragment from an R-type isolate of the pathogen. Specific hybridization occurred only to R-type isolates but not to N, C or S pathotypes of *P. herpotrichoides* or to

P. anguioides (Nicholson et al. 1994). Soybean seed decay is primarily due to *Phomopsis longicolla*, but *Diaporthe phaseolorum* is also commonly associated with *P. longicolla.* The RFLP analysis of PCR amplification products provided reliable basis for the detection and differentiation of these pathogens. Primers Phom.I and Phom.II designed from the polymorphic regions of *P. longicolla* and *D. phaseolorum* isolates from soybean were used for PCR amplification. Bands specific to both pathogens could be recognized. Presence of similar specific bands in DNA extracts of tissues from asymptomatic plants inoculated with *P. longicolla* and *D. phaseolorum* var. *sojae* confirmed the specificity of RFLP analysis in detecting and distinguishing these pathogens (Zhang et al. 1997, 1999). The ITS region of the genomic DNA of the powdery mildew pathogens is considered to be the most appropriate target for identifying anamorphic powdery mildew fungi. The molecular information of the ITS region along with morphological characteristics and host range may provide a sound basis for accurate identification of most anamorphic *Erysiphales* (Cunnington et al. 2003). The identity of a range powdery mildew pathogens occurring in Australia was established by analysing the rDNA ITS region using RFLPs and sequence data. Three broad groups were recognized by RFLP analysis. The anamorphs show affinities to *Golovinomyces* (*Erysiphe*) in the Solanaceae, *Erysiphe trifolii* in Fabaceae (Cunnington et al. 2003, 2004, 2005).

The identity of the etiology of *Glomerella* leaf spot and bitter rot of apple was examined by studying 155 isolates of *Glomerella cingulata,* 42 isolates of *Colletotrichum gloeosporioides* and *C. acutatum* using mitochondrial (mt) DNA RFLP haplotypes. Seven different mt DNA RFLP haplotypes were recognized within isolates of *G. cingulata*, two within isolates of *C. gleosporioides* and two within isolates of *C. acutatum.* For distinguishing isolates of *G. cingulata* pathogenic on both leaves and fruit from those pathogenic only on fruit, application of vegetative compatibility group (VCG) characteristics was a better approach than use of molecular characteristics (González et al. 2006). Anthracanose-like fruits rots in tomato were found to be due to different species of *Colletotrichum, Alternaria, Fusarium, Phomopsis* and *Mucor. Colletotrichum* spp. was most abundant in collections made in Ohio, representing 136 of 187 isolates. The ITS sequence analysis in combination with RFLP pattern analysis was useful in identifying fungal isolates. By using RFLP analysis, the 187 isolates were classified into six groups. This enabled sequence analysis of only those isolates with unique banding patterns. Sequence analysis of amplified products indicated high levels of sequence identity with five different genera. Thus the fungal isolates could be rapidly classified upto genus level (Gutierrez et al. 2006).

2.3.5.3 Amplified Fragment Length Polymorphism

Although many molecular techniques can be used for accurate and rapid identification of *Pythium* spp. causing root rot and damping-off diseases of seedlings, they do not provide required information on their population dynamics. Amplified fragment length polymorphism (ALFP)-finger printing technique was employed to detect

and characterize pathogenic *Pythium* spp. and intraspecific populations. Species diagnostic AFLP fingerprints for *P. aphanidermatum, P. irregulare* and *P. ultimum* were determined, in addition to tentative fingerprints for six other *Pythium* spp. The utility of the fingerprints was revealed by the successful identification of 29 isoaltes of the described species out of 48 blind samples. The missidentification of five isolates of *P. ultimum* based on morphological characters was rectified by the AFLP-fingerprinting procedure (Garzón et al. 2005). Specific PCR primers were developed from AFLP fragments of *Pyrenophora teres* causing net blotch disease on barley leaves. The primers amplified DNA from *P. teres f. sp. teres* (net form) but not from the closely related *P. teres f. sp. maculata* (spot form), indicating the specificity of detection and the possibility of differentiating these two pathogens. The PCR amplification with specific primers generated *P. teres* form-specific products (Leisova et al. 2005).

2.3.5.4 Random Amplified Polymorphic DNA Technique

DNA polymorphisms between two genomes of organisms have been determined by using random amplified polymorphic DNA (RAPD) procedure. Generating information from a RAPD fragment for designing specific primers is an alternative technique that can be useful, if sequences such as ribosomal ITS regions are very conserved and limit the design of a species-specific PCR method. The stone fruit pathogens *Monilinia fructigena, M. fructicola, M. laxa* and *Monilia polystroma* are quarantine fungi with different regulatory status. Timely and precise identification of these pathogens is essential to monitor the imported or exported fruits.

RAPD analysis was applied to generate *M. fructigena*-specific band that was sequenced. Primers were deisgned to amplify bands in the same genomic region of *M. fructicola* and *M. laxa.* A multiplex PCR protocol was formulated using common reverse primer and three species-specific forward primers. This procedure was effective for detection and identification of these pathogens in inoculated or naturally infected apples (Côtè et al. 2004). *Penicillium* spp. involved in blue mold disease were detected in rotten apple and pear fruits and floatation tanks in commercial apple juice facilities and differentiated by RAPD analysis. The involvement of *P. expansum,* known to be a major cause and *P. solitum* reported for the first time were rapidly and reliably identified by RAPD procedure (Pianzzola et al. 2004). The fungal pathogen *Alternaria yaliinficiens* causing chocolate spot of Ya Li pear was detected and identified as a new species by employing RAPD fragment pattern analysis and species-specific PCR assay (Roberts 2005).

The application of molecular markers like random amplified polymorphic DNA (RAPD) for detection and differentiation of sub-specific groups has been shown to be effective. The two overwintering forms of *Uncinula (Erysiphe)necator* (infecting grapevine) have been designated the “flag shoot” and “ascospore” biotypes in European and Australian populations of this powdery mildew pathogen. A third genetic group was reported from India (Délye et al. 1997). Eight RAPD markers specific for flag shoot and ascospore biotypes were identified. They were used

to derive sequence characterized amplified region (SCAR) primers. The SCAR primers are 20–24-mer oligonucleotides designed on the 3′ and 5′ regions of the original sequence. They provide the advantage of higher specificity and reliability than RAPD primers in PCR reactions. Two pairs of SCAR primers UnE-UnF and F6-F6a were specific in detecting and distinguishing the two biotypes, as shown by the test involving 374 *E. necator* isolates consisting of 83 "flag shoot" and 291 "ascospore" biotypes, already characterized by RAPD analysis (Hajjeh et al. 2005). The specificity of detection of *Phytophthora cactorum* infecting agricultural and ornamental crops as well as forest species was improved by designing a new pair of primers (PC1/PC2) derived from a specific RAPD generated fragment. These primers amplified a single product of approximately 450 bp. There was no amplification, when the DNA of *P. pseudotsugae* or *P. idae*i was tested. The detection limit was 6 pg of *P. cactorum* DNA extracted from pure mycelium. *P. cactorum* was detected in infected tissues of pear, potato, strawberry, tomato, pea and walnut trees (Causin et al. 2005).

Rhizoctonia solani with a wide host range causes sheath rot of rice and bare patch disease in cereals, legumes and pastures. At least 13 distinct anastomosis groups (AGs) have been recognized in *R. solani.* Some AGs are compelx and they can be further subdivided into intraspecific groups (Igs) (Carling 1996). AG8 was subdivided on the basis of zymogram patterns into Zymogram groups (ZGs) and five ZGs (1–1 to 1–5) have been identified (MacNish and Sweetingham 1993). Furthermore, vegetatively compatible populations (VCPs) exist within each ZG with AGB (MacNish et al. 1997). RAPD-PCR technique was applied to determine the relationship between four ZGs within AG8. The isolates (79) of *R. solani* AG8 reprsenting four pectic isozyme groups (ZG1-1, 1–2, 1–4 and 1–5) from different locations in southern Australia were analyzed by RAPD-PCR assay. Using six primers the relatedness of the isolates was determined. Within each ZG, four to nine VCPs were recognized. The results indicate that the AG8 populations included four distinct groups that matched the four ZGs, lending support to concept that ZGs are distinct intraspecific groups (MacNish and O'brien 2005).

Detection and differentiation based on the morphological and cultural characteristics of closely related pathogen species has been found to be difficult, as in the case of *Elsinoe fawcetti* (*Ef*) and *E. australis* (*Ea*), causing citrus scab and sweet orange scab diseases respectively. RAPD technique was applied to distinguish *Ef* and *Ea* and the sweet orange and natsudaidai pathotypes within *Ea* by using specific primer sets Efaw-1 for *Ef*; Eaut-1, Eaut-2, Eaut-e and Eaut- for *Ea* and EaNat-1 and EaNat-2 for natsudaidai pathotypes within *Ea* using RAPD products unique to each species or pathotype. Likewise, the Efaw-1 and EFaw-2 primer set efficiently identified *Ef* isolates from Korea, Australia and the US (Florida) and the Eaut-1 to Eaut-5 primer sets identified both sweet orange pathotype isolates of *Ea* from Argentina and natsudaidai pathotype isolates from Korea. *Ef* present in the lesions on leaves and fruits from Korea could be detected by Efaw-1 and Efaw-2 primer sets, whereas *Ea* was detected in lesions on sweet orange fruit from Brazil using primer pairs Eaut-1, Eaut-2, Eaut-3 and Eaut-4 (Hyun et al. 2007).

2.3.5.5 Polymerase Chain Reaction

Detection of fungal pathogens causing vascular wilt and root rot diseases has to be accomplished in early disease development stages and also when their population level in the soil is low, to plan effective disease management strategies to restrict their spread. Polymerase chain reaction (PCR)-based assays provide reliable, rapid and sensitive detection of fungal pathogens in addition to quantification of their biomass in the plants or soil or other substrates. Because of the higher level of sensitivity and reliability of detection of fungal pathogens, PCR-based techniques are employed more frequently than other molecular diagnostic techniques. However, the higher cost and requirement of specialized technical manpower reduce their spectrum of reach for several laboratories/countries. Several fungal pathogens that have been detected using PCR have been indicated in earlier publications (Fox 1998; Narayanasamy 2001).

The use of PCR assay for detection of some of the destructive fungal pathogens is revisited. A region in the ITS specific to *Phytophthroa infestans* causing the potato late blight disease of historical significance, was used to construct a PCR primer (PINF). The pathogen was detected in infected tomato and potato field samples using the pathogen-specific primer (Trout et al. 1997). In another investigation, specific primers were designed based on the sequences of ITS2 region of DNA of *P. infestans* and *P. erythroseptica* (causing pink rot of tubers). These pathogens could be detected in potato tubers as early as 72 h after inoculation well in advance of development of any visible symptoms on the tubers (Tooley et al. 1998). A primer pair (INF FW2 and INF REV) specific to *P. infestans* based on the sequences of ITS region generated a 613-bp product. In a single round PCR assay, 0.5 pg pure *P. infestans* DNA was detectable. Sensitivity was increased to 0.5 pg DNA in a nested PCR using Peronosporales-specific-primers in the first round. *P. infestans* could be detected in leaves, stem and seed potato tubers before expression of symptoms (Hussain et al. 2005).

By using the primers based on sequences of ITS region of ribosomal gene repeat (rDNA), *P. infestans* was detected with greater reliability and sensitivity compared to ELISA format (Bonants et al. 1997). Likewise, *P. fragariae* var. *fragariae* and *P. fragariae* var *rubi* infecting roots of strawberry and raspberry respectively, could be effectively detected by the primers P-FRAGINT and the universal primer ITS4. The pathogens were detectable more effectively in the early stages of infection (1–5 d after inoculation), since the coenocytic mycelium was degraded and production of oospores occurred later (Hughes et al. 1998). Primers (FM75 and FM77/83) spanning the mitochondrially encoded *cox* I and II genes capable of amplifying target DNA from all 152 isolates of 31 species of the genus *Phytophthora* were designed. The amplicons were digested with restriction enzymes for generating species-specific RFLP banding profiles. Digestion with *AluI* alone could detect and differentiate most *Phytophthora* spp. tested. However, single digests with a total of four restriction enzymes were used to enhance the accuracy of isolate identification. *P. capsici, P. palmivora, P. infestans* and *P. megasperma* are some of the important fungal pathogens that were identified by the PCR-RFLP system (Martin and Tooley

2004). The PCR primers that could amplify the DNA of 27 different *Phytophthora* spp. were designed. The amplicons were subjected to restriction enzymes to generate specific restriction pattern or fingerprint unique to each species. This protocol could detect and identify 27 *Phytophthora* spp (Drenth et al. 2006).

Phytophthora infections in tissues of diseased vegetable and fruit crop plants were directly detected by PCR amplifications using primers directed to nuclear rDNA sequences. *P. capsici* was detected in diseased pepper and zucchini plants. *P. infestans* was identified in tomato with late blight symptoms. But buckeye-rot disease-affected tomatoes and strawberries proved to be infected by *P. nicotianae* and *P. cactorum* respectively. *P. citrophthora* and *P. nicotianae* were detected in almost equal frequency in decline diseased clementine trees. *Phytophthora* blight of zucchini was found to be a new disease in southern Italy (Camele et al. 2005). A distinct species of *Phytophthora* was isolated from dying cranberry plant roots, apart from *P. cinnamomi*, most commonly infecting cranberry. DNA containing the complete ITS1, 5.8S rRNA gene and ITS2 was amplified by PCR using universal ITS primers 1 and 4 for all isolates. Both *P. cinnamomi* and *Phytophthora* taxon cranberry isolates had identical but different ITS region sequences. The *Phytophthora* species, did not match any in database. These two species are very different in ITS sequences similarity, apart from their biology and disease cycles (Polashock et al. 2005).

Infection by *Phytophthora ramorum, P. kernoviae*, *P. quercina* along with *P. citricola* forms a serious threat to forest health and natural ecosystems. The development of a multiplex real-time PCR protocol for simultaneous detection and quantification of these four target pathogens in leaf materials was considered to be essential. Specific primers and probes to identify *P. ramorum, P. kernoviae, P. citricola* and *P. quercina* were designed in different regions of *Ypt1* gene and labeled with four different Black Hole Quenchers (BHQ) to allow simultaneous detection of the target pathogens. In order to enhance the sensitivity of the reactions two primers (Yph IF-Yph2R), amplifying DNA from all *Phytophthora* spp. were designed to develop a nested-PCR with a first common amplification with primers Yph 1F-Yph 2R and a second amplification by multiplex real-time PCR. The detection limit of real-time multiplex PCR was 100 fg of target DNA for all four pathogens. Levels of sensitivity and correlation coefficients assessed in separate reaction were as in the multiplex PCR. The over all level of sensitivity obtained with nested PCR was not generally increased over that in single real-time multiplex PCR (100 fg). The presence of plant DNA and coextracted compounds did not significantly affect the amplification of target DNA. The analyses of naturally infected samples showed the presence of *P. ramorum* and *P. kernoviae* infections in 42.8% and 18.2% of analysed samples respectively (Table 2.2). The investigation seems to be the first attempt in developing a multiplex real-time PCR procedure to detect and quantify four important fungal pathogens (Schena et al. 2006).

Phytophthora sojae causes a devastating root and stem rot disease of soybean. The pathogens soilborne and the oospores may survive in soils for several years. A rapid and sensitive technique for the specific detection of *P. sojae* in soil was developed using primers designed based on the sequences of the internal transcribed

Table 2.2 Comparative efficacy of detection of *Phytophthora* spp. in naturally infected leaves by real-time multiplex PCR and conventional isolation methods

Name of pathogen	Molecular detection (Number)	Isolation method (Number)
Phytophthora ramorum		
Positive samples	33	33
Negative samples	44	44
P. kernoviae		
Positive samples	14	15
Negative samples	63	62
P. citricola		
Positive samples	1	1
Negative samples	76	76
P. quercina		
Positive samples	0	0
Negative samples	77	77

Source: Schena et al. (2006)

spacer (ITS) region in *P. sojae*. The primers PS1 and PS2 amplified the DNA sequences only from *P. sojae*, but not from any one of more than 245 isolates representing 25 species of *Phytophthora*. indicating the specificity of the PCR protocol developed in this study. The detection limit of the PCR was 1 fg of pathogen DNA or two oospores in 20 g of soil. The PCR assay combined with a simple soil screening method, allowed detection of *P. sojae* from soil within 6 h, whereas the conventional soybean leaf disk baiting method required at least 15–20 days for detection of this pathogen. Real-time fluorescent quantitative PCR was also developed to detect *P. sojae* directly in soil samples. This PCR assay is being extensively employed in quarantine bureaus in China for effective detection of the pathogen in plants also (Wang et al. 2005).

Availability of high quality DNA is required for performing assays such as PCR and AFLP analysis and ***t***argeting ***i***nduced ***l***ocal ***l***esions ***i***n ***g***enomes (TILLING) for large scale survey of fungal pathogens like *Phytophthora* spp. from natural environments. A strategy to recover high molecular weight genomic DNA from large number of isolates (5000–10,000) of *Phytophthora* spp. was developed [Appendix 17]. The DNA extracted was consistently of high MW with total yields varying based on the amount of starting material used. The DNA isolated could be efficiently used for standard PCR, fluorescently labeled nested PCR, real-time PCR, reverse genetics labeled AFLP applications. This procedure has the potential for processing a large number samples in a relatively short period, using a fraction of total space needed for applying traditional methods. The procedure is less expensive (approximately less than 85% of the cost of the currently available commercial kits). In addition, there is no need to handle hazardous solvents such as chloroform or phenol. This strategy has been successful for DNA extraction from biological materials including plants, fungi and nematodes (Lamour and Finley 2006).

Wilts and root rots are of economic importance due to the significant yield losses caused by them. Specific amplication of a 500-bp DNA fragment by the primers Fov1 and Fov2 designed from ITS sequences occurred in all isolates of *Fusarium oxysporum* f.sp. *vasinfectum* causing cotton wilt disease. This PCR procedure has the potential for use, not only for disease diagnosis, but also for disease monitoring and forecasting program (Morrica et al. 1998). *Rhizoctonia solani* infects a large number of economically valuable crops like rice which suffers seriously because of sheath rot disease. *R. solani*-AG1 IA (anastomosis group) may be detected and identified rapidly by employing primers designed from unique regions within the ITS regions of rDNA. The PCR assay was used to detect the pathogen in infected rice plant tissues and paddy field soils (Matsumoto and Matsuyama 1998). Six primers capable of amplifying sequences of ITS region of AG2 and AG4 of *R. solani* were developed. The DNAs from *R. solani* AG2 and AG4 in infected radish plants and axenic cultures were specifically amplified in PCR assays (Salazar et al. 2000). Rapid PCR assay for the detection and identification of *Verticillium albo-atrum* hop pathotypes PG1 and PG2 were developed. PG-2 and PG-1 specific primers were designed from 16 sequences and polymorphic amplified fragment length polymorphism (AFLP) markers converted into pathotype-specific sequence-characterized amplified region (SCAR) markers. Primer combinations obtained from the AFLP 9-1 marker were specific only for *V. albo-atrum* PG-2 isolates. The pathotype PG-2 was detected in the xylem tissue of hop plants in multiplex PCR and a nested-PCR formats using the highly specific primers. These new SCAR markers provide a valuable tool for rapid identification of *V. albo-atrum* PG-1 and PG-2 hop pathotypes (Radišek et al. 2004).

The Septoria complex affecting wheat crops includes *Stagonospora nodorum* and *Septoria tritici* which cause glume blotch and leaf blotch respectively. Primers designed to conserved sequences on the ribosomal DNA were employed to PCR amplification of target pathogen DNA. The ITS regions of both pathogens were cloned and sequenced. The ITS-derived primers successfully amplified similar-sized fragments from wheat tissues infected by these pathogens (Beck and Ligon 1995). In a further study, ELISA format was integrated into PCR-based assay to develop a microtiter plate format for quantification of disease pressure (Beck et al. 1996b). Likewise a PCR-ELISA protocol was developed for the detection *Pseudocercosporella herpotrichoides,* causing eyespot disease of wheat, rye and barley in temperate region, using the Dig-labeled primers (JB540 and JB 542) that amplified a 413 bp fragment from all isolates of the pathogen. In addition, the pathotypes R and W could be detected and differentiated when they were present in the same sample (Beck et al. 1996a).

Pyrenopeziza brassicae (anamorph-*Cylindrosporium concentricum*) causes a major disease (light leafspot) of winter oilseed rape in UK. Infection remains symptomless for a long time until the first visible necrotic lesions appear. Visual assessment of *P. brassicae* infection is not reliable. Unless plants are first incubated for several days at high humidity, necrotic lesions do not develop. PCR amplification of *P. brassicae* DNA of isolate NH10 using primers Pb1/Pb2 produced a 753-bp amplicon. On other hand, ITS primers Pb ITSF and PbITSR amplified a 461-bp

product. It was possible to detect down to 1 ng of *P. brassicae* DNA with primers Pb1 and Pb2, whereas the ITS primers were more efficient by detecting as little as 1 pg of pathogen DNA. The new ITS primers (PbITSF/PbITSR) were found to be effective for detection of *P. brassicae* in symptomless oilseed rape tissue. The visual assessments, even after incubation with infected leaves, required 2 months more than PCR diagnosis on leaves sampled from field experiments. Infection of leaves by *P. brassicae* was detected immediately after inoculation under controlled conditions. The highly sensitive nature of the PCR assay developed in this study has the potential for large scale application under field conditions (Fig. 2.13) (Karolewski et al. 2006).

Detection of soilborne fungal pathogens has posed formidable problems because of their irregular distribution in the soil and presence along with saprophytes and other microorganisms. Primers targeted to ribosomal RNA genes and ITS regions have been shown to be effective for the detection of *Plasmodiophora brassicae* causing clubroot disease of crucifers in soil and water. The nested PCR assay has detection limits of 0.1 fg (10^{-15} g) for pure template and as low as 1000 spores/g of potting mix (Faggian et al. 1999). In another study reporting a significant improvement of sensitivity of detection, the outer primer PBTZS-2 for amplifying a 1457-bp fragment from *P. brassicae* DNA and nested primers PBTZ-3 and PBTZ-4 for amplifying a 398-bp fragment internal of the 1457-bp fragment were used in a single-tube nested PCR (STN-PCR) format. It was possible to detect even a single resting spore present in one g of soil. When the STN-PCR amplification product was subjected to second PCR amplification (double PCR) using the nested primers, further significant improvement in sensitivity of detection could be achieved (Ito et al. 1999). *Spongospora subterranea* is another important soilborne pathogen infecting potatoes all over the world. The primers Sps1 and Sps2 designed from the sequences of the ITS region of *S. subterranea* DNA amplified a 391-bp product only from this pathogen, but not from any other soilborne microbes, indicating the specificity of detection. The assay was more rapid and sensitive than the immunoassays or conventional bait-plant assays applied earlier. The PCR assay could detect *S. subterranea* DNA equivalent to 25×10^{-5} cystosori or one zoospore per PCR

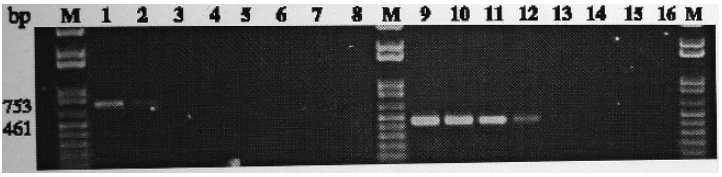

Fig. 2.13 Detection of *Pyrenopeziza brassicae* isolate NH10 in different dilutions using primers Pb 1 and Pb 2 (lanes 1–8) or PbITSF and PbITSR (lanes 9–16)
Pathogen DNA: Lanes 1 and 9: 10 ng; Lanes 2 and 10: 1 ng; Lanes 3 and 11: 100 pg; Lanes 4 and 12: 10 pg; Lanes 5 and 13: 1 pg; Lanes 6 and 14: 100 fg; Lanes 7 and 15: 10 fg; Lanes 8 and 16: Negative controls (water). (Courtesy of Karolewski et al. 2006; British Society for Plant Pathologists, UK)

and it has the potential for use in programs for potato seed stock and disease risk assessment for field soils (Bell et al. 1999).

Smut diseases infect many cereals and millets as well as high value crops like sugarcane accounting for substantial yield loss. Maize is infected by *Sporisorium reiliana* (head smut) and *Ustilago maydis* (*U. zeae*) which can be detected by using primer pairs SR1 and SR3 specific for *S. reiliana* and UM11 specific for *U. maydis.* The extracts of pith, node and shank, but not that of leaves showed the presence of *S. reiliana,* with a detection limit of the PCR assay being 1–6 pg of pathogen DNA, irrespective of host DNA (Xu et al. 1999). The primers based on the sequences of ITS region of rDNA of *U. hordei* were successfully used for the detection of the pathogen in infected leaf tissues of inoculated susceptible and resistant plants at different stages of plant development (Willits and Sherwood 1999). For the detection of *Tiletia caries* (*T. tritici*) causing common bunt disease of wheat, the primer pair Tcar 2A/Tcar 2B was used. The pathogen was detected in the shoots and also in leaves of infected wheat plants. The detection limit was determined to be 16 pg DNA per 100 mg of plant fresh weight. The primer amplified the DNA from the extracts of teliospores, whereas no positive reaction was seen when the spore extract was tested by ELISA (Eibel et al. 2005b).

Establishing the identity of fungal pathogens which do not form sexual spores has been difficult. Furthermore, when sexual spores are formed under in vitro conditions, the relationship between the mycelial mass and sexual spores has to be determined with certainty. *Thecaphora solani* causing potato smut disease was successfully cultured in vitro. The DNA was extracted from the sponge-like mass that developed from the teliospores inoculated on potato-dextrose agar (PDA) medium with cellophane. DNA profiles were generated by PCR using the primers CAG5 and GACA4. The amplicons were separated by electrophoresis. The 5′, region of the nuclear large subunit (LSU) of the ribosomal DNA gene was amplified by PCR using primers NL1 and NL4. The identical DNA profiles among teliospores and sponge-like mycelial mass were revealed by DNA fingerprinting and partial sequencing of the large subunit (LSU) rDNA region (Andrade et al. 2004).

Detection of postharvest pathogens rapidly assumes great importance, because of the short time interval available for applying effective disease management strategies. The strawberry pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* were analyzed by comparing the sequences of 5.8S ITS region by employing species primers to identify isolates of *C. acutatum.* The specificity of detection by PCR assay was demonstrated by the non-amplification of DNA sequences from non-strawberry isolates of *Colletotrichum* (Martinez-Culebras et al. 2003). In another investigation, by adopting a modified DNA extraction procedure and employing species-specific primers in PCR assays, *C. acutatum*, a quarantine pathogen, was efficiently detected on symptomatic and asymptomatic plant parts and in artificially and naturally infected strawberry tissues (Parikka and Lemmetty 2004).

Watermelon *Fusarium* wilt disease caused by *Fusarium oxysporum* f.sp. *niveum* (*FON*) is one of the most destructive diseases. *Mycosphaerella melonis* (MM) causing gummy stem blight is also found together with wilt disease. Two species-specific PCR assays were developed for rapid and precise detection of *FON* and

MM in diseased plant tissues and soil. Two pairs of specific primers *Fn*-1/*Fn*-2 and Mn-1/Mn-2 were designed based on the differences in the ITS sequences. The primer pair Fn-1/Fn-2 amplified only a single PCR band of approximately 320 bp from *FON*, whereas the Mn-1/Mn-2 primers yielded a PCR product of approximately 420 bp from *MM*. The sensitivity of detection was 1 fg of genomic DNA for both pathogens. The sensitivity of detection could be dramatically increased 1000-fold to detect as little as lag (10^{-16}g) by using ITS1/ITS4 as the first-round primers, combined with either Fn-1/Fn-2 and or Mn-1/Mn-2 in two nested PCR procedures. The detection sensitivity was 100 microconidia/g soil for the soil pathogens. A duplex PCR method for detection of *FON* and *MN* in infected plant tissues and a real-time fluorescent quantitative PCR assay to detect and monitor the fungal pathogens directly in soil samples (Zhang et al. 2005) developed in this investigation have the potential for use in detecting other soilborne pathogens.

Fungal pathogens infecting grains, fruits and vegetables not only cause direct spoilage of infected commodities, but also they produce many mycotoxins that can cause chronic and acute diseases affecting humans and animals. The detection of mycotoxins and control of the mycotoxigenic fungi is crucial to prevent the mycotoxins entering food chain. Fumonisins, one of the major group of mycotoxins, are produced by different species of *Fusarium*. *F. verticillioides*, considered as a main source of fumonisins, was detected by using primers designed on the sequences of intergenic spacer region of the rDNA units. The first pair of primers was specific to *F. verticillioides,* while the second pair of primers detected fumonisin-producing strains of *F. verticillioides* strains of *F. verticillioides* (54) from various geographical regions and hosts were tested by using both sets of primers. The specificity, simplicity, rapidity and sensitivity of the PCR assay have provided a sound basis for reliable detection and identification of strains of *F. verticillioides* that pose high risk for human and animal health (Patiño et al. 2004). *F. graminearum,* causing fusarium head blight (FHB) disease of wheat, also produces the mycotoxins deoxynivalenol (DON) and nivalenol (NIV) contaminating wheat grains. A PCR assay was developed using the primer sets to amplify the *Tri3* gene involved in the production of DON. The isolates of *F. graminearum* were grouped into three chemtotypes based on the nature of DON produced by them (Jennings et al. 2004).

Fusarium graminearum and *F. culmorum* are primarily involved in FHB. The biology and infection mode of the two species are very different from each other. A rapid inexpensive, qualitative duplex PCR assay for the simultaneous detection of both pathogens is essential for handling large number of field samples of wheat and barley for quarantine certification. The primer pairs Fg16NF and Fg16NR for *F. graminearum*, OPT18F and OPT18R for *F. culmorum* were employed in the duplex PCR with melting curve analysis performed in a real-time thermocycler in the presence of the fluorescent dye SYBR Green I. This procedure allows the simultaneous identification of *F. graminearum* and *F. culmorum* in one PCR without the use of doubly labeled hybridization probes or electrophoresis. Species-specific PCR products are differentiated by melting curve analysis. The detection limit of each species was 5 pg of genomic DNA. Optimized levels of each pathogen has to be used to avoid impairment of detection limits, since the excess of one species adversely affects the

other, due to competition of the PCR products for binding of fluorescent dye used for detection (Brandfass and Karlovsky 2006).

The seed infection in Tasmanian pyrethrum by *Phoma ligulicola* was detected by applying PCR protocol. The assay could detect infested seed lots down to an incidence of 0.5% and had a detection limit of 800 fg of fungal DNA. Reliable amplification of the target fungal DNA was achieved by adding bovine serum albumin (BSA) to reduce the influence of inhibitors from pyrethrum seed. The percent infection of seeds and viability of *P. ligulicola* varied depending on pyrethrum cultivars (Pethybridge et al. 2006).

Identification of obligate fungal pathogens based on the characteristics of anamorphs often leads to inconclusive results. Molecular evidence was provided for establishing *Oidium neolycopersici* as the cause of tomato powdery mildew disease epidemics in United States and Canada. Whole-cell DNA was isolated from the pathogen mycelium. The nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene was amplified by PCR, using primers ITS5 and P3 for the first amplification and the nested primer set ITS1 and P3 for the second amplification. The internal transcribed spacer sequences of the North American anamorphs were identical with those of three Japanese and four European isolates of *O. neolycopersici*. The study showed that *O. neolycopersici* is a distinct powdery mildew pathogen and it is neither identical for closely related to any known polyphagous species of Erysiphaceae (Kiss et al. 2005). In another study, single germinated conidia were removed from leaves of tomato, using a glass pipette attached to the manipulator of a high-fidelity digital microscope. The conidia were individually transferred to 15 µl of PCR solution in a 200 µl microcentrifuge for amplification of the entire 5.8S rDNA and its adjacent ITS sequences. To enhance the sensitivity level, nested-PCR protocol was followed to amplify the target nucleotide sequences. By employing RT-PCR formats, the transcripts expressed in single conidia were amplified. The conidia were either of *Oidium neolycopersici* or *Erysiphe trifolii* which is non-pathogenic on tomato. This study thus provides a reliable technique for detection of the pathogen as well as molecular method for monitoring gene expression in germinating conidia on leaf surface (Matsuda et al. 2005).

Peronospora tabacina, an oomycete causes blue mold disease of tobacco. The symptoms often are nonspecific under field conditions, making disease diagnosis difficult. By using the primers 1602A and 1602B, the DNA from plant samples was amplified from all DNA samples extracted from tobacco plants with symptoms. This fragment was also obtained from seedlings showing yellowing but neither necrosis nor sporulation on leaves. All suspected plants showed positive reaction. Microscopical examination could detect the pathogen in 6%–50% of the samples tested and the results were not conclusive. Elimination of sonication for DNA extraction made the PCR easier to perform, since fragmentation of DNA was prevented (Caiazzo et al. 2006). In a later investigation, *P. tabacina* was detected and differentiated from other tobacco pathogens by employing PCR with the primers ITS4 and ITS5, sequencing and digestion with restriction enzymes. A specific primer PTAB was used with ITS4 to amplify a 764-bp region of DNA that was specific for *P. tabacina*. The PTAB/ITS4 primers did not amplify host DNA or other 12 tobacco

fungal pathogens including related *Peronospora* spp., but amplified the DNA of only *P. tabcina* at 0.0125 ng level. The primer PTAB has the potential for use in disease diagnosis, epidemiology and regulatory work, since the pathogen can be detected in fresh, air-dried and cured tobacco leaves (Ristaino et al. 2007).

Some of the plant pathogenic fungi, because of their slow growth and poor sporulation, may not be identified accurately in spite of the time required by the conventional diagnostic methods. *Pyrenochaeta lycopersici* causing corky root disease in tomato, is soilborne presenting additional obstacles for detection and identification when present in the soil. Sugiura et al. (2003) demonstrated the existence of two forms designated Type 1 and Type 2 based on mycelial growth-rate, pathogenicity, sequence of rDNA-ITS region and RAPD analysis. The universal primer ITS4 and ITS5 amplified the DNA of all isolates giving a product of 600-bp. The sequence analysis of the rDNA-ITS sequences confirmed the presence two subgroups (Type 1 and Type 2). Two oligonucleotide primer pairs based on the sequences of ITS region of *P. lycopersici* were designed. Specific PCR products of 147-bp and 209-bp were generated from the target DNA of Type 1 and Type 2 respectively. The test was specific and sensitive by detecting 0.7 pg of target DNA. The pathogen was detected in the infected root tissue extracts by performing the nested-PCR protocol. From the field samples, amplicons corresponding to Type 2 isolates only were detected. The results of PCR were in good agreement with tissue isolation method which required about 15 days for disease diagnosis. The nested-PCR procedure needed only 2 days indicating the rapidity of pathogen detection and disease diagnosis (Infantino and Pucci 2005).

Different variants of PCR assay have been developed for the detection of some fungal pathogens.

Reverse Transcription (RT)–PCR

It has been demonstrated that PCR amplicons may be generated from the DNA of cells killed by heat or other treatments at several days after cell death (Josephson et al. 1993). Detection and quantification of the expression of pathogenesis or resistance-related genes of fungal pathogens may be possible by using RT-PCR, as in the case of *Mycosphaerella graminicola* (Ray et al. 2003) and *Alternaria brassicicola* (Schenk et al. 2003). A RT-PCR protocol was developed to detect and identify *Mycospharella graminicola* causing leaf blotch disease in wheat. The specific primer set E1/STSP2R based on the sequences of β-tubulin genes was employed for both one-step and two-step RT-PCR formats. One single fragment was amplified only from the total RNA of *M. graminicola* and infected wheat leaves, but not from either healthy leaves or other fungal pathogens infecting wheat. The one-step RT-PCR was more sensitive (5 ng total RNA) than two-step RT-PCR format (100 pg total RNA). One-step RT-PCR could detect amplicons at 4 days after inoculation, when no visible symptom of infection was exhibited. The band intensities of the amplified RT-PCR products increased as the symptoms developed with increase in the time interval after inoculation (Fig. 2.14) (Guo et al. 2005) [Appendix 18].

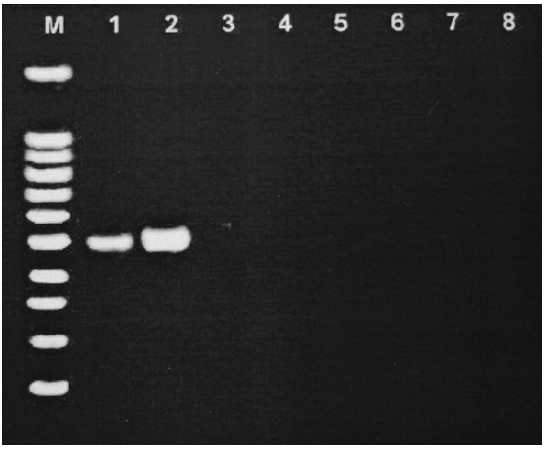

Fig. 2.14 Detection of *Mycosphaerella graminicola* by one-step RT-PCR assay
Lane M: 100-bp DNA ladder; Lane 1: Total RNA of *M. graminicola;* Lane 2: Inoculated wheat leaves; Lane 3: *Stagonospora nodorum;* Lane 4: *Microdochium nivale;* Lane 5: *Fusarium graminearum;* Lane 6: *Fusarium culmorum;* Lane 7: *Pseudocercosporella herpotrichoides;* Lane 8: Healthy leaves. (Guo et al. 2005; Blackwell-Verlag, Berlin, Germany)

Nested-Polymerase Chain Reaction

The sensitivity of detection of fungal pathogens has been enhanced by performing nested PCR assay. A single-tube nested PCR (STN-PCR) was demonstrated to be more sensitive for the detection of *Plasmodiophora brassicae* by employing nested primers PBTZ-3 and PBTZ-4 for amplifying a 398-bp fragment internal of the 1457-bp fragment. A single resting spore of *P. brassicae* present in 1g of infested soil could be detected (Ito et al. 1999). Nested-PCR procedure was applied for the detection of *Phytophthora infestans* in commercial potato seed tuber stocks and soil. The primers DC6 and ITS4 amplified a 1.3 kb PCR product from the oomycetes *Phytophthora* and *Pythium* and downy mildew pathogens in the first-round PCR. *P. infestans*–specific primers were employed for the second-round amplification. The detection limit was 5 fg. A soil assay detected 10 oospores/0.5g of soil. The long-term survival of oospores and sporangia was studied by burying the infected leaf material under natural field conditions. Oospores were consistently detected upto 24 months after burial in soil, while the sporangial inoculum was detectable for only 12 months after burial. This assay has practical application for testing the seed tubers for the presence of *P. infestans.* In most samples that were positive, no symptoms of infection were visible, confirming the usefulness of the assay to reliably detect latent infection. The test can be completed in less than 4 h, including time required for DNA extraction (Hussain et al. 2005). In the case of *Pyrenochaeta lycopersici* causing corky root disease in tomato, amplification of target pathogen DNA with the universal primers ITS4 and ITS5 was possible, if the genomic DNA of the pathogen was tested. However, a nested-PCR format was required for the detection of *P. lycopersici* in plant tissues. The pathogen specific primers were employed for

the second amplification. Specific PCR products from isolates belonging to Type 1 and Type 2 were amplified enabling the unambiguous identification of the isolates of *P. lycopersici* (Infantino and Pucci 2005).

Based on the sequences of microsatellite regions of the genome of *Monilinia fructicola* infecting stone fruits, primers were designed and a nested-PCR protocol was developed for the detection of this pathogen. The sequences of a species-specific DNA fragment were amplified by the microsatellite primer M13. The external and internal primer pairs EM If F + EMf R and IMfF + ImfR amplified 571-bp and 468-bp fragments from *M. fructicola,* but not from any other fungi present in stone fruit orchards. It was possible to detect as little as 1 fg of pathogen DNA from two conidia of *M. fructicola,* demonstrating the high sensitivity level of nested-PCR assay. The additional advantage of nested-PCR format was the possibility of assessing the population in the orchard for epidemiological investigations (Ma et al. 2003). *Phytophthora fragariae* var. *fragariae* causing red stele (root rot) disease of strawberry is a quarantine pathogen requiring a rapid and reliable detection technique to prevent its further spread through infected plant materials. Nested-PCR has been demonstrated to be an effective detection system. Nested-PCR assay detected 100 ag (10^{-16}g) of pure pathogen DNA which is equivalent to ~1/60 part of one nucleus. Such a sensitivity was attained because rDNA is a multicopy gene. In practice, nested-PCR could consistently detect between 5 and 10 zoospores of the pathogen (Bonants et al. 2004).

Multiplex-PCR Assay

The multiplex-PCR assay eliminates the need for the fungal isolation, resulting in significant acceleration of the detection and identification process. A one-tube PCR multiplex protocol was formulated for using the sequences of a repetitive satellite DNA fragment of *Phytophthora infestans* for designing specific primers. These primers were useful to detect all known A1 mating types of *P. infestans* races 1, 3, 4 and 7–11 occurring in Germany and A2 mating types (Niepold and Schöber-Butin 1995). Specific primers designed based on sequences of ITS2 region of DNA were employed to detect *P. infestans* and *P. erythroseptica* (inducing pink rot disease) in potato tubers even before the appearance of visible symptoms (Tooley et al. 1998).

A multiplex-PCR procedure was developed for the detection and identification of *Monilinia* spp. and *Monilia polystroma* infecting stone fruits. In a single tube reaction, appearance of a specific PCR band may help to identify the pathogen concerned. The multiplex-PCR assay consistently produced a 402-bp PCR product for *M. fructigena,* a 535-bp product for *M. fructicola* and a 351-bp product for *M. laxa.* The target DNA of *Monilia polystorma* amplified a 425-bp PCR product. The identification was based on the PCR product amplified directly from inoculated apples and the PCR band was specific to inoculated *Monilinia* or *Monilia* species. The pathogen(s) present on the naturally infected stored fruits were also identified by this protocol (Côtè et al. 2004). The etiology of bull's eye rot disease of pear was established as *Neofabrae alba* by applying a multiplex-PCR assay using

species-specific primers. This new fungal pathogen was more frequently detected in the sample from Oregon and California, whereas samples from Washington showed infection by another species *N. perennans.* In addition, these pathogens involved in bull's eye rot were detected also in nine European pear cultivars, Asian and quince (Henriquez et al. 2004).

Specific sequence-characterized amplified region (SCAR) primer pairs were employed in multiplex PCR for detection and differentiation of the two pathotypes of *Verticillium albo-atrum* infecting hop. Three pairs of SCAR primer pairs viz., 9-21-For/9-21-Rev, 11-For/11-Rev and 9-21-For-/9-21-Rev were used for more specific diagnosis of *V. albo-atrum* hop PG1 and PG2 pathotypes. The amplified PCR products corresponded to the SCAR markers and hence the specificity of the primers was not affected by the multiplex reaction. Identification of PG1 and PG2 pathotypes by SCAR markers was further improved by the development of multiplex PCR which increased the specificity in the diagnosis of the pathotypes by means of simultaneous amplification of two specific loci for PG2 and one locus for PG1. Thus the multiplex-PCR can make the pathotype screening assays more reliable (Radišek et al. 2004).

Phaeomoniella chlamydospora, an endophyte associated with esca (decline) disease of grapevine occurring in Europe and NorthAmerica was detected in samples collected from Vineyards by employing a species-specific marker (Ridgway et al. 2002; Whiteman et al. 2002). A potential molecular marker in the New Zealand isolate A21, the 1010-bp marker band was characterized. Sequencing of the region flanking the 1010 bp product revealed a single nucleotide polymorphism in the 3′ border of the marker band. Primers were designed to amplify a 488-bp fragment from all 53 *P. chalamydospora* isolates. The primers were specific for *P. chlamydospora*. The detection limit of the isolate-specific PCR was 5 pg. The nested-PCR markedly improved the sensitivity and a detection level of 50 fg (equivalent of 1spore/g of seed) was achieved. The nested-PCR was more rapid than the conventional methods involving isolation and culturing of the fingus from infected plant material. The nested PCR-RFLP procedure was applied to detect the pathogen in the nursery soil. The DNA from the soil inoculated with viable spores could be detected after 119 days (Ridgway et al. 2005).

2.3.5.6 Real-Time PCR Assay

Among the PCR-based techniques, real-time PCR has been shown to be simple and reliable for detection and quantification of viral, bacterial and fungal plant pathogens. This technique uses a fluorescence detection system to measure a cycle threshold (Ct) value that can be transferred into a quantitiy of DNA after comparison with a DNA calibration curve. The DNA of a specific target organism can be quantified by measuring the intensity of fluorescence with time during the exponential phase of DNA amplification. Quantification of DNA in unknown samples may be done by direct comparison with standards amplified in parallel reactions. The principal advantage of real-time PCR is that amplification products may be monitored, as they are accumulated in the long-linear phase of amplification. Compared

with conventional PCR, it is more accurate, less time-consuming and preferable, especially if the aim is to discriminate between slightly different levels of infection (Schaad et al. 2003; Vandemark and Barker 2003).

Detection of seed-borne fungal pathogens is an important of component of seed health assessment programs. The rapidity, reliability and reproducibility in addition to sensitivity and specificity are the criteria that determine the suitability of detection method(s) to be applied in large scale. A real-time PCR procedure was tested for the detection of *Alternaria brassicae* in cruciferous seed, since the standard PCR suitable for the detection of *A. brassicola* or *A. japonica*, did not provide satisfactory results for *A. brassicae.* Specific oligonucleotides 115 sens and 115 rev complementary to a portion of ORF1 were designed. A standard curve was plotted using known quantities of *A. brassicae* DNA in a serial dilution followed by real-time PCR and the Ct values were calculated. The mean Ct values obtained with 100 and 10% aritifically contaminated radish seed batches were 25.56 and 32.55 respectively, corresponding to approximately 250 and 3.5 pg of *A. brassicae* DNA respectively. Real-time PCR was more sensitive than conventional PCR format and it is readily amenable for automation. The pathogen can be accurately and realiably detected in infected seed in about 50 h (Guillemette et al. 2004).

The presence of *Tilletia caries* causing stinking or common bunt disease of wheat was detected and the level of contamination in wheat seed lots was quantified by applying a quantitative real-time PCR assay using TaqMan chemistry. This assay detected 44 pg of pathogen DNA equivalent of less than one spore/seed. It allows an increase in test throughput and provides a sensitivity level required for an advisory threshold of one spore per seed (McNeil et al. 2004). *Fusarium* head blight (FHB) is a disease complex of wheat and small-grain cereals. Depending on the season, crop and geographical location, the components of the disease complex (over 17 *Fusarium* spp.) may differ. A real-time PCR procedure was developed to monitor and quantify the major *Fusarium* spp. involved in FHB complex during the growing season. Taq Man primers and probes showed high specificity for *F. avenaceum, F. culmorum, F. graminearum, F. poae* and *Microdochium nivale* var. *majus*. The concentrations of genomic DNA of each fungal pathogen in leaves, ears and harvested grains were accurately determined. The correlation between Ct value and known DNA contents was high for *F. graminearum* ($r^2 = 0.9987$) and also for other species. All *Fusarium* spp. of FHB complex were quantifiable between 0.9 and 9000 pg. Quantitative monitoring of fungal species of the FHB complex can be a powerful tool for the development of preventive disease management strategies that are generally directed towards reduction of pathogen inoculum (Waalwijk et al. 2004).

The fusaria in malting barley are invariably assessed by analyzing the percentage of kernels contaminated. The plating method used for this purpose is both laborious and time-consuming and information concerning toxigenic species can not be derived. To meet this requirement a rapid and simple quantification method like real-time PCR is required to evaluate the mycotoxin risk in cereals used in cereal-based industry. High level of specificity due to the use of fluorigenic sequence-specific probes, rapid analysis of data and a wider dynamic range compared to end-point

PCR are advantages of real-time PCR assay. In the investigation for quantification of trichothecene producing *Fusarium* species as well as the highly toxigenic *F. graminearum* present in barley and malt was taken up. The TMTR1 assay and TMFg 12, two variants ofTaqMan were employed to quantify trichothecene-producing Fusarium DNA and *F. graminearum* DNA present in barley grain and malt samples respectively. Both variants exhibited required sensitivity and reproducibility. The amounts of *Fusarium* DNA estimated with TMTR1-trichothecene assay reflected the deoxynivalenol (DON) contents in barley grains. In addition, the TMFg12 assay for *F. graminearum* estimated the DON content reliably in the north American barley and malt samples. In contrast, the DON content in Finnish samples could not be determined satisfactorily by TMFg12 assay, probably because of the low DON contents in these samples (Sarlin et al. 2006).

Three species of *Botrytis* viz. *B. aclada, B. allii* and *B. byssoidea* associated with onion seeds, cause neck rot of onion bulbs later. A real-time fluorescent PCR assay using SYBR Green chemistry was developed to quantify the pathogens in the onion seeds. The ribosomal intergenic spacer (IGS) regions of target and nontarget *Botrytis* spp. were sequenced, aligned for designing a primer pair specific to *B. aclada, B. allii* and *B. byssoidea.* The designed primer reliably detected 10 fg of genomic DNA per PCR reaction extracted from pathogen cultures. Real-time PCR assay was employed to quantity pathogen DNA in 23 commercial seed lots. This assay appeared to be more sensitive than the conventional agar assay, since 5 of the 23 seed lots that tested negative using agar assay, were found to carry the pathogens as indicated by PCR-based protocol developed in this investigation. However, no relationship was evident between the incidence of seed infection and neck rot disease incidence in the field (Chilvers et al. 2007).

As the true seeds and vegetatively propagative materials form primary sources of infection, they spread the disease-causing microbes to new areas and to subsequent generations. A direct method of testing potato tubers for detection of *Spongospora subterranea* causing powdery scab disease was developed. This method combines a fast two-step automated approach to DNA extraction with a sensitive TaqMan® PCR assay for rapidly processing the samples. The ITS-regions were used to design real-time PCR primers and a probe. The assay was species-specific for *S. subterranea* and it showed broad utility by its ability to detect *S. subterranea* directly on a range of different potato cultivars from different geographical locations within UK. Furthermore, the assay could detect the pathogen in tubers at more stages in its life cycle and not at the cystosori stage only as in the case of ELISA. The TaqMan® PCR was shown to be at least 100 times more sensitive than either ELISA or conventional PCR (Ward et al. 2004). The microtiter plate-based (MTP)-PCR-ELISA method and real-time PCR technology were applied for the detection of *Fusarium* spp. implicated in causing potato dry rot disease worldwide. *F. sulphureum* is the most common pathogenic species associated with tuber dry rot in North America and some European countries. The ITS regions (ITS/and ITS 2) of the rDNA gene of the isolates of *F. coeruleum, F. sulphureum, F. avenaceaum* and *F. culmorum* were amplified with the universial primers ITS5/ITS4. Regions of dissimilarity were used to design primers for the specific detection of *F. coreuleum* and *F. sulphureum*.

Additional sets of species-specific primers and probes were designed in the ITS regions of all *Fusarium* spp. tested to meet the optimal requirements for fluorescent PCR technology based on amplicons between 50 and 150 bp in length. By using PCR-ELISA assay, specific signals from 50 to 100 femtograms (fg: 10^{-15}) of genomic DNA from pathogenic *Fusarium* spp. The detection limits for seed peel extracts (0.5 ml) for PCR-ELISA assay were determined to be 12.5–25.0 macroconidia (depending on *Fusarium* spp). On the other hand, TaqMan technique could reliably detect attogram (10^{-18}g) levels of genomic DNA extracted from the pathogenic isolates. Real-time PCR assay detected all the four *Fusarium* spp. either singly in combination in potato seed stocks sampled from commercial stores. Increased DNA levels were correlated with increased disease severity between 8 and 12 week of storage. Both PCR-ELISA and real-time PCR assays exhibited high sensitivity and specificity and allowed detection of *Fusarium* spp. on potato tubers and produced similar results. Regarding level of sensitivity, real-time PCR had detection limits in attogram DNA levels (10^{-18}g) well below that of PCR-ELISA (10^{-15}g) DNA levels. But real-time requires more expensive equipments than PCR-ELISA assay, whereas the reagent costs of the PCR-ELISA are higher per assay than for real-time PCR assay (Cullen et al. 2005).

Fusarium solani f.sp. *glycines,* a soilborne pathogen, causing sudden death syndrome (SDS) in soybean, is difficult to detect and quantify, due to its slow-growing nature and variable phenotypic characteristics. Real-time quantitative-PCR (QPCR) assay was developed for both absolute and relative quantitification of *F. solani* f.sp. *glycines.* QPCR assays were performed in a 96-well plate format. The pathogen was detected and quantified based on detection of the mitochondrial small-subunit rRNA gene. As low as 9.0×10^{-5} ng of *F. solani* f.sp. *glycines* could be detected in soybean plants with or without SDS foliar symptoms using the absolute QPCR assay. The relative QPCR may be used to elucidate the fungus-host interaction in the development of SDS or screen for host resistance (Gao et al. 2004).

Different species of *Phytophthora* are responsible for blight or root rot diseases affecting many economically important crops. *Phytophthora fragariae* var. *fragariae* causing red stele (root rot) disease is present in most European countries and it is an "A2" quarantine organism necessitating adoption of methods to prevent its further spread. As the infected planting material is the principal method of long-distance dispersal, a sensitive and reliable test to detect low levels of pathogen populations in plants is essential. TaqMan™ technology provided real-time measurements in a closed-tube system. The target pathogen DNA could be quantified, the Ct values giving a good indication of the amount of *P. fragariae* present in plant tissues tested (Bonants et al. 2004). *Phytophthora capsici* causing root rot disease of pepper crops has to be detected and identified rapidly to minimize the loss due to the disease. Early detection of *P. capsici* using specific primers based on ITS region of rDNA in PCR assay in artificially inoculated and naturally infected pepper plants was attempted (Silvar et al. 2005b). In order to quantify pathogen biomass, a real-time PCR format was developed for the detection of *P. capsici* in pepper tissues. Using SYBR Green dye [double-stranded DNA-binding dye (as DNA is amplified more dye is bound and hence fluoresces)] and specific primers for

P. capsici, the minimal amount of pathogen DNA quantified was 10 pg and it could be detected as early as 8 h post-inoculation in susceptible pepper cultivar. Among the plant tissues affected, the pathogen biomass was maximum in stems (Silvar et al. 2005a).

Phytophthora ramorum, the causative agent of sudden oak death disease, can become a serious threat to forest ecosystems, if effective measures are not in place. The availability of rapid and accurate detection methods for *P. ramorum* is considered as a critical factor for management of this pathogen. Real-time PCR assays provide advantages of speed, accuracy and sensitivity over conventional PCR methods. A single round TaqMan PCR assay was developed for the detection of *P. ramorum* within 2 h under field conditions. Specific primers *Pram*- 114 F and *Pram*-190 R and probe (*Pram* probe) and generic 5.8S TaqMan primers (5.8S F and 5.8S R) and probe (5.8S probe) were designed based on ITS sequences. The protocol developed in this study, has been applied outside the laboratory to extract and test DNAs from healthy and infected plants at disease outbreak sites several hundred miles from the diagnostic laboratory and it can be performed right in the field using equipment powered by a generator. The results obtained were comparable to those of real-time PCR testing in the laboratory conditions (Tomlinson et al. 2005).

In a later investigation, a real-time PCR detection method based on the mitochondrial DNA sequence with an ABI Prism 7700 (TaqMan) Sequence Detection System was developed. Primers and probes were developed for detection of *P. ramorum* and *P. pseudosyringae* that causes symptoms similar to *P. ramorum* on certain host plant species. The lower limit of detection of *P. ramorum* DNA was one fg of genomic DNA, which is lower than that can be attained with other PCR procedure for detecting *Phytophthora* spp. It was also possible to detect both pathogens, in addition to plant DNA in a single tube simultaneously by applying a three-way multiplex format. The multiplex assay could detect *P. ramorum* in infected California field samples from several plant hosts. This real-time PCR is highly sensitive and specific in addition to offering several advantages over conventional PCR assays in detecting *P. ramorum* in nurseries (Tooley et al. 2006). Since *P. ramorum* was reported to infect many trees and shrubs in parts of Europe and North America, efforts were made to improve the specificity and sensitivity of detection techniques. A single-round real-time PCR assay based on TaqMan chemistry was developed. Primers were designed based on the sequences of ITS1 region of the nuclear ribosomal (nr) RNA gene for the detection of *P. ranomorum* in plant materials. This procedure has the distinct advantage over other PCR variants: absence of post-amplification steps or multiple rounds of PCR. This protocol was applied to detect *P. ramorum* successfully in 320 plant samples from 19 different plant species. In addition, the assay amplified the DNA of *P. ramorum* only, but not from 28 other *Phytophthora* spp. tested, indicating the high specificity of the protocol (Hughes et al. 2006).

In another investigation for the detection of *P. ramorum*, 5 (prime) fluorigenic exonuclease (TaqMan) chemistry was used to detect and quantify the fungal pathogen in infected plant tissues. The limit of detection was 15 fg of target DNA when used in a nested design or 50 fg when used in a single-round of PCR. The specificity of

the assay was revealed by the absence of amplification of DNA of 17 other *Phytophthora* spp. tested. Furthermore, the nested methods were found to be significantly more sensitive than non-vested methods. However, the host substrate influenced the sensitivity of all assays. Field testing showed that the nested TaqMan protocol detected *P. ramorum* in 255 of 874 plants in California as against few positive reactions provided by single-round TaqMan procedure (Hayden et al. 2006).

Phoma tracheiphila causing a tracheomycotic disease designated "mal secco" in citrus is of quarantine concern. Hence, it was considered that a rapid, specific and sensitive technique for the detection and identification of *P. tracheiphila* was essential. A specific primer pair and a dual-labeled fluorigenic probe were used in a real-time PCR with the Cepheid Smart Cycler II system in addition to the regular PCR assay. The sensitivity of real-time PCR was greater (10 to 20-folds) with the detection limit of 500 fg of pathogen DNA, while the PCR assay required a minimum of 10 pg pathogen DNA for successful detection. A simple and rapid procedure to obtain suitable DNA samples from citrus plants to be tested enabled diagnosis of "mal secco" disease to be completed within about 10 min using real-time PCR protocol developed in this investigation (Licciardello et al. 2006). Species-specific primers based on the variable regions of ITS of rDNA were employed on real-time PCR assay for the detection and quantification of nine *Pythium* spp. in eastern Washington. Among the *Pythium* spp., *P. irregulare* and *P. ultimum* are important as pathogens causing destructive diseases in several economically important crops (Schroeder et al. 2006).

The importance of detecting ancient microbial DNA in historical samples has been realized for infectious diseases of humans, animals and plants (Ristaino et al. 2001; Zink et al. 2002; May and Ristaino 2004). A long-term dynamics of *Phaeospora nodorum* and *Mycosphaerella graminicola* populations were investigated by using PCR assays in wheat samples maintained in wheat archieves since 1843 at Rothamsted, UK. Quantitative real-time PCR assays were applied to determine the amounts of *M. graminicola*, *P. nodorum* and wheat DNA present in a set of samples covering a 160 year period of wheat production. Changes in amounts of DNAs of the two pathogens showed that *M. graminicola* was the most abundant in the mid-19th century. *P. nodorum* DNA was more abundant than *M. graminicola* DNA for much of the 20th century with a peak ≈1970. The ratio of the DNA of the two pathogens correlated well with the ratio of severity of the two Septoria diseases, estimated from the survey data during 1970–2003. A close relationship between changes in the ratio of the two pathogens and changes in UK atmospheric SO_2 emissions over the 160 year period (Bearchell et al. 2005).

Phytophthora ramorum causing the devasting sudden oak death disease is spreading fast and quarantine regulations are enforced to restrict its introduction and dissemination. The molecular methods developed earlier appear to be inadequeate in providing reliable differentiation of *P. ramorum* from other closely related species. By using sequences of ITS, β-tubulin and elicitin gene regions, three different reporter technologies viz., molecular beacons, TaqMan and SYBR Green were compared. These assays differentiate *P. ramorum* from 65 other species of *Phytophthora* tested. The pathogens could be detected in all 48 infected samples by all three

real-time PCR assays. However, assays based on detection of ITS and elicitin regions using TaqMan appeared to be more sensitive in detecting and differentiating *P. ramorum* (Bilodeau et al. 2007).

A real-time quantitative PCR was developed to detect and quantify *Verticillium dahliae* causing potato early dying (PED) or wilt disease, by employing the primer pair VertBt-F/VertBt-R derived from the sequence of the β-tubulin gene. This primer was found to be more efficient (>95%) than monoplex Q-PCR and duplex methods using the primers PotAct-F/PotAct-R designed from the sequence of actin gene. It was possible to detect and quantify as few as 148 fg of *V. dahliae* DNA which is equivalent to five nuclei. *V. dahliae* in naturally-infected air-dried potato stems and fresh stems of inoculated plants was detected by this Q-PCT protocol. Furthermore, *V. dahliae* was detected by Q-PCR in 10% of stem samples that were considered to be free of infection by isolation on culture medium. As the Q-PCR assay is more sensitive and rapid, the response of breeding lines of potato to colonization by *V. dahliae* may be reliably assessed for resistance breeding programs (Atallah and Stevenson 2006; Atallah et al. 2007).

Some of the fungi may vector plant viruses in addition to their potential to become pathogens as in the case of *Polymyxa* spp. *Soil-borne wheat mosaic virus (SBWMV), Barley yellow mosaic virus* (BaYM) and *Beet necrotic yellow vein virus* (BNYVV) are transmitted by *Polymyxa* spp. Primers and probes were designed based on sequences of ITS2 region of rDNA and they amplified *P. betae* and *P. graminis* DNA extracted from infested soils, using real-time PCR and TaqMan chemistry. Primers based on ITS2 region was found to be useful for direct detection and quantification of *Polymyxa* DNA and it can be completed in less than a day. Further this protocol sensitive enough to detect *Polymyxa* from as little as 500 mg of soil (Ward et al. 2004).

The real-time PCR assay has been shown to be effective for the detection of obligate pathogens in some host plant species. Detection and differentiation of four rust pathogens *Puccinia graminis, P. striiformis, P. triticina* and *P. recondita* was achieved by duplex real-time PCR assays. Variable ITS1 region of the nuclear rDNA gene to distinguish between species and the conserved 28S region as an internal control were used to design the primer/probe sets. Species-specific ITS1 primer/probe sets were highly specific and capable of detecting as low as <1 pg of rust pathogen DNA. The 28S primer/probe combination was very effective in detecting all *Puccinia* spp. tested in multiple collections representing a range of races and formae speciales within a species. The assays were useful to identify rust fungi infecting pasture grasses also (Barnes and Szabo 2007).

2.3.5.7 Kinetic-PCR (kPCR) Assay

The biotrophic plant pathogens causing destructive diseases such as rusts, powdery mildews and downy mildews present certain obstacles not encountered in the case of culturable pathogens. The kinetic-PCR (kPCR) has been effective for the detection and quantification of *Melampsora* spp. causing leaf rust disease of poplar in North America and Europe. The ability of kPCR to accurately quantify pathogen DNA

allows construction of growth curves that provide details of pathogen infection that were earlier unattainable. Since DNA replication is intimately linked to cell division, quantification of pathogen DNA present in leaf tissues over time was possible by kPCR procedure. Growth curves from inoculation through the final stages of uredinial maturation, as well as pathogen monitoring before symptoms become visible could be documented. The variations in growth parameters such as period of latency, generation time in logarithmic growth and the increase in DNA mass at saturation were determined in compatible, incompatible and nonhost interactions. Differentiation by kPCR assay of the rust pathogens *Melampsora medusae* f.sp. *deltoidae* and *M. larici-populina* using species-specific primers, when present in a mixed populations in infected leaves is yet another advantage over conventional PCR assay. Pathogen detection was not significantly affected by the presence of other DNAs, since the Ct values for specific pathogen were nearly identical for all DNA mixtures containing the same amount of the pathogen. The kPCR has the potential to be a means of monitoring of microorganisms in their environment, whether it be in planta or in soil (Boyle et al. 2005).

2.3.5.8 Padlock Probes–Multiple Detection System

Although PCR-based detection techniques are generally effective, they target only a single pathogen per assay, making comprehensive screening of samples laborious and time-consuming. Padlock probes (PLPs) provide a means of combining pathogen-specific molecular recognition and universal amplification, thereby increase sensitivity and multiplexing capabilities without limiting the range of potential target organisms. The PLPs are long oligonucleotides with ~100 bases, containing target complementary regions at both their 5′ and 3′ ends for recognizing adjacent sequences on the target DNA. The universal primer sites and a unique sequence identifier known as Zipcode are located between these segments. The ends of the probes get into adjacent position upon hybridization. They can be joined by enzymatic ligation. Only when both ends recognize their target sequences correctly, ligation and consequent formation of circular molecule can occur. By treating with exonuclease, noncircularized ones are removed, whereas the circularized ones may be amplified by using universal primers. The target-specific products are detected then by a universal complement Zipcode (cZipcode) microarray. Since nondegenerate universal primers and uniform size of the amplicons are used, bias in the universal amplification step will be limited. In addition, the targeted sequences and the probes on the array are independent, making the assay easily modifiable and extendable to include new target pathogens.

The genomic DNA of the test fungal pathogen was fragmented by digestion using *Eco*RI, *Hind*III and *Bam* HI (New England Biolabs) for 30 min and used as template. Cycled ligation was performed in the reaction mixture containing *Taq* ligase. Amplification of ligated PLPs was followed in real-time using an ABI prism 7700 Sequence Detector System (Applied Biosystems) and the PCR kit (Eurogentec). The ligation conditions for *Phytophthora fragariae* consisted of PLPP-frag

that targeted its ITS region and of the corresponding synthetic, target and non-target oligonucleotide. The discriminatory areas within the ITS regions of rRNA operons of *P. fragariae* because of their high copy number which could significantly increase the sensitivity of the assay. A genes-specific PLP was designed to target all *Phytophthora* spp. and to discriminate them from related oomycetes. After selecting the target-complementary regions, they were combined with the universal primer site sequences and a unique Zipcode sequence for each probe was selected. High levels of specificity and sensitivity (5 pg of pathogen DNA) were discernible in all assays (Szemes et al. 2006).

2.3.5.9 Molecular Beacon Technology

The principles of molecular beacon assay have been indicated elsewhere (Chapter 2, Section 2.3.1.5). The molecular beacon (MB) technique was applied for the detection of *Phytophthora fragariae*. A molecular Beacon™ is an oligonucleotide probe, with a central region complementary to the target amplicon and a 607 bp sequence (one end labeled with a quencher and the other with a fluorescent dye) that complement each other at the 3′ and 5′ ends. A molecular Beacon™ probe was developed to detect *P. fragariae* PCR amplicons in a quantitative manner similar to TaqMan™. The pathogen genomic DNA concentration detected was linear with dilutions from 100 attogram (ag) to 1 pg. The sensitivity of MB was similar to that of TaqMan assay. The MB probe detected amplicons in samples with as little as 100 ag of genomic cDNA and as few as 25 zoospores of *P. fragariae,* in addition to pathogen DNA in water and not samples with controlled level of infection. Moelcular Beacon™ as well as TaqMan™ provide "real-time" measurements in a closed-tube system on the ABI 7700 and they are capable of assessing the amounts of target DNA with Ct values giving the good indication of the quantity of *P. fragariae* present in the sample. However, with its "nil tolerance" limit quantification may not be an important factor for consideration, while choosing a diagnostic test for application (Bonants et al. 2004).

2.3.5.10 DNA Array Technology

Most molecular diagnostic assays target one specific pathogen. As crops are infected by several pathogens, it is desirable to use assays that are able to detect multiple pathogens simultaneously. Multiplex PCR-based techniques and real-time PCR procedures have certain technical limitations. On the other hand, array hybridization technology offers the possibility to add a multiplex aspect to PCR-based detection. DNA arrays, originally developed to study gene expression or to generate single-nucleotide polymorphism (SNP) profiles, have been demonstrated to be useful for detecting an unlimited number of different organisms in parallel (McCartney et al. 2003). *Synchytrium endobioticum* causing wart disease of potatoes is an important quarantine pathogen world wide existing as at least 30 biotypes, defined based on their virulence on different potato cultivars. Once introduced into

the field, the resting spores produced by *S. endobioticum* can survive for a period of 30–70 years (Hampson 1993). Since no sequence information was available for *S. endobioticum*, its 18S rDNA sequence was analysed by extracting DNA from the resting spores exracted from infected potato tubers. The sequences of 18S rDNA of different species of *Synchytrium* were conserved and showed high homology. Specific oligonucleotide probes were designed and arrayed onto glass slides for detection of the pathogen. In the case of viruses and viroid infecting potatoes *Andean potato latent virus, Andean potato mottle virus, Potato black ringspot virus* and *Potato spindle tuber viroid,* nucleic acid sequences were available in the databank. Probes for specific detection of these viruses were designed based on the nucleic acid sequences of the different viruses. In order to determine the sensitivity and specificity of the oligonucleotide probes, total RNA from infected plants was reverse-transcribed, labeled with Cyanine 5 and hybridized with the microarray. A significant number of the oligonucleotide probes showed high specificity in detecting the fungal and viral pathogens. The results indicated the potential of microarray-based hybridization for identification of multiplex pathogen targets which would be highly useful for quarantine laboratories where parallel testing for diverse pathogens is required (Abdullahi et al. 2005).

A DNA-microarray technique using a combination of low-density arrays and a single universal PCR for identification of target sequences has been developed. The detection of the amplicons on the capture probes of microarray is facilitated by the presence of biotinylated nucleotides in the PCR amplification. The microarray could detect and differentiate 14 trichothecene-and moniliformin-producing *Fusarium* spp. by using capture probes corresponding to sequences of the translation elongation factor-1-α (TEF-1α). A consensus PCR amplifcation of a part of the TEF-1α was followed by hybridization to the *Fusarium* chip and the results are visualized by a colorimetric silverquant detection method. The limit of detection was less than 16 copies of genomic DNA in spiked commercial wheat flour. This *Fusarium* chip technique including DNA extraction from several samples may provide the results within 1–2 days, whereas the conventional methods may need 7–21 days. Further, this microarray-based system was shown to be cost-effective (Kristensen et al. 2007).

Appendix 1: Electrophoretic Characterization of Strains of Bacterial Pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) (Bouzar et al. 1994)

A. Culture Maintenance

i. Preserve strains of *Xcv* with different origins at −70°C in aqueous glycerol (15%).
ii. Multiply the strains on nutrient agar (Difco Laboratories, Detroit, MI) to the required level for analysis.

B. Electroporesis of Whole-Cell Proteins of Xcv

i. Transfer each strains to be tested to nutrient broth at 28°C and place them on an orbital shaker (200 rpm) for 18 h.
ii. Take samples (1.5 ml each) and pellet the bacterial cells by centrifugation at 16,000 g for 10 min; wash the cells in sterile deionized water twice and resuspend the cells (about 20 mg wet weight) in 180 µl of sorbitol (10%).
iii. Mix the bacterial cell suspension with equal volume of double-strength lysis buffer containing Tris–hydrochloride (125 mM, pH 6.8), 2-mercapto-ethanol (10%), glycerol (20%) and bromophenol blue and heat the mixture at 95°C for 10 min prior to loading.
iv. Use a discontinuous gel (10 min of stacking gel and more than 100 mm of separation gel).
v. Prepare the separation gel consisting of total acrylamide (10%), Tris–hydrochloride (375 mM, pH 8.8) and sodium dodecyl sulfate (SDS) (0.1%) and the stacking gel comprising of total acrylamide (5%), Tris–hydrochloride (125 mM, pH 6.8) and SDS (0.1%).
vi. Aerate the solutions for 15 min and initiate polymerization by addition of ammonium persulfate (0.05%) and tetramethyl ethylenediamine (0.005%).
vii. Load the wells with samples (10 µl) and controls containing protein molecular weight standards.
viii. Perform electrophoresis at 4°C in a Protean II double slab vertical electrophoresis cell (BioRad Laboratories, Richmond, CA) filled with electrophoresis buffer composed of Tris (250 mM), glycine (192 mM) and SDS (0.1%).
ix. Apply a constant current of 25 mA per gel until the bromophenol blue reaches the separation gel (0.75 h) and then increase the current to 35 mA per gel until the tracking dye migrates about 100 mm through the separation gel (about 2.75 h).
x. Use silver staining kit (Sigma silver stain kit, Sigma Chemcial Co., St. Louis, MO) or a combined Coomassie blue-silver staining procedure. In the combined procedure, stain the protein first with Coomassie blue; destain with a mixture of methanol (50%) and acetic acid (10%) followed by staining with silver staining kit.

Appendix 2: Detection of Virus-Specific Protein in Infected Leaves by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Seifers et al. 1996, 2005)

i. Grind samples (1.0 g) of tissues from healthy and infected (with *Wheat yellow head virus*) plants in a mortar with pestle after adding (at the rate of 1:7 w/v) ammonium citrate buffer (0.1 M), pH 6.5 containing mercaptoethanol (0.25%), sodium diethyl dithiocarbamate (0.1%) and polyvinylpyrrolidone (1.0%) and filter through cheese cloth.

ii. Centrifuge for 10 min at 5900×g; remove the supernatant carefully and add Triton X-100 to give a final concentration of 7.4%.
iii. Centrifuge through a 1 cm layer of sucrose (0.988 M) in ammonium citrate buffer at 85,000×g for 2 h.
iv. Resuspend the pellet formed in the centrifuge tube in 200 µl of sodium dodecyl sulfate (SDS) treatment buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol): heat the mixture at 100°C for 4 min, reduce and alkylate.
v. Electrophoresce aliquots of 25 µl in 10% polyacrylamide gels, along with standard molecular markers (Sigma Markers – Sigma-Aldrich).
vi. Stain the gels with Coomassie Blue R-250.
vii. Scan the gels to calculate relative molecular mass using a Höefer GS 300 densito meter to determine electrophoretic mobility.
viii. Process the data using Höefer GS-365 data analysis software; calculated values for proteins using a linear regression will fit to protein standards used on the gel.
ix. Repeat the analyses at least three times with extracts from different samples.

Appendix 3: Indirect ELISA for Assessing Titers of PABs and MABs Specific to *Callalily chlorotic spot virus* (CCSV) and *Watermelon silver mottle virus* (WSMoV) (Lin et al. 2005)

i. Coat 96-well microtiter plates with diluted (50-folds) crude extracts of CCSV and WSMoV-infected plants (*Nicotiana benthamiana*) in coating buffer containing sodium carbonate (0.05 M, pH 9.6) buffer and sodium azide (0.02%).
ii. Dispense PABs (rabbit antiserum) to CCSV-nucleo-capsid protein (NP) at 2-fold serial dilutions commencing from a 10^{-3} and then add alkaline phosphatase (ALP) – conjugated goat anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 2×10^{-4} dilution as the secondary antibody.
iii. Apply PABs to WSMoV at a dilution of 2.5×10^{-4} in place of CCSV in another set of microtiter plates.
iv. In the case of ascitic fluids (from BALB/cByJ mice) containing MABs to CCSV and WSMoV, use 10-fold serial dilutions starting from 10^{-3} dilution and ALP-conjugated goat antimouse IgG 2×10^{-4} dilution as the secondary antibody.
v. Add ALP substrate (p-nitrophenyl phosphate at concentration of 1 mg/ml in diethanolamine buffer, pH 9.8 at room temperature) and allow the reaction for 30 min.
vi. Stop the reaction by adding NaOH (3.0 M) at 50 µl/well.
vii. Record the absorbance values at 405 nm using a microplate reader (Bio-Tek instruments, Winooski, VT).

Appendix 4: Detection of *Citrus psorosis virus* (CPsV) by Direct Tissue Blot Immunoassay (DTBIA) (Martin et al. 2002)

i. Prepare tissue prints by cutting young shoots, petioles or rolled leaf blades and gently press the freshly cut surface onto nitrocellulose membrane (NCM) of 0.45 µm pore size (Bio-Rad, Madrid, Spain) or nylon (Amersham Pharmacia, Barcelona, Spain or Roche Diagnostics, Barcelona, Spain).
ii. Air-dry the prints and block and nonspecific areas present on the membrane surface in TBS buffer (10 mM Tris–HCl, pH 7.4, 0.15 M NaCl) containing defatted milk powder (50 g/l) (TBS-milk buffer); or in PBS buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.14 M NaCl, 3 mM NaN_3, pH 7.4) containing Triton X-100 (20 g/l) and defatted milk powder (50 g/l)(PBS-milk buffer).
iii. If PBS-milk buffer is used, wash the membranes three times in distilled water prior to incubation with antibodies.
iv. Incubate the membranes in appropriately diluted antiserum solution (1:10,000–1:50,000 of ascites fluid containing monoclonal antibodies (MAB 13 C5 or 2A3) in TBS-milk or PBS-milk buffer for 90 min.
v. Wash the membranes thrice for 15 min each with TBST or PBST [TBS or PBS + Tween (3 g/l)] and wash twice with distilled water (the first and last).
vi. Incubate the membranes in TBS-milk or PBS-milk buffer (1:20,000 dilution) with appropriate alkaline phosphatase (AP) conjugated antibody for 90 min.
vii. Wash the membranes as done earlier and equilibrate in substrate buffer containing Tris–HCl (0.1 m, pH 9.5) for 5 min and add the substrate.
viii. For direct action, block the membranes as indicated earlier [step (ii)]; incubate in A322 antibodies (1:10,000 dilution) conjugated with AP in TBS – milk buffer and wash and equilibrate in substrate buffer as mentioned earlier.
ix. All incubations are performed at room temperature.
x. Detect the enzyme activity with chromogenic substrate BCIP/NBT or with chemiluminiscent substrates CSPD or CPD-star (Roche Diagnostics) as per the instructions of the manufacturer.

Appendix 5: Detection of *Potato virus Y* (PVY) and *Cucumber mosaic virus* (CMV) in Tobacco by Immunostaining Technique (Ryang et al. 2004)

i. Prepare pieces of stem tissues of tobacco plants infected with PVY and doubly-infected with CMV; immerse them immediately in a fixative consisting of ethanol (50%), acetic acid (5%) and formalin (3.7%) and leave them in the fixative over night at 4°C.
ii. Transfer the stem pieces to a graded series of ethanol solutions (50%, 70%, 90% and 100%) for dehydration and infiltration in each concentration for 30 min.

iii. Embed the tissues in paraffin (Paraplast-Plus, Sigma) and cut sections (12 µm thick) using a rotary microtome (Yamato Kohki).
iv. Transfer the sections to glass slides (Matsunami Glass); dewax the slides in xylene and wash in ethanol (100%).
v. Hydrate the sections using a graded series of ethanol solutions (70%–50% and 30%) followed by distilled water for 10 min each solution.
vi. Incubate the sections in PBST and BSA (1%) for 1 h and then in PVY CP-specific antibody (diluted 1:200 in PBST/BSA) for 2 h at 37°C and wash the sections in PBST.
vii. Incubate the sections in alkaline phosphate-conjugated goat anti-rabbit IgG (Sigma) (diluted to 1:200 in PBST/BSA) at 37°C for 2 h and wash the sections thrice in PBST for 10 min each.
viii. Stain them with BCIP/NBT liquid substrate system and wash the sections with distilled water.
ix. Observe the sections under the microscope (x-50) for development of color.

Appendix 6: Detection of *Citrus tristeza virus* (CTV) by In Situ Immunoassay (ISIA) (Lin et al. 2000)

i. Prepare transverse sections (100–200 µm thick) from fresh stems (of young shoots), petioles or veins from healthy and virus-infected plants, using a razor blade and maintain four to six replicates for each sample.
ii. Transfer the sections with forceps to 24-well plastic plates (Corning Glass Works, Corning, NY); fix the tissues (sections) with 70% ethanol (300–1000 µl/well) for 5–20 min at room temperature.
iii. Pipette out the ethanol from the wells, incubate the sections at 37°C with specific antibodies (300–500 µl) of PABs or MABs in PBST containing NaCl (0.15 M), sodium phosphate (0.15 M), Tween-20 (0.05%) and fetal bovine serum or bovine serum albumin (BSA) (3.0%) for 30–60 min.
iv. Wash the sections with PBST-PVP (PBST+ polyvinyl pyrrolidone) (2.0%) for 5–10 min and incubate the sections with alkaline phosphatase (AP) conjugated with goat antimouse Ig for sections allowed to react with MABs or AP-conjugated with antirabbit IgG in the case of sections exposed to PABs for 30–60 min at 37°C.
v. Wash the sections with PBST-PVP for 5–10 min: then wash the sections with TTBS buffer [Tris (hydroxymethyl) aminomethane (20 mM), NaCl (500 mM) and Tween 20 (0.05%) pH 7.5] for 5–10 min.
vi. Incubate the sections with freshly prepared NBT-BCIP substrate mixture composed of 66 µl of nitroblue tetrazolium (0.3 mg/ml) and 33 µl of 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml) in 10 ml of sodium carbonate buffer (0.1 M), pH 9.8 for 5–15 min.
vii. Remove the substrate solution from the wells to stop the reaction with enzyme and add water (500–1000 µl) to each well.
viii. Transfer the sections to glass slides using forceps and examine them under a light microscope (×100 magnification).

ix. Observe the development of purple color in phloem tissues indicating a positive reaction.

Appendix 7: Detection of Potyvirus by Western Blot Analysis (Larsen et al. 2003)

A. Preparation of Total Proteins

i. Macerate the infected plant tissues in 150 mM Tris–HCl, pH 6.8 containing sodium dodecyl sulfate (20%) (SDS), 2-mercaptoethanol (5.0%) and glycerol (10.0%), boil the suspension for 5 min and chill on ice.
ii. Centrifuge at 12,000 × g for 5 min.

B. Western Blot Analysis

i. Resolve the preparation on 12% discontinuous SDS-polyacrylamide gels and electroblot onto 0.45 µm nitrocellulose membrane.
ii. Probe the blots overnight at room temperature with potyvirus group-specific monoclonal antibody at a dilution of 1:1000.
iii. Wash the blots and probe with goat antimouse alkaline phosphatase (Bio-Rad, Hercules, CA) at a dilution of 1:2000.
iv. Wash after 1 h and stain with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate substrate (Sigma Chemical Co., St. Luis, MO).
v. Use low-molecular-weight prestained protein standards (Bio-Rad) for determination of molecular weights of proteins in the sample.

Appendix 8: Detection of Bacterial Pathogens by Enzyme-Linked Immunosorbent Assay (ELISA) in Seeds (Lamka et al. 1991; Pataky et al. 2004)

A. Antiserum Preparation

i. Prepare polyclonal antibodies (PABs) to *Erwinia stewartii* strain SS104 using New Zealand white rabbits by immunization with pathogen (bacterial) cells.
ii. Prepare monoclonal antibodies (MABs) to live cells of *E. stewartii* using BAL B/c mice and purify the MABs by using protein A chromatography.

B. Indirect-ELISA

i. Cultivate the pathogenic bacteria on nutrient agar (Difco) containing antifoam B (0.05%) (Sigma); flood the culture and flush the cells from the agar surface

with phosphate-buffered saline (PBS) (containing 0.02 M sodium phosphate and 0.85% NaCl, pH 7.2) and wash by centrifugation three times.

ii. Resuspend the final pellet in carbonate buffer consisting of 0.05 M sodium carbonate, pH 9.6.

iii. Dispense predetermined aliquots of bacterial cells to wells in microtiter plate (Immulon I plates, Dynatech Laboratories, Inc., Chantilly, VA) and incubate at 20°C for 1 h or at 4°C overnight for firm binding of cells to the wells.

iv. Block the well surface with BLOTTO (5% nonfat dry milk prepared in PBS containing 0.05% Tween 20 (PBS-T), 0.03% antifoam A and 0.02% NaN_3 and add dilute hybridoma culture medium (approx 1:50 dilution with PBS-T).

v. Add alkaline phosphatase (ALP)-labeled rabbit antimouse IgG (Sigma) and then add the substrate phosphate (1 mg/ml) of *p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8).

vi. In the case of PABs, dispense the bacterial cell suspensions into three wells per plate and replicate over three plates; add ALP-conjugated PABs followed by addition of substrate phosphate.

C. Preparation of Seed Samples for ELISA

i. Surface sterilize the samples of 500 seeds selected at random from plants either inoculated *E. stewartii* or from uninoculated plants treated with 0.05% NaOCl for 1 min, rinse the seeds in sterile water thrice and soak them overnight in 300 ml of PBS containing 0.02% NaN_3 at 4°C.

ii. Grind the seeds in a food blender for 1 min and filter the suspension through a single layer of cheese cloth.

iii. Centrifuge 50 ml subsamples at 1085 × g for 10 min at 5°C; decant the supernatant and resuspend the pellet in small volume (approx. 1/8 of the original) of PBS.

iv. Test four 100-µl samples from PBS soak solution, the ground seed suspension, the supernatant and the resuspended pellet to determine which sample preparation would yield maximum positive/negative (P/N) ratio.

Appendix 9: Detection of *Ultilago nuda* Barley Seeds by DAS-ELISA (Eibel et al. 2005a)

A. Preparation of Antigens from Pathogen Mycelium

i. Cultivate the fungal pathogen in appropriate liquid medium as shake cultures [*U. nuda*) in malt exact broth (MPB) containing malt extract (30.0g), peptone from soybean (3.0g) per litre] at 15°C.

ii. Separate the mycelial mass using a Büchner funnel fitted with a filter paper circle (No.595, Schleicher and Schwell, Dassel,Germany); pulverize with quartz sand and liquid nitrogen and homogenize with a low volume of extraction buffer [PBS containing Tween-20 (0.05%) and polyvinyl-pyrrolidone-40,000 (0.2%)] in a mortar.
iii. Centrifuge the homogenate at 30,000g for 10 min at 4°C; centrifuge again at 45,000g for 30 min at 4°C and store the supernatant at –20°C until required for analysis.

B. Preparation of Antigens from Culture Filtrate (CF)

i. Take the culture filtrate (approx, 400 ml) after the removal of fungal mycelium (step A (iii) above); dialyze against pre-cooled distilled water overnight at 4°C followed by lyophilization of the CF.
ii. Resuspend the lyophylisate in approx. 4 ml of PBS [consisting of NaCl (8.0g), KH_2PO_4 (0.2g) Na_2HPO_4, $2H_2O$ (1.44g), KCl (0.2g), NaN_3 (0.2g) and distilled water 1000 ml, pH 7.2–7.4] and dialyze the suspension against PBS at 4°C overnight.

C. Preparation of Seed Extracts

i. Place test seeds singly in the wells of microtiter plates (96-wells); dispense extraction buffer (3000 µl/well) and incubate for 24 h at 4°C.
ii. Cut the seeds into small pieces (3 mm diameter) in the well using small sharp scissors; add another aliquot of 300 µl of extraction buffer/well and shake the plates gently for about 5 min using the shake mode of microplate reader.
iii. Pipette out the suspension from the well into reaction tubes; centrifuge at 20,000g for 20 min at 4°C and use the supernatant for ELISA testing.

D. Double Antibody Sandwich (DAS)-Enzyme-Linked Immunosorbent Assay (ELISA)

i. Dispense 100 µl of IgGs (specific to the pathogen- *U. nuda*) at a dilution of 1:1000 in coating buffer [containing Na_2Co_3 (1.59g), $NaHCO_3$ (2.93g), NaN_3 (0.2g), distilled water (1l), pH 9.6) to each well in 96-well microtiter plates (U 96 maxisorp, Nuric, Wiesbaden); incubate overnight at 4°C and wash.
ii. Transfer to each well 200 µl of blocking buffer [coating buffer +BSA (0.2%) (Fraction V, Serva, Heidelberg)] to block non-specific adsorption of proteins to the well surfaces; incubate for 2 h at room temperature and wash.
iii. Add seed/plant/fungal extracts at 100 µl/well; incubate the plates overnight at 4°C and wash.

iv. Add 100 µl of biotinylated IgGs (diluted to 1:1500 in PBS-Tween (PBS +0.05% Tween 20) + BSA (0.2%); incubate overnight at 4°C and wash.
v. Add 100 µl of streptavidin-alkaline phosphatase (StrAP, Roche, Mannheim) solution (at dilution of 1:7500 in PBS-Tween + 0.2% BSA) to each well; incubate the plates at 37°C for 30 min and wash.
vi. Wash the wells thrice (3 min each) with half strength of PBS-Tween after every incubation in steps above.
vii. Add 100 µl of enzyme substrate, para-nitrophenyl phosphate (pNPP) (Serva, Heidelberg) solution (1 mg pNPP per 1 ml of substrate buffer (1 M diethanolamine in distilled water adjusted to pH 9.8 with HCl) to each well.
viii. Develop the color reaction by incubating the plates at room temperature in the dark for 2 h.
ix. Record the absorbance values at 405 nm using a microplate reader (Spectra Mini, Tecan, Craillsheim, Germany).
x. Maintain at least duplicates for each sample and work out the mean for comparison.

Appendix 10: Detection of Plant Viruses by Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) Assay (Huang et al. 2004; Spiegel et al. 2004)

A. Extraction of Nucleic Acid from Plant (Citrus) Tissues

i. Extract the total RNA from dried plant samples by using Qiagen RNeasy Total RNA Kit (Qiagen, Charsworth, CA) or by placing the tissue samples (approx 250 mg) in a canister and pulverize with liquid N for 40 s using a KLECO Model 4200 Pulverizer (Kinetic Laboratory Equipment Company, Visalia, CA).
ii. Collect the pulverized material into a sterile Eppendorf tube (1.5 ml); suspend it in nucleic acid extraction buffer consisting of Tris–HCl (0.1 M, pH 8.0), EDTA (0.05 M), NaCl (0.5 M), and *N* – lauroyl sarcosine (1.0%) and incubate for 1 h at 55°C.
iii. Centrifuge for 5 min at 6000 rpm; treat the supernatant (800 µl) with 100 µl of NaCl (5.0 M) and 100 µl of 10% hexadecyl-trimethyl-ammonium bromide (CTAB) at 65°C for 10 min and extract with chloroform/isoamyl alcohol mixture (24:1).
iv. Separate the aqueous supernatant and reextract with phenol/chloroform/isoamyl alcohol (25:24:1).
v. Percipitate the nucleic acid from the aqueous layer with 0.6 volume of isopropanol at room temperature for 15 min.
vi. Centrifuge at 12,000 rpm for 10 min; collect the precipitate; wash with ethanol (70%); dry and resuspend in 20 µl of TE buffer, pH 8.0.

B. Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

i. Amplify the desired nucleic acid fragment (243-bp fragment in the CP coding region of *Plum pox virus*) using the appropriate primers (P1 and P2); combine the total RNA (approx. 4 µg) in 5 µl double-distilled diethyl-pyrocarbonate (DEPC)-treated H_2O with 4 µl of antisense P1 primer (5 µM stock) and incubate for 5 min at 72°C.
ii. Perform RT reaction at 54°C for 30 min in a 20 µl volume by adding 11 µl of RT mix consisting of 4 µl of 5× First strand buffer (Invitrogen, Burlington, Ontario, Canada), 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP Mix, 0.5 µl of RNase Block (Stratagene, La Jolla, CA), 1 µl of Super Script II (Invitrogen) and adjust to a final volume of 11 µl with DEPC H_2O.
iii. Prepare PCR mix consisting of 0.5 µl of 10 mM dNTP mix, 0.75 µl of 50 mM $MgCl_2$, 0.2 µl of sense primer (P2, 5 µm stock), 0.25 µl of antisense primer (P1, 5 µM stock) 0.25 µl of *Taq* DNA polymerase (Invitrogen), 0.25 µl of Taq Extender PCR additive (Stratagene) and adjust to a final volume of 20 µl with DEPC H_2O.
iv. Add the PCR mix to 1 µl of cDNA (generated by RT) and adjust to 5.0 µl with DEPC H_2O for final reaction volume of 25 µl.
v. Denature the mixture at 92°C for 10 min and carryout amplification at a cycling scheme of 92°C for 1 min, 62°C for 20 s and 72°C for 1 min for 35 cycles.
vi. Electrophoresce aliquots (10 µl) in 2% agarose gels in Tris-borate (TBE) buffer (0.09 M Tris base, 0.09 M boric acid, 0.002 M EDTA, pH8.0); stain the gels, with ethydium bromide at 0.5 µg/ml and analyze using a BIO imaging system (Syngene, Frederick, MD. Apply suitable ladder as a nucleic acid marker.

Appendix 11: Detection of Virus (*Potato virus Y*) by Reverse Transcription – DIAPOPS System (Nicolaisen et al. 2001)

A. RNA Extraction

i. Homogenize plant tissue samples (0.3g) in 0.9 ml of extraction buffer containing 0.2 M Tris–HCl, pH 8.5, 15g/l lithium dodecylsulfate, 0.375 M LiCl, 10g/l sodium deoxychlotate, 1% Igepal CA-630 (Sigma, St. Louis, MO, USA) and 10 mM EDTA in a plastic bag using an electric roller (Bioreba AG, Basel, Switzerland).
ii. Precipitate using 750 µl 5 M potassium acetate, pH 6.5 and centrifuge at 14,000 rpm (20,000g) for 10 min at 4°C.
iii. Add to the supernatant (600 µl) cold isopropanol (500 µl) and centrifuge at 14,000 rpm for 20 min.
iv. Wash the pellet in ethanol 70% and finally resuspend in 20 µl of sterile double-distilled water.

B. Reverse Transcription of RNA

i. For the synthesis of cDNA for 1 h, use 1.4 µl RNA (0.5 µg/l) at 37°C in a final volume of 10 µl containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MyCl_2$, 10 mM dithiothreitol, 0.5 mM of each dNTP, 20 pmol oligo dT_{20}, 4 units of RNasin (Promega, Madison, WI, USA) and 26 units of M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA).
ii. Include RNA from virus-free tissues and RNA-free reactions as negative controls for each experiment.

C. Amplification

i. Coat NucleoLink™ Strips (NUNC A/S) by adding 100 ng of phosphorylated D1 in 100 µl 10 mM 1-methyl imidazole 10 mM 1-ethyl-3-(3-dimethylaminopropyl)-carobodiimide; incubate for 5 h at 50°C and wash seven times with NaOH (0.4 N) followed by washing in distilled water thrice (as per manufacturer's instruction).
ii. Block the strips with bovine serum albumin (BSA, 10g/l in Tris–saline buffer containing 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl and 0.1% Tween-20) for 30 min at room temperature (20°C).
iii. Perform amplification in 27.5 µl using 2.5 µl cDNA in 10 mM Tris–HCl, pH 8.0, 0.1% Tween-20, 50 mM KCl, 1.5 mM MgCl2, 0.15 mM of each dNTP, 0.5 µM R5, 0.6 µM D1 and 0.5 units of *Taq* polymerase (Life Technologies).
iv. Maintain the following cycle conditions: an initial denaturation at 94°C for 2 min, followed by 20 cycles at 94°C, 1 min; 72°C, 1.5 min; 20 cycles at 94°C, 1 min; 65°C, 1 min; 72°C, 1 min and a final extension for 10 min at 72°C, wash the plates in 0.2 N NaOH, 0.1% Tween–20 and then eash with Tris–saline buffer.

D. Detection

i. Perform hybridization with 100 nM biotinylated detection probe R7 (biotin is linked to the probe at the carbon – 5′ of the ribose via a C6 – phosphate linker) in 100 µl 6× SSC (900 mM NaCl, 90 mM sodium citrate), 5× Denhardt's solution (1 g/l Ficoll, 1 g/l polyvinyl pyrrolidone (PVP-360, Sigma Uppsala, Sweden), 1 g/l (BSA) at 50°C for 2 h.
ii. Wash the strips three times with 0.1× SSC, 0.1% Tween-20; incubate at 37°C for 15 min and then wash thrice with the same buffer.
iii. Incubate the stripe with horseradish peroxidase (HRP)-labeled streptavidin (DAKO A/S Glostrip, Denmark) diluted 1:5000 in Tris–saline buffer at room temperature for 1 h; Wash in Tris–Saline buffer.

iv. Add 100 µl TMB substrate (3, 3′, 5, 5′-tetramethyl-benzidine, hydrogen peroxide and incubate for 30 min at room temperature.
v. Stop the reaction by adding 100 µl of 0.1 M H_2SO_4 and determine the absorbance at 450 nm using a microplate reader (Multiskan MCC 1340, Labsystems, Helsinki, Finland).
vi. Maintain duplicates for all samples.

Appendix 12: Detection of *Grape fan leaf virus* (GFLV) in Nematode Vector *Xiphinema index* by RT-PCR (Finetti-Sialer and Ciancio 2005)

A. RNA Extraction from Nematodes

i. Extract the total RNA from nematodes (1–30) by following the single – step RNA isolation method with a monophasic solution of phenol and guanidine isothiocynate (TRIzol) (according to manufacturer's instruction); add 0.2g of acid-washed glass beads (425–600 µm diameter) to nematode suspension (50 µl) in tubes ; vortex for 5 min in the presence of 250 µl of TRIzol and centrifuge.
ii. Dissolve the RNA pellets in 10 µl of diethylpyrocarbonate (DEPC)-treated water and store at −70°C until required.

B. Reverse Transcription–Polymerase Chain Rection (RT-PCR)

i. Design the primers on the basis of highly conserved regions of the sequences and the downstream primer (5′-GCT CCT GCA AAA TTCCCAA-3′) complementary to nucleotide positions 3402–3420 with AMV transcriptase.
ii. Amplify with downstream primer mentioned above and the upstream primer (5′-TATAACCGGATAACTAG-3′) homologous to nucleotide positions 2264–2280 in a 50 µl mixture 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 100 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM MgCl2, 1.25 units of *Taq* polymerase and 500 mM of each primer.
iii. Incubate the mixtures in a programmable thermocycler (Promega Corp, Madison, WI) with the following cycling conditions: 4-min denaturation step at 94°C followed by 35 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 2 min and finish with a 6-min elongation at 72°C.
iv. Analyze the amplicons by electrophoresis in 1.2% agarose gels in Tris-borate-EDTA.

Appendix 13: Detection of *Potato virus Y* by Reverse Transcription Loop-Mediated Isothermal Amplification DNA (Nie 2005)

A. RNA Extraction from Plant Tissues

i. Mix six drops (approx. 200 µl) of tissue sap (extract) with 300 µl of extraction buffer containing 0.1 M Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$ and 0.65% Na_2SO_3 and incubate for 10 min at 37°C.
ii. Extract the mixture with 300 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v/v); collect the aqueous phase after centrifugation (12,000×g) at 4°C for 10 min; add isopropanol (500 µl) and 3 M sodium acetate (50 µl, pH 5.2) and incubate at −20°C overnight.
iii. Collect the precipitate after centrifugation (12,000×g) at 4°C for 15 min; wash the pellet with 70% ethanol and dry under vacuum.
iv. Dissolve the pellet in 200 µl (for tuber tissues) or 1000 µl (for leaf tissues) of sterile water.

B. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

i. Incubate the nucleic acid extract (2.5 µl) for 8 min at 65°C and chill on ice for 3 min.
ii. Add 7.5 µl of RT mixture to provide a final concentration of 20 ng/µl of the reverse primer Y4A (TGGTGTTCG TGA TGTGACCT), 50 mM Tris–HCl, pH8.3, 75 mM KCl, 10 mM dithiothreitol (DTT), 2.5 mM MgCl2, 10 mM of each dNTP (dATP, dTTP, dCTP and dGTP), 5U of RNasin Ribonuclease Inhibitor (Promega Corp., Madison WI) and 100U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega corp) and incubate at 42°C for 1 h and at 95°C for 2 min. Use the resulting cDNA as template for PCR or two-step RT-LAMP.
iii. Perform uniplex PCR with primer pair Y3S (ACG TCC AAAATGAGA ATGCC) on a Peltier Thermal Cycler (MJ Research, Watertown, MA) in a 25 µl volume contouring 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCL), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 ng of each Y35 and Y4A primers, 0.625U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) and 2 µl of cDNA or 20 ng plasmid DNA.
iv. Amplify the samples for 30 cycles of 30 s at 92°C, 30 s at 57°C and 1 min at 72°C followed by a final extension of 10 min at 72°C.
v. Electrophorese amplified product (8 µl) in a 1.2% agarose gel containing ethidium bromide at 0.5 µg/ml.
vi. Photograph under UV illumination with Alpha Innotech IS 1000 imaging system (San Leandro, CA).

C. Loop-mediated Amplification (LAMP) and RT-LAMP

i. Incubate cDNA or plasmid DNA (2 µl at 10 ng/µl at 95°C for 5 min; chill on ice; add 23 µl of LAMP mixture to have a final concentration of 20 mM Tris–HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2$ SO_4 0.1% Triton X-100, 0.8 M Betaine (Sigma-Aldrich, Oakville, Ontario, Canada), 2–10 mM $MgSO_4$, 0.2–1.0 mM each dNTP, 0.2 µM each of one outer forward primer and one outer reverse primer, 0.8 µM each of primer FIP (GTTTGGCGAGGTTCC-ATTTTC) – TGT-GATGAATGGGGCTTATGGT) and BIP (TGAAACCAATCGT TGA GAA TG –ATGTGCCATGAT-TTGGCTAAG) and 1–12U of *Bst* DNA polymerase (New England Biolabs, Beversely, MA).
ii. Incubate the mixture for 1 h at 65°C followed by incubation at 80°C for 2 min.
iii. Analyze the LAMP products (8 µl) by electrophoresis (steps B(v) and B(vi) above).

D. One-Step RT-LAMP

i. Use RNA as template; prepare the reaction mixture of 2.5 µl containing 2 µl of RNA, 100U of M-MLV reverse transcriptase or 1.25U of Avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corp) in addition to the components of LAMP using primers BIP and FIP (see step C(i) above), F3 (ATACGA-CATAGGAGAAACTGA) and B3 (ACGCTTCTGCAACATCTGAG); add also RNasin Ribonuclease Inhibitor (5U) and 5 mM DTT.
ii. If AMV reverse transcriptase is used, incubate the mixture at 65°C for 1.5 h followed by incubation at 80°C for 2 min; if M-MLV reverse transcriptase is used, incubate at 65°C for 5 min and chilled on ice, add the mixture containing the components of RT-LAMP to the denatured RNA solution.
iii. Incubate the solution at 42°C for 1 h and then at 65°C for an additional 1 h followed by 2 min at 80°C.
iv. Analyze reaction products either by gel electrophoresis or spectrophotometrically using a Microplate Reader (Molecular Devices, Sunnyware, Cambridge, UK) at 600 nm.

Appendix 14: Detection of Fruit Tree Viroids by a Rapid RT-PCR Test (Hassen et al. 2006)

A. Extraction of RNA from Plant Tissues

i. Treat leaf and bark tissue samples (0.2g) with liquid N and powder; homogenize the powdered material with 1 ml of 2× sodium chlororide–sodium citrate (SSC) buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 1% sodium sulfite and centrifuge at 16,000×g for 30 min.

ii. Mix the supernatant with 500 µl of 10% nonionic CF-11 cellulose (Whatman, Maidstone, UK); shake for 2 h; collect the cellulose after centrifugation and wash twice with 1 ml of sodium chloride–HCl EDTA (STE) buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0) containing 35% ethanol.
iii. Wash the cellulose twice with 300 µl of STE to release the adsorbed RNA and precipitate with 700 µl of isopropanol and 64 µl of sodium acetate (5 M, pH 5.2).
iv. Dry the pellet in a Speed-Vac (Savant, Farmingdale, NY, USA) and suspend in 30 µl of diethylpyrocarbonate (DEPC)-treated water.
v. Assess the concentration and extraction purities by determining the absorbance of preparations at 260 and 280 nm with a spectrophotometer (Ultrospec II, LKB Biochrom, Cambridge, UK).

B. Preparation of Plant Tissue (Crude Sap) Extract

i. Transfer the pulverized tissue, after grinding the leaf/bark tissues (0.2g) in a mortar with liquid N, to Eppendorf tube (1.5 ml) and mix with 1 ml of extraction buffer 2× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 1% sodium sulfite as an antioxidant.
ii. Collect the supernatant, after centrifugation at 16,000×g for 30 min; prepare different dilutions (1:10, 1:50, 1:100, 1:500, 1:1000) and store at −20°C).

C. Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Assay

i. Adopt one-tube RT-PCR format (Titan Kit, Roche Diagnostics, Penzberg, Germany) to allow RT and amplification to be performed sequentially in the same tube.
ii. Mix samples (either 2 µl aliquot containing 200 ng nucleic acid extract or 2 µl of diluted crude sap extract) with 0.4 µ mol complementary primer; heat the mixture for 5 min at 100°C and chill immediately on ice.
iii. Prepare RT-PCR reaction mixtures (total volume of 25 µl) containing 5 µl of 5× RT-PCR buffer, 1.25 µl of 100 mM dithiothreitol (DTT), 0.5 µl of 10 µM dATP, dCTP, dGTP and dTTP each, 0.4 µ mol of homologous primer, 0.5 µl of enzyme mix (AMV reverse transcriptase and high fidelity *Taq* polymerase) and DEPC-treated water to a volume of 22.5 µl.
iv. Add denatured extract/complementary primer mixture; subject the RT-PCR reaction mixtures to 30 amplifications cycles (1 h at 50°C for cDNA synthesis, denaturing at 95°C for 3 min (first cycle) or 30 s (following cycles), primer annealing at 60°C for 45 s and extension at 72°C for 45 s] followed by a final extension at 72°C for 7 min.
v. Include a water control and extracts from viroid-free fruit trees as healthy controls.

vi. Electrophorese aliquots (10µl) of each amplification product in ethidium bromide-stained agarose gel (2%) in 1× TAE buffer (40 mM Tris-actate, 1 mM EDTA, pH 8.0).

Appendix 15: Membrane BIO-PCR Technique for Detection of Bacterial Pathogen (*Pseudomonas syringae* pv. *phaseolicola*) (*Schaad* et al. 2007)

A. BIO-PCR Technique (Schaad et al. 1995)

i. Spread samples (100 µl) onto each of eight KB and MSP agar plates and incubate for 32–34 h and 48–50 h respectively at 28°C for the development of pin-head size bacterial colonies.
ii. Wash five plates each KB and MSP agar plates three times with 1 ml of water for each washing and pool the washings into a single sample of 15 ml or wash each plate twice with 1 ml of water and keep the washings of each plate separately.
iii. Keep the samples on ice and use 2.8 µl immediately for direct PCR (without extraction of DNA) or store at −20°C.
iv. Incubate the three plates for each medium for an additional 24–48 h for determining mean CFU/ml and/or recovery of the bacterial pathogen.

B. Membrane BIO-PCR Technique

i. Use a specially designed 96-well microplate fitted with surfactant-free 0.45 µ pore size cellulose nitrate filter (Sartorius Corp) to allow the membrane to be flush with the bottom of the plate and allow complete contact with agar medium.
ii. Dispense aliquots of 400 µl of each sample into triplicate wells of each of the two plates and place plates on a 96-well vacuum section device (Whatman Polyfiltronics) to remove the liquid and repeat the procedure after adding two additional 400 µl aliquots so that 1.2 ml of the sample may be filtered through each well.
iii. Spread 100 µl of each dilution onto each of five plates of KB and MSP agar plates and incubate under the same condition to determine mean CFU/ml of each sample.
iv. After filtering the samples, place one of the 96-well plates onto KB or MSP soft agar in the original sterile plastic container; slightly tap to allow contact with agar and place the lid to keep the plate sterile.
v. Alternatively, place the plates onto soft agar in a 20 × 40 cm Pyrex baking dish and cover with aluminimum foil.
vi. After incubation for 32–34 h for KB and 48–52 h for MSP, wash each well of the microtitre plate with 200 µl of sterile water using a multi-channel micro-pipette.
vii. Transfer the sample to a microfuge tube for PCR or store at −20°C.

viii. Use the second 96-well plate for membrane PCR without incubating on agar media.
ix. Wash each well and use 12.8 µl for the real-time PCR.

Appendix 16: Detection of Bacterial Pathogens by DNA Array Technology (Fessehaie et al. 2003; Scholberg et al. 2005)

A. DNA Extraction from Bacterial Cutlures

i. Harvest bacterial cells of *Erwinia* spp., *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) and *Ralstonia solanacearum* (*Rs*) in late log phase.
ii. Extract the genomic DNA from 50 to 100 mg fresh weight of cells using a commercial DNA Purification Kit (BIO 101, LaJolla, CA) as per manufacturer's instruction and elute DNA in 100 µl volumes and store at −20°C until required.

B. PCR Amplification

i. Amplify the DNA extracted from the bacterial pathogens using PCR in 25 µl volumes containing 10–20 ng of DNA, 1× buffer (75 mM Tris, 20 mM $(NH4)_2SO_4$, 0.01 Tween 20), 1.6 mM $MgCl_2$, 1.5 mM dNTP mix, 0.4 µM each, forward and reverse primers specific to the pathogen(s) and 1 unit of Ultra Therm DNA polymerase (TetraLink International, Buffalo, NY).
ii. Add 1.5 mM concentration of Dig-dUTP mix to the reaction mixture to label the PCR product with digoxigenin (Dig) (Roche Diagnostics GmbH, Mannheim, Germany) and use appropriate primers to amplify the DNA.
iii. Perform amplification on a GeneAmp 2400 thermal cycler (Applied Biosystems, Foster City, CA) with variable cycle conditions depending on the primers used: 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min and a final extension cycle of 72°C for 7 min.
iv. Purify the amplification products using Qiaquick PCR purification kit (Qiagen Inc., Mississauga, ON, Canada) for removing unused PCR reagents.
v. Determine the concentration of amplification products on a 1.5% agarose gel with Low Mass Ladder (Invitrogen, Gaithersburg, MD).

C. Selection of Oligonucleotides

i. Select taxon-specific oligonucleotides from the sequences of ribosomal DNA genes-from 16S to 23S rDNA IGS regionsof *Erwinia* spp., from 16S to 23S rDNA sequences of *Cms* (to serve as immobilized ologonucleotides within the

array) and sequences of species-specific oligonucleotide probes for *Rs* based on GenBank data.

ii. Design the selected nucleotides for optional and uniform hybridization kinetics using Oligo 6.3 software (1998, Molecular Biology Insights, Inc., West Cascade, Co) and generate C6-amino – terminated oligonucleotides with a DNA synthesizer (Beckman Oligo 1000;Beckman Instruments Inc., Fullterton, CA).

D. Preparation of the DNA Array

i. Prepare a template of 16- to 24-mer amino-terminated oligonucleotides in the required array format and dilute the DNA oligonucleotides from 200 µM stock to 40 µM sodium bicarbonate buffer (0.4 µM, pH 8.0) in a sterile 384-well microplate.

ii. Spot the oligonucleotides with a VP384 multi-Blot Replicator (V&P Scientific Inc., San Diego, CA) in three rows of 18 on Immunodyne ABC membranes (PALL Europe Limited, Portsmouth, England) cut into 3 × 9 cm strips; spot the duplicates of the same oligonucleoides on the diagonal by repeated printing and air-dry the printed membranes for 10 min.

iii. Transfer the membranes into blocking solution [2× standard saline citrate (SSC) (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate)] 0.5% casein (BDH Biochemical, England] and 0.05% Tween 20) and agitate for 15 min.

iv. Wash the blots in 2× SSC for 30 min; store short-term in 2× SSC or air dry and keep them in a envelope at room temperature for long-term storage.

E. Hybridization to DNA Arrays

i. Prehybridize the membranes, before use, for 1.5 h with hybridization buffer (6× SSC, 0.1% N-lauroyl-sarcosine (Sigma, L4509), plus 1% casein (BDH Biochemical, England).

ii. Denature dig-dUTP-labeled DNA target in boiling water for 10 min; perform hybridization overnight at 54°C with 6 ml of hybridixation buffer containing approximately 60 ng of digoxigenin dUTP-labeled DNA target per membrane; and wash twice with stringency buffer (4× SSC, 0.1% SDS) at hybridization temperature.

iii. Use anti-Dig alkaline phsophatase conjugate (Roche Diagnostics GmbH, Mannheim, Germany) and the chemiluminescent substrate CDP-Star (Roche Diagnostics) as per the manufacturer's instructions.

iv. Perform multiple exposures of sealed membranes for 15–120 min on film (X-Omatic K, Kodak, Rochester, NY) on the day after adding the CDP-Star.

v. Select films that show the maximum number of dots before the background becomes too dark (>45 min exposure) for scanning and analyses and deter-

mine gray values for each spot, using Genepix Pro 3.0.6 (AXON Instruments, INC., CA).

Appendix 17: Extraction of Genomic DNA from Fungal Pathogens (*Phytophthora* spp.) (Lamour and Finley 2006)

A. Cultivation of Fungal Pathogens and Disruption

i. Multiply the pathogen (*P. sojae*) in appropriate medium kept in petriplates and gently scrap the mycelium from the top surface of the medium.
ii. Load 24-deepwell (DW) Uniplate microtiter plates (Whatman Inc., Clifton, NJ) containing 10 ml wells, at the rate of 1 ml PARP-V8 broth [containing pimaricin 25 ppm, ampicillin 100 ppm rifampicin 25 ppm and pentachloronitrobenzene (PCNB) 25 ppm); seed the wells with wefts of mycelium scrapped from fungal culture plates; cover the plates with rayan breathable tape and incubate the plates at room temepature for 6 days.
iii. Harvest the fugnal colonies from the deepwells; transfer them into a 96-well 2 ml DW plate containing three 3 mm glass balls per well; use a Millipore dry dispensing plate to dispense the glass balls prior to transferring fungal colonies cover plates with Aeraseal rayon breathable tape (PGC Scientifics, Frederick, Ma) and freeze the contents at −80°C for at least 1 h.
iv. Lyophilize the samples for a period of 48 h; use the Labconco stoppering tray drying systems (STDS) (Labconco Corp., Kansas City, Missouri) with incubation chamber at 0°C for 24 h, followed by 24 h with incubation chamber at 23°C.
v. Remove the samples from chamber and immediately apply a capmat to deepwell plates with a capmat applicator (CMA) (Fisher Scientific).
vi. Disrupt the samples with MM300 for a total of 2 min on the highest setting of 30 rpm; rotate the 96-well deepwell plate 180 degrees, after bashing 1 min and bash again an additional min.

B. Extaction of DNA (Adaption of QIAGEN DNeasy 96 Plant Kit)

i. Centrifuge the plates containing pulverized dried mycelium at 4600g for 5 min and remove the capmats carefully.
ii. Add a total of 400 µl of lysis cocktail containing 100 mM Tris (pH 8.0), 50 mM EDTA, 500 mM NaCl, 1.33% SDS with 0.8% Fighter F antifoaming agent (Lovel- and Industries, Greely, Colorado) and 0.2 mg/ml RNase A to each well using the Apricot and apply a new capmat.
iii. Agitate vigorously by inverting the plate 5–10 times and incubate them in a 65°C chamber for 20 min.
iv. Centrifuge the plates at 4600g for 2 min; gently remove the capmat; add 150 µl of 5 M potassium acetate using the Apricot and apply a new capmat.

v. Agitate the inverted plates vigorously 5–10 times; incubate at −20°C for 30 min to overnight and centrifuge the plates at 4600g for 30 min.
vi. Transfer 400 µl of the supernatant to a new 2 ml DW plate containing 600 µl of a 0.66 M guanidine hydrochloride and 63.3% ethanol solution using the Apricot (Take care while handling guanidine hydrochloride which is a dangerous irritant and use eye protection mask) and apply a new capmat.
vii. Agitate the plates as done earlier to mix the solutions; transfer 1 ml of the mixture to a Nunc spin column plate (Nalge Nunc Int., Rochester, NY) sitting on a 2 ml DW plate and centrifuge at 4600g for 5 min.
viii. Discard the flow through; wash the membrane by adding 500 µl wash solution consisting of 10 mM Tris (pH 8.0), 1 mM EDTA, 50 mM NaCl and 67% ethanol and centrifuge at 4600g for 5 min.
ix. Wash the membrane again by adding 500 µL of 95% ethanol; centrifuge at 4600g for 5 min and incubate the spin column plate at 65°C for 10 min to dry the membrane.
x. Add 200 µl of 10 mM Tris (pH 8.0) to each well using the Apricot and incubate plates at room temperature for 30–60 min.
xi. Elute the DNA into a clean 1 ml DW plate by centrifuging at 4600g for 2 min and assess the quality of DNA by separation on a 1% agar gel.

Appendix 18: Detection of *Mycosphaerella graminicola* in Wheat Using Reverse Transcription (RT)-PCR (Guo et al. 2005)

A. Extraction of Total RNA

i. Homogenize samples of fresh mycelium or wheat leaves (2g) to powder in liquid N; add 2.5 ml of homogenization burfer containing 0.9 NaCl and 0.6g insoluble PVP-36 and grind and transfer to prechilled centrifuge tubes (50 ml).
ii. Add 5 ml of prewarmed extraction buffer and mix well by vortexing.
iii. Elute the mixture 2 or 3 times with 5 ml acidic phenol (pH 4.5–5.0); chloroform: isoamylalcohol (25:24:1); precipitate the supernatant with $\frac{1}{4}$ volume of 10 M LiCL overnight at 4°C and centrifuge.
iv. Wash the pellet with 1 ml of 2 M LiCL followed by washing with 70% ethanol twice; dry in vacuum and resolve in 100 µl diethylpyrocarbonate (DEPC)–treated water.

B. One-Step RT-PCR Assay

i. Use a commercial kit (Access RT-PCR Introductory System-Promega, Madison, WI, USA); employ specific primers (E1 and STS2R).
ii. Treat total RNA with DNase (Promega) as per the manufacturer's instructions.

iii. Use the reaction mixture; 500 pg to 2 µg total RNA, 10 mM Tris–HCl (pH 8.8), 3 mM MgCl2, 0.3% (v/v) Triton X-100, 30 pmol of each primer, 200 µM of each dNTP, 5U Avian Myeloblastosis Virus (AMV) reverse transcriptase and 5U *Tfl* DNA polymerase in a final volume of 50 µl.
iv. Provide the following reaction conditions: 45 min at 48°C, 2 min at 94°C, 40 cycles of 30 s at 94°C, 1 min at 60°C and 2 min at 68°C, followed by a final step of 7 min at 68°C.
v. Analyze 20 µl of RT-PCR product on a 2% ethidium bromide-stained agarose gel.

C. Two-Step RT-PCR Assay

i. Synthesize the first strand of cDNA using the following reaction components; 2 µg total RNA of pathogen, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM 1,4-dithiothreitol (DTT), 500 µM dNTP, 25U rRNasin® Ribonuclese inhibitor (Promega, MA, USA), 40 pmol of E1 and STS 2R primers and 100U M-MLV reverse transcriptase (Promega) in a final volume of 25 µl.
ii. Incubate the reaction mixture at 42°C for 60 min and dilute the cDNA solution in a concentration series equal to 100 ng, 10 ng, 1 ng, 100 pg, 50 pg, 10 pg, 5 pg and 1 pg total RNA respectively.
iii. Add to the 25 µl PCR mixture consisting of 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl2, 200 µM of each dNTP, 20 pmol of each primer and 1U *Taq* DNA polymerase (Promega).
iv. Analyze 20 µl PCR product on a 2% ethidium bromide-stained agarose gel.

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Chapter 3
Molecular Variability of Microbial Plant Pathogens

Abstract Microbial plant pathogens are known to exist in the form of different strains, varieties, races or biotypes that differ in their pathogenic potential and host range. It is necessary to determine the influence of genetic and environmental factors that favor disease incidence and spread. The gene-for-gene relationship, phenomenon of avirulence, production of new strains and selection of certain strain(s) due to environment and agricultural production conditions have to be studied, in order to plan effective disease management strategies. Identification of strains or races based on biological properties or pathogenicity using sets of differential hosts or crop varieties requires long time and large greenhouse space. The usefulness and applicability of molecular methods for assessing the extent of variability in pathogenicity of fungal, bacterial and viral pathogens is discussed with appropriate examples.

The ability of a microorganism to infect a plant species depends on the presence of pathogenicity genes (or virulence factors) in the microbe and the genes controlling susceptibility in the plant species. This type of interaction is known as compatible. The gene-for-gene relationship first defined for flax rust and proposed by Flor (1946) has been studied intensively in several host-pathogen interactions. A pathogen may be avirulent (inability to infect) on a plant species, if it has an avirulence factor matching a specific host resistance factor. This kind of interaction is termed incompatible, since there is a matching pair of resistance (in host plant) and avirulence factor (in the pathogen). Variations in characteristics required for the pathogenenicity which distinguish the pathogens from saprophytes have been observed. There is an imperative need to understand the influence of genetic and environmental factors that favor the infection of plant hosts by pathogen (s) and the development of disease(s). The knowledge of this basic information will be required to plan effective disease management systems.

In a compatible interaction, the intensity of infection (infection type) may vary depending on the virulence of the pathogen and the host genotype. A set of host varieties (differential varieties) with different specific resistances has to be inoculated to determine differences in the virulence of pathotypes. Likewise, the pathogen may be able to infect a range of host plant species or it may infect only a few plant species.

P. Narayanasamy, *Molecular Biology in Plant Pathogenesis and Disease Management: Microbial Plant Pathogens, Vol. 1*.

This host range (wide or narrow) of a pathogen is a critical factor that determines its survival in the absence of crop hosts. The expression of specific resistances has been reported to be influenced by environmental factors. The specific resistance of wheat cv. Axona to *Erysiphe graminis* f.sp. *tritici* causing powdery mildew disease was expressed only under a set of day/night temperature regime (Clarkson and Slater 1997). The pathogenic potential also is markedly affected by environmental conditions. The molecular variability of the pathogens contributing to successful infection or failure is discussed in this chapter.

In the studies related to microbial pathogen variability, molecular markers have been applied primarily (i) to follow the progress of disease and associated pathogen populations and their genes, (ii) to study the heritability of genes and (iii) to prepare genetic maps-location, isolation and characterization of genes and their products, especially those controlling pathogenicity, host-specificity and resistance to chemicals. Two types of marker systems have been followed: (i) dominant and (ii) codominant markers. The dominant markers are derived from random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP). These markers give complex patterns of bands in which each band can be considered to represent a locus. If a band is amplified in some, but not in all individuals, it is polymorphic. In the case of codominant markers such as random fragment length polymorphism (RFLP) and simple sequence repeats (SSRs), each allele is revealed as a unique band (SSR) or number of bands (RFLPs). Codominant markers are considered to be better for population studies, since populations of pathogens can be distinguished and the extent of gene flow may be determined more precisely.

Among the applications of molecular markers, identification of genotypes (biotypes, races, strains, varieties, pathovars and subspecies) by molecular tests may effectively replace conventional pathogenicity tests. For the development of an informative marker, the first step is to survey a large number of DNA fragments. As a preliminary step, RAPD and AFLP techniques are suitable. A marker that can reliably discriminate desired groups is selected and cloned. The selected marker may be used as a probe or in an RFLP system. It is commonly sequenced, at least at the ends of the clone, so that specific PCR primers that amplify the informative DNA fragment can be designed. The RFLP or PCR marker may be used to study population variation in host range. Banding patterns obtained with RAPDs or AFLPs can be used for determining relatedness between pathogen isolates. DNA polymorphism analysis through use of molecular markers has been responsible for significant advancement in inheritance studies. It is possible to saturate genomes rapidly with molecular markers. Conventionally, RFLPs were used initially as markers, then came the use of RAPDs. AFLPs and SSRs are now preferred. Marker methods which exploit the remarkable advantages of polymerase chain reaction (PCR) are more frequently employed.

3.1 Molecular Basis of Variability of Fungal Pathogens

Molecular techniques have been demonstrated to be useful for detection (Volume 1, Chapter 2) and differentiation of microbial plant pathogens. An understanding of

the pathogen genetics and its population dynamics is essential for predicting the sustainability of crop disease management strategies. The usefulness and the feasibility of applying marker technology to understand the molecular basis of variability of some of the selected destructive fungal pathogens that cause economically important crop diseases is highlighted.

3.1.1 Isozyme Variation

Phytophthora infestans, the causative agent of late blight disease was considered to be the primary cause for the Irish potato famine due to which millions of humans perished and many more migrated to other countries. *Phytophthora* has a tremendous range of mechanisms for creating and maintaining genetic diversity. The knowledge of spatial and temporal distribution of A1 and A2 strains (mating types) of *P. infestans* is the basic requirement to study the generation and maintenance of genetic diversity and to determine the disease aetiology. *P. infestans* is heterothallic and the sexual spores (oospores) are formed, when the mating types A1 and A2 are brought together.

The enzymes produced by the pathogen are separated by electrophoresis in a horizontal starch gel. Isozyme patterns are recorded according to their relative mobility and each band is considered as an allele to specific locus. The bands are then labeled alphabetically from the slowest to the fastest. Isozyme analysis is less expensive, yielding data amenable for population genetic analysis. However, despite testing many isoenzymes, only glucosephosphate isomerase could offer useful differentiation of isolates of *P. infestans* for large scale application (Spielman et al. 1990; Fry et al. 1992; Goodwin 1997). Based on the isozyme analyses, *Phytophthora cambivora*, *P. cinnamomi* and *P. cactorum* were distinctly separated and each species could be further subdivided into electrophoretic types (ETs). Three enzymes-phosphoglucose isomerase, malate dehydrogenase and lactate dehydrogenase – when fractionated by cellulose acetate electrophoresis appear to possess diagnostic potential, permitting clear differentiation of these three species. The studies to establish intraspecific diversity and interspecific relatedness of different papillate species of *Phytophthora*, revealed that *P. medii* and *P. botryosa* clustered together, indicating a very close genetic relatedness. *P. kasturae* and *P. heveae* also formed a single cluster. while *P. capsici* and *P. citrophthora* formed another distinct cluster (Oudemans and Coffey 1991a, b).

Allozyme genotypes of *Phytophthora infestans* could be resolved precisely by using cellulose acetate electrophoresis (CAE), at two loci glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*). The rapidity and accuracy of CAE system in predicting mating types and metalaxyl sensitivities of the genotypes existing under field conditions are the distinct advantages. The CAE system required only 15–20 min as against 16–18 h needed for starch gels (Goodwin et al. 1995). Isolates grouped into eight genotypes based on variations in allozyme banding patterns at two loci *Gpi* and *Pep* with markers for mating type, cultural morphology and metalaxyl sensitivities. Five of the genotypes existing in Canada exhibited similarities to the genotypes

[US-1, US-6, US-7, US-8 and g11 (or US-11)] present in the United States. Significant correlations among characteristics considered were also seen (Peters et al. 1999). Another investigation revealed that 85 isolates of *P. infestans* obtained from tomato and potato fields in North Carolina, belonged to four different allozyme genotypes at *Gpi* and *Pep* loci (Fraser et al. 1999). *P. infestans* isolates (401) were collected from Asian countries China, India, Indonesia, Korea, Nepal, Taiwan and Thailand. Polymorphisms of three isozymes viz., glucosephosphate isomerase (*Gpi*: EC 5.3.7.9), peptidase (*Pep*: EC 3.4.3.1) and malic enzyme (*Me*: EC 1.1.1.40) were determined. Three genotypes (*Gpi*, *Pep* and *Me*) were identified in the A1 and A2 mating type isolates from Japan (Nishimura et al. 1999; Gotoh et al. 2005).

Differentiation of highly virulent and weakly virulent strains of *Leptosphaeria maculans* causing canola (*Brassica napus*) black leg or stem canker disease was possible based on isozyme analysis. The isozyme glucose phosphate isomerase (*Gpi*) present in 68 of the 92 isolates of *L. maculans* moved 70 mm in starch gel after 11 h of electrophoresis, whereas the isozyme *Gpi* of other isolates moved only 65 mm. Thus the isolates could be classified into two electrophoresis types (ET) 1 or 2 reflecting fast or slow movement of *Gpi*. The isolates with fast moving *Gpi* were highly virulent and belonged to ET1 group, whereas the weakly virulent isolates with slow isozyme constituted the ET2 group (Sippell and Hall 1995). In another study, the possibility of employing the GPI electrophoresis on starch gels for reliable detection and differentiation of *L. maculans* from *Pseudocercosporella capsellae* was demonstrated. The electrophoresis was performed directly on the extracts of leaf lesions induced by the fungal pathogens. Four ET patterns ET1, ET2, ET3 and ET4 of allozymes were recognized. ET1 and ET2 were present in highly virulent and weakly virulent isolates of *L. maculans* respectively, while a few typical and atypical leaf lesions induced by *L. maculans* yielded ET3 pattern. The lesions caused by *P. capsellae* had the fastest ET4 allozyme, thus providing a reliable basis for the differentiation of these two pathogens (Braun et al. 1997). The intraspecific population diversity in fungal pathogens, such as *Colletotrichum gloeosporioides*, infecting a range of plant species causing anthracnose diseases was studied by assessing isozyme variations. The isozymes of nicotinamide adenine dinucleotide dehydrogenease (NADH) and diaphorase (DIA) produced maximum number of electrophoretic phenotypes that clustered on the basis of host origin. Three major ETs (I, II and III) and four sub groups (IA, IB, IIIA and IIIB) of *C. gloeosporioides* were differentiated (Kaufmann and Weidemann 1996).

3.1.2 Immunological Assay

Gibberella zeae (anamorph-*Fusarium graminearum*) causing head blight or scab disease of wheat and barley is responsible for serious losses in grain yield, in addition to health hazards due to contamination of grains with the mycotoxins produced by this pathogen. Further, there is a close relationship between aggressiveness of and production of deoxynivalenol (DON) by *F. graminearum* and *F. culmorum*

(Miedaner et al. 2000; Mesterhazy 2002). The isolates show variations in host colonization which was found to be predictive and a sensitive indicator of the presence of DON in the infected plant tissues (Lamper et al. 2000). An immunoassay was developed based on exoantigens (ExAgs), a soluble mixture of extracellular fungal products by Kaufman and Standard (1987). The contents of EXAg formed the basis for measuring fungal biomass within host tissues. Later a linear correlation between ExAgs of *F. graminearum* as determined by an indirect ELISA, and DON contents was observed. The progenies (50) from a segregating population of *G. zeae* were inoculated onto a susceptible winter wheat cultivar. Two ELISA formats were applied for determining fungal colonization, ExAg contents and DON production. Significant genotypic variation was observed for all traits studied. Correlation between DON production and ExAg content across environments (year-location combinations) was high ($r = 0.8$, $P = 0.01$) (Cumagun et al. 2004).

3.1.3 Dot-Blot Hybridization Assay

Rhizoctonia solani, a soilborne pathogen has a wide host range including rice and several vegetable crops which suffer from root rot and damping-off diseases. A plasmid DNA fragment designated PE-42 hybridized to DNA of all isolates of *R. solani* AG-2-2-IV inducing large patch disease of *Zoysia* grass. There was no hybridization to the DNA of other pathogens infecting *Zoysia*, indicating the specificity of reaction between the probe and target DNA. As a marker employed in the Southern hybridization, the PE-42 plasmid DNA fragment could distinguish *R. solani* AG-2-2-IV from other intraspecific groups of *R. solani* in addition to its ability to be used for the diagnosis of large patch disease infecting *Zoysia* grass (Takamatsu et al. 1998). A specific DNA probe (pG158) exhibited differential hybridization intensity. Hybridization of pG158 with pathogenic isolates of *Gaeumannomyces graminis* var. *tritici* was strong, while it was moderate with *G. graminis* var. *avenae*. There was no hybridization at all to nonpathogenic isolates of *G. graminis* var. *tritici* (causing wheat take-all disease) and other soil fungi. A basic requirement to relate the population of soil fungi to incidence of wheat take-all disease is the ability to differentiate pathogenic isolates from the morphologically similar nonpathogenic isolates. The use of pG158 as a marker for intraspecific differentiation of *G. graminis* isolates was shown to be a reliable technique to assess the population of pathogenic isolates (Harvey and Ophel-Keller 1996).

3.1.4 Restriction Fragment Length Polymorphism

The restriction fragment length polymorphism (RFLP) method is used to assess the extent of natural variations in the genomes of different species, biotypes, strains or races of fungal pathogens. Deletions of or insertion in the DNA sequences may lead to variations (polymorphisms) in fragment sizes. A specific set of fragments,

considered as a fingerprint for a strain/species, may be formed following digestion with desired restriction enzymes that cleave the genome at specific sties. The specific sites may be identified by Southern blot analysis or viewed directly by staining the gels with ethidium bromide, under UV light. The RFLP patterns may be helpful in determining the genetic diversity of the pathogen population and also in estimating the extent of relatedness of the pathogen groups/strains.

The sensitivity and reliability of the assay may be enhanced by employing highly repetitive DNA sequences as probes, as the signal is present in multiple copies. Repetitive DNA fragments of 12 species of *Phytophthora*, in agarose gels appeared as continuous discrete bands over a faint smear, when stained with ethidium bromide. However, different species of *Phytopthora* showed different patterns. *P. cryptogea* and *P. dreschleri* which are morphologically similar, could be differentiated by the repetitive DNA profiles. The DNA profiles of *P. megasperma* indicated its heterogenous status, whereas the complete homogeneity of 12 isolates of *P. parasitica* was revealed by the DNA profile analysis. The taxonomic problems associated with *Phytophthora* spp. may be resolved by the DNA profile analysis, since the method is relatively sample (Panabieres et al. 1989; Drenth et al. (2006)).

By employing the moderately repetitive RFLP probe RG57, a fingerprint of 25–29 bands was produced from the DNA of *P. infestans*. The RFLP technique has been found to be a valuable tool for monitoring the genetic diversity of this pathogen. Finger-printing of many thousands of isolates and establishment of an international database of the results of RFLP analysis have been useful for significant advancement in understanding the population dynamics of this destructive pathogen (Forbes et al. 1998; Zwankhuizen et al. 2000). However, the requirement of large amounts of pathogen DNA, long time required, in addition to difficulty to interpret the data are the disadvantages of this technique (Cooke and Lees 2004). RFLP analyses of 125 isolates using the RG57 probe were performed by digesting the pathogen DNA with *Eco* RI followed by agarose gel electrophoresis. Twenty different RG57 fingerprints were recognized. The fingerprints of the isolates occurring in the Asian countries were closely similar or identical with those of isolates such as Japanese A1-A and Japanese A1-B, JP-1, US-1, OS-1.2 and US-1.3 (Gotoh et al. 2005).

RFLP analyses of both mitochondrial and nuclear DNA of *Phytophthora capsici* were performed on isolates from widely different geographical locations, years and hosts located in a worldwide collection. No patterns of similarity in mtDNA could be discerned based on host or geographical location. But RFLP analysis of nuclear DNA using a lowcopy number probes of 15 *P. capsici* isolates indicated high nuclear DNA diversity. The study was useful to better understand how natural populations of *P. capsici* were distributed in space and time (Förster et al. 1989). Isolates of *Phytophthora parsitica* var. *nicotianae* causing tobacco black shank show distinct variation, in their ability to produce elicitin (TE) which is known to induce initiation of resistance reaction in tobacco. The isolates producing elicitin (TE^+) have low virulence on tobacco, but pathogenic on tomato. In contrast, TE^- isolates, that do not produce elicitin are generally highly virulent and specialized to

tobacco. The TE^+ and TE^- isolates could be differentiated by RFLP analysis of both mitochondrial and nuclear DNA. Loss of the ability to produce elicitin may possibly lead to increased virulence and this may be an important factor in the development of effective crop disease management systems (Colas et al. 1998).

A genotype-specific *Eco* RI RFLP profiles was constructed by using the probe specific for a dispersed repeated DNA sequence (called MGR). The MGR-DNA fingerprints for the field isolates of *Magnaporthe grisea* causing rice blast disease prevalent in the United States, were prepared. The MGR-DNA fingerprints served as the basis of differentiating major pathotypes of *M. grisea*, identifying the pathotypes accurately, and defining the organization of clonal lineages within and among pathotype groups (Levy et al. 1991). The RFLPs in nuclear DNA were reported to have some relationship with the prevailing races of *M. grisea* in Korea. However, there was no clear cut relationship between RFLP in nuclear DNA and virulence of *M. grisea* (Ko et al. 1993). The genetic relationships among isolates of *Pyricularia grisea* from rice and other hosts were analysed by RFLP analsyis by using the repetitive probe MGR 586. Rice blast isolates were grouped by using set of differential rice varieties into four distinct races. These isolates produced multiple bands hybridizing to the probe MGR 586 (Han et al. 1995). The discovery of the MGR DNA sequence provided a powerful method for studying the genetic variation in *M. grisea*. This repeated DNA element was found in 30–50 copies in rice pathogen, but only in one or two copies in other *M. grisea* host-limited forms. The common belief that new rice blast pathogens evolved from isolates infecting grass hosts found at the edges of rice fields was proved to be incorrect. Grass pathogens generally have one or two copies of MGR 586, indicating that they are a distinct population and not closely related to rice pathogens. On the other hand, *M. grisea* strains pathogenic to wheat (found in Brazil) had only a low copy number of MGR 586 suggesting that they would have evolved from grass pathogenic forms of *M. grisea* rather than rice pathogens (Talbot 1998).

The applicability of the results of RFLP analysis in fungal taxonomy has been brought out by several workers (Coddington et al. 1987; Förster et al. 1987; Manicom et al. 1987; Klich et al. 1993). Genomic DNA RFLPs combined with random probes could be employed for differentiating species, formae speciales races and isolates of *Fusarium* (Kim et al. 1993). The genetic similarity of the isolates (39) of *Fusarium oxysporum* encompassing five formae speciales that infect cucurbits causing vascular wilts was assessed. The total pathogen DNA was subjected to digestion by endonucleases (*Pst* I, *Hind* III and *Eco* RI) followed by Southern blotting and hybridization with a mtDNA polyprobe from *F.o. niveum*. Unique RFLP patterns for each formae specialis was identified. *F.o. cucumerinum* exhibited maximum genetic divergence, while it was the least in the case of *F.o. niveum* (Kim et al. 1993). Total DNAs of 28 isolates of *F. oxysporum* that infected different plant species were digested with restriction enzymes. A probe (p449) derived from a 3.38-kb mtDNA fragment obtained from *F.o*. f.sp. *cubense* causing banana wilt disease was employed to determine the RFLP patterns in test isolates. Mitochondrial DNA polymorphisms within and between different formae speciales were discernible (Bridge et al. 1995).

Cotton wilt pathogen *F. oxysporum* f.sp. *vasinfectum* is a forma specialis which has been subdivided into pathotypes or races defined by differential pathogenicity to differential cotton varieties. At least 12 vegetative compatibility groups have been identified within *F.o*. f.sp. *vasinfectum*. Molecular techniques have been applied to demonstrate the presence of the two mating type (MAT) idiomorphs in *F.o*. f.sp. *vasinfectum* and other formae speciales (Arie et al. 2000). The MAT idiomorphs were used as a molecular marker to identify the trait. Seventeen isolates of *F.o*. f.sp. *vasinfectum* were characterized using RFLP of the ribosomal intergenic spacer (IGS) region. Seven different combinations of patterns representing seven IGS types were identified among the 46 isolates. IGS type 14 was the most abundant, as they were found in 19 of the 46 isolates analyzed (Abo et al. 2005).

Fumonisins are a group mycotoxins produced by different *Fusarium* spp. and they have shown to be toxic to humans and animals. DNA-based strategies have been applied to detect and differentiate the fungal species or strains with potential to produce fumonisins. Different approaches to develop reliable genetic markers and diagnostic assays to detect toxigenic species of *Fusarium* such as (i) the use of nuclear genetic markers unrelated to toxin biosynthesis and (ii) the use of genes related to toxin production have been attempted. A combination of PCR-RFLP methods applied to variable regions of genes (introns) or to the internal transcribed spacer (ITS) and intergenic spacer (IGS) of the rDNA units has been frequently used, due to its simplicity and reproducibility (Donaldson et al. 1995; Edel et al. 1997; Mirete et al. 2004). Attempts were made to identify specific diagnostic sequences for the detection of trichothecene-producing *Fusarium* spp. Some of the studies indicated cross-reaction with closely related species/strains, making the results unreliable (Edwards et al. 2001, 2002). In a later investigation, two sets of primers based on the IGS sequence, VERT-1 and VERT-2 were employed for specific detection of strains of *F. verticillioides.* The second set of primers VERTF-1 and VERTF-2 detected those *F. verticillioides* isolates that produced fumonisins (Patiño et al. 2004).

The relationship of two host-adapted pathotypes of *Verticillium dahliae*, causing wilt diseases in different crops like cotton and peppermint was examined by RFLP analysis. The isolates obtained from and adapted to peppermint constituted a subgroup (M) distinct from the non-host adapted subspecific group A of *V. dahliae.* Another group (D) was formed to enclose isolates of *V. dahliae* from cruciferous host plant species. The isolates infecting crucifers were distinguishable based on the variation in polymorphisms by employing two specific probes (Okoli et al. 1994). The complete intergenic spacer (IGS) region of the nuclear rRNA gene (rDNA) and the β-tubulin gene were amplified and sequenced. The sequences of the complete IGS region and the β-tubulin gene alone or in combination formed the basis for determining the genetic relationships among different isolates of *Verticillium* spp. Four distinct groups comprising isolates of *V. albo-atrum*, *V. tricorpus* and *V. dahliae* from cruciferous and noncruciferous hosts were recognized. Isolates of *V. dahliae* from cruciferous hosts exhibited the closest affinity to *V. dahliae* from noncruciferous hosts. In addition, isolates of *V. dahliae* from noncruciferous hosts could be further subdivided into four groups based on the sequence similarity within the IGS region (Qin et al. 2006).

The grapevine endophyte *Phaeomoniella chlamydospora* is the most important pathogen associated with "esca" (decline) disease in Europe and North America. Genetic variation analysis of New Zealand and Italian strains of *P. chlamydospora* detected a potential molecular marker in New Zealand isolate A21. Of the 53 isolates of *P. chlamydospora* tested, the primer 3-2 produced a bright, clear and robust 1-kb amplimer only in isolates A21 (Fig. 3.1). Characterization of the 1010 bp marker band revealed that it had 50% identity to *moxY*, a gene involved in the aflatoxin biosynthetic pathway of *Aspergillus parasiticus*. Amplification of a 950-bp region of 1-kb marker band indicated that the DNA fragment was present in all *P. chlamydospora* isolates. Amplification of the regions flanking the marker band determined that a single nucleotide polymorphism in the 3′ binding site for UP-PCR primer 3-2 had generated the unique band (Ridgway et al. 2005). The genetic structure of *Pyrenophora graminea* causing barley leaf stripe disease was studied. Genetic variation in the Syrian populations of *P. graminea* was high. Of the 366 scorable DNA bands, 290 bands were polymorphic and the genetic distances among all isolates ranged from 0.01 to 0.74 with a mean of 0.29 (Jawahar and Arabi 2006).

Various species of *Colletotrichum*, cause anthracnose diseases in several crops such as banana, mango, strawberry, papaya and avocado which suffer heavy losses. Variations in strains of *C. acutatum*, *C. fragariae* and *C. gloeosporioides* isolated from infected strawberry plants were determined by rDNA and mtDNA restriction patterns. The rDNA restriction patterns were generated by digesting with the restriction enzyme *Eco*RI. The strains of *C. acutatum* and *C. fragariae* could be separated into four and two groups respectively, whereas all strains of *C. gloeosporioides* produced an identical pattern with each of the four enzymes used. RFLP patterns

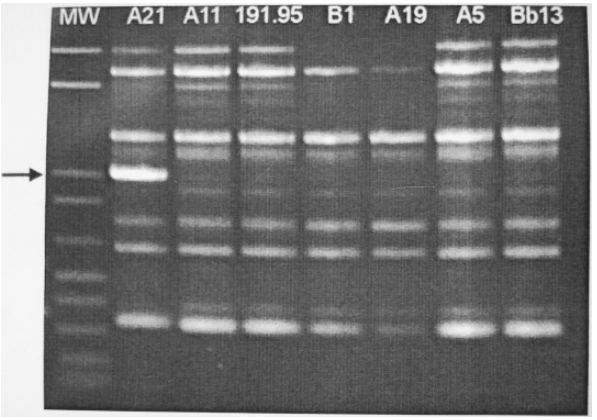

Fig. 3.1 Identification of a unique polymorphic band (indicated by arrow) only in the isolate A21 of the endophyte *Phaeomoniella chlamydospora* infecting grapevine that can be used as isolate-specific marker detected by PCR/RFLP system
Lane MW: 1 kb Plus DNA ladder. For other lanes: Different isolates are indicated by their designated numbers. (Courtesy of Ridgway et al. 2005; The Mycological Society of America, Lawrence, USA)

formed from mtDNA of the pathogens were different. Based on the polymorphisms obtained from mtDNA, five groups in *C. acutatum* and two groups in *C. gloeosporioides* could be formed based on restriction patterns generated by the strains of these fungal pathogens. High levels of polymorphism in rDNA and mtDNA were recognized in the strains of *C. gloeosporioides* from the fruit crops avocado, papaya and banana. The banding patterns of all mango strains originating from the United States, West Indies, Sri Lanka, Malaysia and Australia produced identical banding patterns with all restriction enzymes (Mills et al. 1998).

Water yam (*Dioscorea alata*) foliar anthracnose disease is due to *Colletortichum gloeosporioides* which occurs in four forms viz., the slow-growing grey (SGG), the fast-growing salmon (FGS), the fast-growing olive (FGO) and the fast-growing grey (FGG) forms. The identity of the yam anthracnose pathogen (s) was established by 18S rDNA polymorphism, PCR-RFLP and sequence analysis of ITS region of the rDNA in addition to the cultural characteristics and fungicide sensitivity. FGG isolates produced unique ITS RFLP banding patterns, while FGS, FGO and SGG isolates produced RFLP patterns identical to those of *C. gloeosporioides* reference isolates, but distinct from other *Colletotrichum* spp. Restriction fragments generated by the endonuclease *Alu I*, *Hha I* and *Hae* III could be used for rapid differentiation of FGG isolates from other forms of *Colletotrichum* from yam (Abang et al. 2001, 2002). The FGG group of isolates described as *C. gloeosporioides* based on morphological characters, formed distinct ITS RFLP group and exhibited only limited ITS sequence similarity (<86%) to *C. gloeosporioides* reference isolates (Abang et al. 2003).

3.1.5 Polymerase Chain Reaction

Polymerase chain reaction (PCR) has been applied to detect, identify, differentiate and quantify microbial plant pathogens in planta, in their natural habitats and vectors involved in the spread of the diseases caused by them, in addition to the detection in axenic culture. PCR has been applied to amplify sequences of specific regions such as ITS or rDNA with universal primers and to differentiate the pathogens like *Pythium* spp. that are difficult to identify on the basis of morphological characteristics. Probes based on the tandem arrays of 5S genes unlinked to the rDNA repeat unit present in *Pythium* spp. were prepared to target the genomic DNA of 92 species of *Pythium*. Probes specific for *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiferum* exhibited species – or variety specificity. The sequences of 5S rRNA gene spacer have the potential for use in defining species boundaries in the genus *Pythium*, since these sequences diverged rapidly after speciation (Klassen et al. 1996). Species-specific primers were designed on the sequences of the variable regions of the ITS of the rDNA for the detection, quantification and differentiation of nine *Pythium* spp. present in the soils in eastern Washington. The primer pairs for *P. ultimum* and other species were used in real-time PCR and standard curves were generated for each species. Isolates (77) of different *Pythium* spp. were extracted from the soil and screened. The populations of *P. irregulare* group I, *P. irregulare* group IV and *P. ultimum* in the soils could be correlated with the quantities of DNA

amplified from the same soil using the standard curves for each species of *Pythium.* This technique is rapid and accurate and has the potential for quantification of these pathogens present in the soils (Shroedder et al. 2006).

By using primers based on sequences of ITS1 region of rDNAs of *P. ultimum* and *P. aphanidermatum* associated with leak syndrome in potato tubers, it was possible to differentiate, these two pathogens present in infected tubers (Triki et al. 2001). *Phytophthora infestans* and *P. erythroseptica* causing late blight and pink rot diseases in potato tubers could be detected and differentiated by employing primers designed from the sequences of ITS2 region of their respective genomic DNA (Tooley et al. 1998). Primers developed based on ITS4 and ITS5 sequences were employed to amplify 5.8S rDNA gene in a PCR assay. Six taxonomic groups of *Phytophthora* spp. including *P. infestans* could be differentiated using a PCR procedure (Liew et al. 1998).

Classification of the genera *Pythium* and *Phytophthora*, using criteria other than morphological characteristics is considered more reliable and useful to plant pathologists. Among the range of genetic markers, the ITS regions of rDNA, the sequences of cytochrome oxidase II (*CoxII*) gene and β-tubulin gene were examined for their usefulness in investigating the relationships within each genes. Fifty eight isolates representing 39 species of *Pythium* and 17 isolates representing 39 species of *Pythium* and 17 isolates representing nine species of *Phytophthora* were included for studying the intra- and inter-genic relationship based on the sequence analysis of three genomic areas. The ITS1 and ITS2 regions including the 5.8S gene of rDNA were amplified with the universal primers ITS1 and ITS4 using standard PCR format. By using the primer pair FM66 and FM58, the 563 bp of *CoxII* gene was amplified for *Pythium* and FM75 and FM78 pair was used for *Phytophthora*. The 658-bp partial β-tubulin gene was amplified with the forward primer BT5 and reverse primer BT6. These analyses grouped the isolates into four major clades, reflective of sporangial morphology. The phylogenetic relationship between *Pythium* and *Phytophthora* is yet to be clearly established. The results indicated the higher genetic divergence within *Pythium* than within *Phytophthora*. Furthermore, *Phytophthora* was found to be a monophyletic group, whereas *Pythium* is a polyphyletic one suggesting that *Phytophthora* is a relatively recently evolved genus having not yet radiated into many forms as compared to *Pythium* (Villa et al. 2006; Schroeder et al. 2006).

Anastomosis groups (AGs) within the morphological species *Rhizoctonia solani* have been recognized. For the amplification of the sequences of 5.8S rDNA and part of the ITS region of *R. solani* AG2, primers in combination with the universal primers ITS 1F and ITS 4B were employed. Six primers specifically amplified *R. solani* AG2, AG2-2 and AG2-3 and the ecological type AG2-t, suggesting that PCR-based methods may be employed for the reliable identification of different anastomosis groups of *R. solani* (Salazar et al. 2000). Wheat stem-base disease complex is due to *Rhizoctonia cerealis*, *Fusarium* spp., *Tapesia* spp. and *Microdochium nivale*. A PCR procedure was applied to rapidly differentiate the components of the disease complex at early growth stages of wheat crop, before chemical control program was initiated (Morgan et al. 1998).

The involvement of different species of *Fusarium* in foot rot and head blight diseases of small grain cereals and grasses and also ear and stalk rot of corn has been reported. PCR assay for differential detection and differentiation based on amplification of sequence characterized amplified regions were developed for *Fusarium culmorum*, *F. graminearum* and *F. avenaceum*. The molecular variability of 37 *Fusarium culmorum* isolates from the Pan-Northern Hemisphere was examined by PCR assay, using primer pairs specific for *Tri7* and *Tri13* genes. The isolates (30) producing deoxynivalenol and 3-acetyl-deoxynivalenol belonged to chemotype I, whereas chemotype II included isolates (7) producing nivalenol and/or fusarenone X. Nivalenol production was correlated with the presence of a functional *Tri7* gene. Chemotype I isolates were generally more virulent in in vitro assessments than chemotype II isolates (Tóth et al. 2004)

Several DNA sequences have been used to analyze intraspecific variability including intron regions of histone, the β-tubulin and calmodulin genes (O'Dennell et al. 2000b; Steenkamp et al. 2002) and transduction elongation factor EF-1α (O'Donnell et al. 2000a, b). The intergenic spacer (IGS) region of rDNA has been shown to be a highly variable noncoding sequence that may be useful for differentiation of populations of fungal pathogens. Suitable primers were employed for PCR amplification of the entire IGS region of *Fusarium verticillioides* from banana fruits, phylogenetic analyses, and the partial amplification of the *EF-1α* gene. The partial sequences of the IGS region and the translation factor *EF-1α* gene of representative sample (48 strains) of *F. verticillioides* isolated from diverse hosts, geographical origins and with different levels of fumonisin production were analyzed. Genetic variability detected by both sequences was found to be high. The phylogenetic analysis showed the existence of two distinct clusters of strains within *F. verticillioides*. The first group called FP group enclosed a major population with wide geographical distribution, wide host preferences and the ability to produce fumonisins (FP group). The second group is confined to strains associated with banana located in Central America.They were unable to produce fumonisns and designated FNP group (Mirete et al. 2004).

Various molecular markers in different techniques to detect and differentiate formae speciales of *Fusarium oxysporum* have been used. Polygalacturonase (PG) is one of the important cell wall-degrading enzymes (CWDE) secreted by *Fusarium* spp. involved in pathogenesis. It may be possible to study the genetic diversity of pathogen populations by comparing nucleotide sequences from PG genes of isolates. A PCR-based technique targeting PG genes was developed to identify pathogenic types of *F.oxysporum* on/in a tomato plant with wilt symptoms in the field. The partial nucleotide sequences of *endo* PG (*pg1*) and *exo* PG (*pgx4*) genes from isolates of *F. oxysporum* f.sp. *lycopersici* (*FOL*) and *radicis–lycopersici* (*FORL*) from Japan were compared. Based on the nucleotide differences noted among the pathogenic types, specific primer sets (uni and sp13; sp23 and spr1) were designed. PCR with the uni primer set amplified a 670∼672-bp fragment from all isolates of *FOL* and *FORL.* With the sp13 primer set, an amplicon 445-bp was detected only when the DNA from isolates of *FOL* race 1 and 3 was amplified. A 518-bp fragment was amplified, when sp23 primer set was used for the PCR assay in isolates of *FOL* race 2 and 3. The DNA of isolates of *FORL*, but not *FOL* yielded a 947-bp

fragment following PCR amplification using the spr1 primer set. The pathogenic types of *F. oxysporum* in tomato can be precisely differentiated by a combination of amplifications with the primer sets developed in this study (Hirano and Arie 2006).

A rDNA fragment of *Gaeumannomyces graminis* amplified by PCR was labeled and used as a probe which hybridized to *Eco*RI digests of target DNA. The three varieties of *G. graminis* viz., *tritici*, *avenae* and *graminis* exhibited distinct differences in banding patterns consistently. The results indicated that the selected probe(s) could be employed for detection and differentiation of the varieties/subspecies of fungal pathogens (Ward and Gray 1992). The intraspecific genetic variation in the nuclear rDNA of *Phialophora gregata* was used as a criterion for differentiating isolates from soybean, mungbean and adzuki bean. A unique banding pattern, following digestion of PCR-amplified ITS and the 5′ end of the large subunit of rDNA with restriction enzymes was generated by all 79 isolates. Isolates from United States and Brazil had identical ITS sequences, whereas the adzuki bean isolates from Japan showed 98% homology with soybean isolates (Chen et al. 1996).

The genetic diversity of *Verticillium dahliae* infecting artichoke crop has been investigated based on the vegetative compatibility and molecular methods. Vegetative compatibility refers to the genetically controlled ability of individual fungal strains to undergo hyphal anastomosis and form stable heterokaryons. Vegetatively compatible isolates of a fungal species are included in the same vegetative compatibility group (VCG). As *V. dahliae* reproduces only by vegetative reproduction, this pathogen isolates in different VCGs are thought to be genetically isolated populations each having the potential to share a common gene pool. *V. dahliae* isolates from artichoke (109 isolates) and cotton (three isolates) were characterized by VCG and specific PCR assays using three sets of primer pairs that differentiate the cotton-defoliating (D) and non-defoliating (ND) *V. dahliae* pathotypes. Two subgroups of isolates were identified in VCG2B based on heterokaryon in amplification of 334- and 824-bp DNA fragments which are markers of the D and ND pathotypes respectively. The molecular subgrouping of VCG2B determined by amplification of these molecular markers correlated with virulence of isolates to the two hosts of *V. dahliae* (Jiménez-Díaz et al. 2006).

By PCR amplification of a portion of the 18S rDNA gene, ITS1, and ITS2 and the 5.8S rDNA gene, the genetic variations in different species of *Venturia* were resolved. The optional group I intron in the 18S rDNA gene of *V. inaequalis* was detected in 75% of 92 strains collected world wide. The sequence and restriction analysis of rDNA of four intron alleles were determined. Most of the strains of *V.inaequalis* possessed at least three intron alleles. The strains of *Venturia* spp. were classified into three monophyletic groups depending on the sequences of ITS1-5.8S–ITS2. The populations of *V. inaequalis* could be subdivided using the intron and ITS1 alleles (Schnabel et al. 1999). The PCR primers specific to the 3′ regions of the intron (located only in *Monilina fructicola* infecting plum fruit) together with the small subunit (SSU) rDNA primer NS5, *M. fructicola* was differentiated from two other related fungal pathogens *M. fructigena* and *M. laxa* (Fulton and Brown 1997).

Many species of *Cercospora* described from diverse hosts are morphologically indistinguishable and hence they are referred to as *C. apii* sensu lato. A new species

C. apiicola isolated from celery was investigated for its phylogenetic relationship with closely related *Cercospora* spp. using the sequences of five different gene areas viz., the ITS of 5.8S rRNA gene, the actin gene (ACT), the translation elongaton factor 1-α gene (EF), the calmodulin gene (CAL) and the histone H3 gene (HIS). Two separate analyses were performed. The first combining ITS, EF, ACT and CAL sequences and the second using only HIS sequences. These two analyses showed that *C. apiicola* isolates clustered together in a well-defined clade. On the other hand, *C. apii* and *C. beticola* sensu stricto isolates clustered into well defined clades and they had wider host ranges. But *C. apiicola* was specific to celery and the *Cercospora* strain isolated from other hosts did not show any similarity in sequences of the five genes used in this study (Groenewald et al. 2006).

Colletotrichum gloeosporioides causing leaf anthracnose in water yam, occurs in SGG, FGS, FGO and FGG forms. Molecular differentiation of SGG and FGS populations using genetic markers will be useful for epidemiological investigations. PCR-RFLP analysis of the entire ribosomal DNA ITS-1–5.8S-ITS.2 region did not show any polymorphism between SGG and FGS isolates. However, the genetic analysis based on 52 microsatellite-primed PCR (MP-PCR) markers indicated highly significant differentiation between the SGS and FGS population infecting water yam. These results suggest that the SGG and FGS morphotypes (differing in the growth rate) represent genetically differentiated populations (Abang et al. 2003, 2005). Microsatellite-primed (MP)-PCR assay was applied to assess the amount and distribution of genetic variation within and among populations of *C. gloeosporioides* originating from different yam species and non-yam hosts in Nigeria. Fifty two scorable bands were generated by the four microsatellite primers, revealing a high level of polymorphism. The polymorphic loci identified by the four MP-PCR markers differed depending on the origin of *C. gloeosporioides* populations. High level of genetic diversity (GD) of isolates from yam and other hosts was observed. The correlation between the pathotypes (determined by using differential varieties) and MP-PCR haplotype was weak, suggesting a lack of association between genetic polymorphism and virulence (Abang et al. 2006).

The diversity and distribution pattern of *Colletotrichum* spp. populations, their relatedness, variability in pathogenic potential and host preference (range) were investigated by using arbitrarily by primer (AP)-PCR. Isolates of *Colletotrichum* (131) from olive were analyzed by employing AP-PCR markers. Distinct band patterns generated by 128 isolates indicated that they were similar to *C. acutatum* reference isolates, whereas three isolates grouped with *C. gloeosporioides* reference isolates. In addition, based on the nucleotide sequence generated for the variable region of *tub2* gene, PCR primers specific for *C. acutatum* (TBCA) and *C. gloeosporioides* (TBCG) were designed. Species-specific PCR assays by employing the *tub2* primers for *C. acutatum* identified 128 isolates from olive as *C. acutatum*. Likewise, three of 131 isolates were considered as *C. gloeosporioides*, indicating predominance of *C. acutatum* among the fungal populations causing olive anthracnose. The comparative analysis of the isolates with rRNA gene ITS based specific PCR for *C. acutatum* and *C. gloeosporioides* entirely confirmed the results obtained from PCR assays (Talhinhas et al. 2005).

The studies on the French and Chilean populations of *Botrytis cinerea* (teliomorph, *Botryotinia fuckeliana*), showed that *B. cinerea* is composed of two sympatric species, *transposa* and *vacuma* (Giraud et al. 1999; Muñoz et al. 2002). No difference in pathogenicity between *transposa* and *vacuma* isolates could be seen. The transposable elements *Boty* and *Flipper* were present in *transposa* isolates, whereas they were absent in *vacuma* isolates (Diolez et al. 1995; Levis et al. 1997; Martinez et al. 2003). The information on genetic structure of *B. cinerea* population will be useful to plan effective disease management strategies. The microsatellite-primed (MP)-PCR technique was applied to assess the genetic variation in the populations of *B. cinerea*, because of its greater reliability compared to the use of random amplified polymorphic DNA (RAPD) markers. The PCR primer pair F300 and F1550 was used to detect the *Flipper* element, whereas the primer pair BotyF4 and BotyR4 was employed to detect *Boty* element. A total of 16 microsatellite primers were tested for their ability to generate polymorphic bands from *B. cinerea* isolates. Among the 234 isolates tested, *Boty* and *Flipper* elements were present in 195 isolates indicating that *transposa* isolates were predominant (83.3%) in the sampled populations of *B. cinerea*. Only one isolate belonged to the *vacuma* type and 38 isolates had only *Boty* element. Six microsatellite primers generated stable MP-PCR band profiles for each isolate in four independent amplifications. Based on the PCR profiles generated, 99 haplotypes were found from 234 isolates tested. The populations of *B. cinerea* from grape, kiwifruit, squash and pea did not exhibit any significant genetic variation. But the population from figure was genetically different from grapevine. However, the genetic differentiation between these two populations was very low (Ma and Michailides 2005) [Appendix 1].

The ITS region has been shown to be polymorphic and the differences in the sequences may be valuable for the differentiation of taxa especially on and below generic level. Attempt was made to amplify the ITS-region from *Plasmopara halstedii* causing sunflower downy mildew disease. The study revealed that the ITS region of this fungus was quite long with 2587 bp, in contrast to about 900 bp reported for other members of Peronosporaceae. The presence of a single *Eco*RI-site allowed a restriction-ligation procedure to amplify parts of the ITS fragment separately. The presence of four copies of randomly arranged negative element in the ITS-2 region which accounted for 2212 bp made this fragment probably to be the longest. The high degree of variation of the repetitions might also allow the specific detection and diagnosis of different *P. halstedii* strains. This will help to understand the pathogen's genetic potential for development of epidemics (Thines et al. 2005). The sequence data for the ITS 1 and 2 regions and the 5.8S rRNA gene regions of *Ceratocystis fimbriata*, causing a serious decline disease of mango were compared with the sequence data from several other hosts and geographic locations. The isolates from Oman were closely related to an isolate from mango in Brazil, suggesting that the mango pathogen in Oman might have originated in Brazil (Van Wyk et al. 2005).

Molecular genetic analyses have indicated that *Pyricularia* spp. isolated from rice and other hosts are genetically distinct (Borromeo et al. 1993; Schull and Hamer 1994; Kato et al. 2000). Based on the sequence analysis of three genes, viz., *actin*, beta-tubulin and calmodulin, the telomorph *Magnaporthe oryzae* (infecting

rice and other cultivated grasses) was described as a species distinct from *M. grisea* (infecting the grass genus *Digitaria*) (Couch and Kohn 2002). In a later investigation, the phylogenetic relationships among 41 isolates of *Pyricularia* and related genera were determined by analyzing complete sequences of the ITS regions, including the 5.8S rRNA gene. The potential of rDNA sequences in the analysis of anamorph-teleomorph relationships of the genereic level or using sequence analysis of rDNA combined with PCR-fingerprinting for establishing the connection between an anamorph species and an ascomycete has been established. The combination of morphological and molecular characters, such as spore morphology and ITS rDNA sequences data suggested that conidial shape may be used as a primary character to distinguish *Pyricularia* from related genera. *Pyricularia zingiberis* and *Gaeumannomyces amomi* isolated from Zingiberaceae plants were strongly grouped and closely related to other *Gaeumannomyces* spp. from grasses (Bussaban et al. 2005).

The repetitive-based (rep)-PCR generates DNA fingerprints by amplifying sequences between randomly dispersed copies of the repeat element in a pathogen genome. This technique combines the simplicity of PCR with detection of polymorphism by RFLP. A rep-PCR protocol was first applied by George et al. (1998) to amplify *Pyricularia oryzae* transposable (Pot) elements present in the genome of *M. grisea.* A good correlation between the dispersal repetitive element MGR 586-RFLP and rep-PCR based DNA fingerprinting groups was discernible. DNA fingerprinting by *Pot2* rep-PCR was demonstrated to be an efficient method of monitoring the population dynamics of *M. grisea*. In a later investigation the extent of genetic and pathogenic diversity in *M. grisea* populations in China was determined based on DNA-fingerprinting using rep-PCR markers and differential hosts. The DNA fingerprinting analysis using rep-PCR of 381 haplotypes (482 isolates) revealed that the *M. grisea* populations cannot be delineated into region-specific groups. A comparative analysis of pathotypes (based on infection types on differential rice cultivars) and DNA fingerprinting of the haplotypes in China was made. There was only a poor correlation between DNA fingerprinting groups and pathotypes (Chen et al. 2006).

The obligate fungal pathogens have been responsible for substantial yield losses in cereals, fruit and ornamental crops. The ribosomal DNA ITS region of powdery mildew fungi have been investigated for identification and differentiation of these pathogens. The ITS sequences were considered as a basis for resolving relationships between the genera *Sphaerotheca* and *Podosphaera*, leading to the reduction of *Sphaerotheca* to synomy with *Podosphaera* (Takamatsu et al. 2000; Braun and Takamatsu 2000). Monophyletic classification of the three major species in *P. fusca* complex was possible based on the ITS sequences (Braun et al. 2001). *Podosphaera tridactyla* var. *tridactyla* infecting fruit trees in the genus *Prunus* is a morphologically variable species. To assess the genetic variation within this species, the ITS region of rDNA was amplified from 29 specimens from a range of *Prunus* species cultivated in Australia, Switzerland and Korea. Six groups were formed based on the RFLP analysis of the PCR products (Cunnington et al. 2005).

The rusts (*Uredinales*) from a large group of diseases caused by obligate biotrophic organisms with a complex life style. A fragment of the mitochondrial

cytochromae *b* (*cytb*) gene of plant pathogenic Basidiomycetes was sequenced. The relatedness of rust pathogens was investigated by examining the deduced sequences (residues 142–266), as compared to other Basidiomycete fungi (Grasso et al. 2006a). In addition, the relatedness was also studied at nuclear level using the ITS of rDNA. Based on the amino acid sequences and ITS sequences, the *Puccinia* spp. infecting cereals, such as *P. recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis* and *P. sorghi* together with *P. horiana* infecting *Chrysanthemum* were very closely related to each other. *Puccinia arachidis* causing peanut rust disease was closely related to *Uromyces appendiculatus* infecting beans. On the other hand, soybean rust pathogen *P. pachyrhizi* and coffee rust organism *Hemileia vastatrix* were outside the *Puccinia* cluster. The results show that the amino acid sequence of mitochondrial cytochrome b is a valid basis to study phylogenetic relatedness among the pathogenic fungi belonging to Basidiomycetes. However, the ITS sequences, due to their high variability, were able to discriminate *Puccinia* species, which were identical on the basis of the cytochrome b amino acid sequence. ITS sequences may be able to bring out the differences among species or within a species. In contrast, cytochrome b is more suitable than ITS for phylogenetic inference at family or genus level (Grasso et al. 2006b).

3.1.6 Random Amplified Polymorphic DNA Technique

The random amplified polymorphic DNA (RAPD) technique is a PCR procedure involving arbitrary primers. It can be applied to differentiate races, strains and pathogenic or nonpathogenic isolates of fungi. Generally very short (10 or fewer bases) pieces of DNA from the selected source are used as primers. It may be expected that these primers may probably find some complementary sequences in the target DNA, forming a mixture of DNA fragments of different sizes. When the amplified products from such a reaction are analyzed on electrophoresis gel, unique banding patterns are seen. These patterns may reflect the differences characteristic of certain species or varieties or strains. Some patterns may be useful for detection and diagnosis of some microbial pathogens. The unique bands representing the fungal pathogenic species may be cut out of a gel and sequenced to produce specific primers for more accurate PCR analysis or probes for dot hybridization and other detection procedures. The RAPD method is simple, sensitive and rapid for the detection, differentiation and determination of phylogenetic relationship between isolates of a morphological species of several pathogens (Narayanasamy 2001).

The RAPD technique was applied to distinguish the pathogenic and nonpathogenic isolates of *Fusarium oxysporum* f.sp. *dianthi* causing wilt disease of carnation. The genetic markers were identified and four amplification groups among the isolates of the pathogen were also identified based on the RAPD banding patterns generated by the primer OPA17. The nondianthi isolates were discriminated by this primer. However, no relationship between the RAPD patterns and races

of the pathogen identified using differential host varieties could be established (Hernandez et al. 1999). The RFLP technique using five different restriction enzymes and RAPD analysis based on four different primers were applied to differentiate pathogenic and nonpathogenic isolates of *F. oxysporum* f.sp. *phaseoli* (FOP). There was correlation between the banding patterns and the vegetative compatibility groups (VCGs), but not between banding patterns and pathogen races. However, RFLP and RAPD markers differentiated nonpathogen and self-incompatible isolates of FOP (Woo et al. 1996). In a later investigation, twenty isolates of *F. oxysproum* from Brazil pathogenic and nonpathogenic to common bean were analyzed by employing RAPDs to study the genetic diversity. In this technique 23 oligonucleotides were used for the amplification of 229 polymorphic and seven monomorphic DNA fragments ranging from 234 to 2590 bp. High genetic variability was observed among the isolates with distances varying between 8 and 76% among pathogenic, 2% and 63% among the nonpathogenic and 45% and 76% between pathogenic and nonpathogenic isolates. The analysis of genetic distance data showed that the pathogenic isolates tended to cluster together in one group, whereas the nonpathogenic isolates constituted another group (Zanotti et al. 2006).

The pathotypes of *Fusarium oxysporum* f.sp. *ciceris* (FOC) were differentiated by applying the genetic fingerprinting and RAPD assays for amplification of DNA. Wilt-inducing and yellowing pathotypes of FOC could be recognized, as confirmed by the results obtained by inoculating chickpea differentials (Kelley et al. 1994). In a further study RAPD and PCR techniques were combined to differentiate these two pathotypes of FOC. The wilt-inducing pathotype possessed a 1.6-kb RAPD fragment that was absent in yellowing pathotype. The specific primers based on the sequences of this DNA fragment were employed for the detection of FOC in symptomless chickpea plants at 16 days after inoculation. The root and stem tissues, but not leaves, showed the presence of PCR amplicons (Kelley et al. 1998).

The genetic diversity among isolates of a worldwide collection of *Fusarium culmorum* and *F. graminearum* was assessed by RAPD fingerprinting (Schilling et al. 1994). RAPD technique was applied for reliable differentiation of *F. culmorum*, *F. graminearum* and *F. avenaceum.* Species-specific fragments were identified and used to develop primers for selective amplification of *F. culmorum* and *F. graminearum*. Based on the sequences of the selected fragments, pairs of 20-mer oligonucleotide primers were designed to yield distinguishable amplicons of different molecular weight. Single fragments were specific for *F. culmorum* and *F. graminearum* (Schilling et al. 1996). RAPD analysis has been useful to identify the amplification products that form the basis of differentiating *F. culmorum* and *F. graminearum* infecting wheat stem base and grains respectively. The wheat grains showed the colonization by the isolates of *F. graminearum* producing trichothecene predominantly. The results suggested that the mycotoxin trichothecene may act as a virulence factor in colonizing wheat grains (Nicholson et al. 1998).

Elsinoe fawcetti, *E. australis* and *Sphaceloma fawcetti* var. *scabiosa* involved in citrus scab disease, do not show distinct differences in their morphological characteristics, making it very difficult to differentiate them. The sequence analysis of ITS region of rDNA and restriction analysis of amplified ITS with several endonucleases

provided sound basis for the differentiation of *E. australis* from *E. fawcetti* and *E. fawcetti* var. *scabiosa*. The relatedness of isolates should be established by RAPD analysis. *E. fawcetti* isolates from Australia and Florida were more closely related to each other than to *E. australis* isolates from all Florida isolates. The RAPD profiles showed close correlation with the results of pathogenicity tests as evidenced by the pathotype identification by RAPD technique (Tan et al. 1996).

The blue mold disease of apple is due to *Penicillium expansum* and the isolates of *P. expansum* were characterized by employing RFLP of the region including ITS1 and ITS2 and the 5.8S rRNA gene in ribosomal DNA region and RAPD primers. The isolates of *Penicillium* spp. recovered from rotten apple and pear fruits as well as from water and floatation tanks in commercial apple juice facilities were rapidly and reliably identified and grouped into *P. expansum* and *P. solitum*. The involvement of *P. solitum* in blue mold disease was reported for the first time in this study (Pianzzola et al. 2004). The RAPD and amplified fragment length polymorphism (AFLP) techniques were employed to determine the genetic relationship of *Botrytis cinerea* causing gray mold disease of apple populations. The pathogen showed greater genetic diversity, as determined by RAPD analysis, compared with RFLP analysis, as RAPD generated more polymorphisms per loci and the genetic relationships between isolates may be more precisely deduced using RAPD analysis (Moyano et al. 2003).

The RAPD technique was applied to detect early infections of sweet cherry by *Monilinia fructicola*, causing brown rot disease. The primer sets were developed from a RAPD fragment (X-09 int F3/X-09R) that specifically amplified DNA from isolates of *M. fructicola* and *Monilinia* spp. No detectable amplification of DNA from *Botrytis cinerea* (causing gray mold) and other fungi associated with sweet cherry, indicating the specificity of detection of the pathogen in question (Förster and Adaskaveg 2000). In a later investigation, the imported and exported fruits were monitored for the presence of quarantined pathogens *M. fructigena*, *M. fructicola*, *M. laxa* and *Monilia polystroma* by using RAPD analysis. Primers were designed based on the sequences of the DNA fragment that was unique to each species to be detected. Based on all three sequences, a multiplex PCR protocol was used to differentiate these three pathogen species (Côté et al. 2004b). The presence of group I intron in the small-subunit (SSU) ribosomal DNA gene of *M. fructicola*, but not in *M. fructigena* or *M. laxa* was used on the basis of species differentiation. RAPD was employed to confirm the identification of *M. fructicola* strains. All *M. fructicola* strains showed identical or nearly identical patterns when compared to *M. laxa* strains (Fig. 3.2) (Côté et al. 2004a).

Simultaneous identification and differentiation of *Alternaria alternata*, *Fusarium semitectum*, *F. roseum* and *Penicillium viridicatum* infecting melon fruits causing postharvest decay by employing RAPD-PCR analysis was demonstrated. These pathogens could be differentially detected and identified and the results were reliable and reproducible (Chen et al. 1999). RAPD-PCR analysis of 216 isolates *Alternaria* (earlier considered as *A. alternata*), using total genomic DNA and three different primers resulted in inclusion of these isolates in *A. gaisen*, *A. longipes*, the *tensuissima* group, *arborescens* group, and *infectoria* group (Roberts et al. 2000).

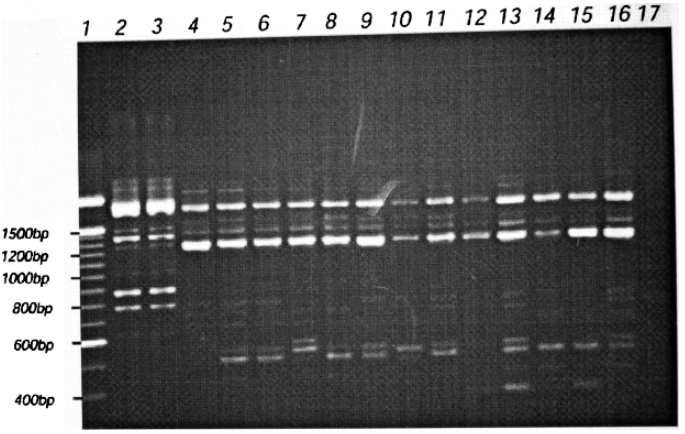

Fig. 3.2 Differentiation of strains of *Monilinia fructicola* and *M. laxa* by RAPD amplification Lane 1: 100-bp ladder (standard); Lanes 2 and 3: Two strains of *M. laxa*; Lanes 4–16: Thirteen strains of *M. fructicola*; Lane 17: PCR negative control. Note identical or near identical patterns of strains of *M. fructicola* compared to strains of *M. laxa*. (Courtesy of Côte et al. 2004; The Mycological Society of America, Lawrence, USA)

By using primers based on the sequence of a cloned RAPD fragment of *Alternaria radicina* infecting carrot seeds, the pathogen was detected efficiently (Pryor and Gilbertson 2001). *Mycosphaerella fijiensis* and *M. musicola* causing the destructive sigatoka disease of banana, in addition to *M. musae* and *M. minima* also occurring on banana, were differentiated by RAPD procedure. Distinct RAPD banding patterns were generated from the DNAs of these pathogens with the PCR primers tested (Johanson et al. 1994). The isolates of *Uncinula necator* causing grapevine powdery mildew disease were collected from Europe and India. Based on RAPD analysis, three main groups were recognized. The isolates from Europe were placed in one group enclosing 53 isolates and the second group included nine isolates. The isolates (15) from India formed a subgroup of one of the group with European isolates and the remaining isolates (13) constituted a distinct group (Délye et al. 1997).

The RAPD technique was applied to assess the genetic diversity among rice blast pathogens. The analysis of RAPD polymorphism on *M. grisea* isolates exhibited high level of polymorphism indicating a wide and diverse genetic base. A repeat sequence designated MGR 586 present in the genomic DNA of rice infecting strains of *M. grisea* and another retrotransposon named as *fosbury* have been used for differentiation of isolates of *M. grisea* (Schull and Hamer 1994). The isolates from Bangla Desh lacked both MGR 586 and *fosbury* (Schull and Hamer 1994). In a later investigation the RAPD markers were used to find out the density of genetic relationships. Screening of RAPD markers (128) using DNA from three isolates of *M. grisea* resulted in the selection of 33 primers that gave reproducible results. The phylogenetic grouping based on the RAPD data did not seem to be congruent with geographical locations. A high genetic diversity was evident among the isolates

due to natural and stress induced transposition which may be the main reason for genetic diversity. Diversity within isolates from Andhra Pradesh, Maharashtra and Karnataka states in India could be due to different crop seasons. The results indicate that populations of *M. grisea* in India are heterogenous genetically and the RAPD technique can be effectively used to assess the interrelationship among the isolates (Chadha and Gopalakrishna 2005).

Septoria musiva (teleomorph, *Mycosphaerella popularum*) causing leaf spot and canker diseases of *Populus* spp. suitable for production of fuel and fiber has isolates widely varying in aggressiveness and sporulation. The five primers employed generated a total of 126 amplified DNA fragments. The genetic relatedness among isolates of *S. musiva* was 80%, suggesting a high degree of relatedness. The RAPD analyses of 52 isolates of *S. musiva* collected from five states of US revealed that there was a large degree of genetic similarity although each isolate had a unique RAPD pattern. The isolates did not exhibit any relationship in respect of molecular genetic distance, host clone percentage or taxonomic classification section and location (Ward and Ostry 2005). The genetic diversity in the isolates of *Cercospora canescens* causing cercospora leaf spot disease in legume was determined by using RAPD marker technique and variations in the ITS region of rDNA. RAPD profiling showed that the isolates of *C. canescens* grouped into three clusters. Significant genetic diversity was exhibited by the isolates from the same geographical location. The comparison of results obtained from the two techniques indicated that RAPD technique was more efficient in determining the genetic diversity and differentiation of *C. canescens* isolates (Joshi et al. 2006).

Phytophthora erythoseptica inducing potato pink rot disease exists as isolates pathogenic to various crops such as tomato, raspberry, clover, pea, lupine and white calla. The genetic diversity among the isolates of *P. erythroseptica* collected from North America was studied using the RAPD procedure. Randomly chosen decanucleotides primers were employed to amplify regions of DNA to reveal polymorphisms among templates (RAPD). The isolates of *P. erythroseptica* differed in their geographic origin and in their sensitivity to mefenoxam. Three primers yielding polymorphisms were used to screen 106 isolates of *P. erythroseptica*. No significant variation was discernible suggesting that the absence of genetic diversity among the isolates tested may be attributed to the relatively recent introduction of a small population in North America (Peters et al. 2005).

3.1.7 Amplified Fragment Length Polymorphism Technique

The molecular tools such as isozyme analysis, RFLP and RAPD techniques have been useful to identify and differentiate microbial plant pathogens. However, they have been found to be time-consuming and expensive, providing little information beyond species identity. Hence, the necessity to develop a technique with a higher resolution at population level and its dynamics was realized. Amplified fragment length polymorphism (AFLP), a versatile technique has been shown to be useful

for performing fingerprinting analyses, mapping and other genetic studies on a wide range of microorganisms including *Pythium* spp., *Phytophthora* spp. and *Peronospora* spp. The AFLP technique involves selective amplification of restriction fragments from a digest of total genomic DNA using PCR. The DNA fragments designated AFLP markers can be resolved using a polyacrylamide gel. If the PCR primers are labeled with a fluorescent dye, a DNA sequencing machine has to be used. Reproducibility and sensitivity of AFLP are distinct advantages, since as many as 50–75 AFLP markers can be resolved per reaction as in the case of (one isolate of) *Phytophthora capsici* (Blears et al. 1998). Oospore (sexual spore) progenies (107) from a laboratory cross between parents with different AFLP genotypes were characterized. The progenies were all recombinant and the AFLP markers segregated as Mendelian characters (Lamour and Hausbeck 2001a). The natural populations of *P. capsici* occurring in Michigan, United States were analyzed. The isolates (70%) had unique AFLP profiles. In total, 94 AFLP markers were recognized, but no single population exhibited the presence of all 94 markers, the number of markers present varying between 68 and 80. Genetic similarity of isolates was more based on geographic locations (Lamour and Hausbeck 2001b). The pools of genetic diversity remained stable. The clonal lineages of *P. capsici* were confined in space to single fields and in time to single years (Lamour and Hausbeck 2002; Hausbeck and Lamour 2004).

The isolates of *Phytophthora infestans* from potato cultivars carrying *R2* gene were characterized for pathogenicity and molecular fingerprints to assess the chances of development of virulent isolates of *P. infestans* populations, if *R2* has to be deployed extensively in French commercial potato crops. A total of 89 bands were scored within the collection of *P. infestans* isolates tested. The isolates were distributed among the five AFLP genotypes. Virulence was detected against all resistance genes tested except *R5*. The simplest race carried three virulences, whereas half of the isolates had seven or eight virulences. No relationship between AFLP genotype and race (identified by differential varieties) could be observed (Pilet et al. 2005).

The AFLP technique was applied to generate diagnostic AFLP fingerprints for *Pythium* spp. affecting greenhouse crops and to assess the extent of the intraspecific variation among the isolates of *P. aphanidermatum*, *P. irregulare* and *P. ultimum* using AFLP fingerprinting. A total of 399 fragments in a range of 50–499 nucleotides were generated for nine *Pythium* spp. and three *Phytophthora* spp. The study provided diagnostic AFLP fingerprints of *P. aphanidermatum*, *P. irregulare* and *P. ultimum* and tentative fingerprints for the other six *Pythium* spp. were also described. Accurate identification of the 29 isolates without any misidentification indicated the reliability of the results obtained. The incorrect identification of five isolates done earlier was also rectified by using AFLP fingerprinting. Furthermore, the DNA fragments that constitute each of the bands of the diagnostic fingerprints could also be employed to design probes for real-time PCR procedures. The results of AFLP fingerprinting analysis were supported by analysis of ITS sequences (Garzón et al. 2005) [Appendix 2].

The temperate and tropical isolates of *P. capsici* from a wide range of host species were characterized based on cultural properties, AFLP, and DNA sequence analyses of ribosomal ITS region and mitochondrial cytochrome oxidase II (*CoxII*) genes. A strong bootstrap support for separation of temperate and tropical isolates using AFLP was inferred. The majority of temperate isolates clustered within a single clade with low variation regardless of host or geographical origin, while the tropical isolates were more variable and grouped into three distinct clades. RFLP analysis of the ITS regions separated the temperate and tropical isolates, as in the AFLP and ITS phylogenetic analyses. However, RFLP analyses of *Cox I* and *II* gene cluster did not distinguish the temperate and tropical isolates of *P. capsici*. The results did not lend support to the suggestion of separating temperate and tropical isolates into different species (Bowers et al. 2007).

Amplified fragment length polymorphism (AFLP) genotyping is considered to be one of the most reliable and reproducible DNA fingerprinting methods (Vos et al. 1995; Duncan and Cooke 2002). AFLP analysis of 132 isolates of *P. cactorum* prevalent in California obtained from almond (30) strawberry (36) walnut (5) and from other hosts (11) including 22 isolates of 15 other *Phytophthora* spp. from various hosts, was carried out. The analysis using 12 primer pairs among all 132 isolates of *P. cactorum* showed a high degree of similarity within the species. There was no clear relationship among cluster members, isolate hosts and geographical sources of isolates. However, the isolates within the populations varied greatly in aggressiveness in almond and strawberry and host specialization was evident among almond and strawberry populations, as indicated by pathogenicity experiments. The results pointed out practical implications. The variation in aggressiveness and virulence among isolates of *P. cactorum* suggested the importance of careful selection of isolate suitable for evaluation of resistance of genotypes of host plants (Bhat et al. 2006).

Two major genotypes of *Phytophthora infestans*, an A2 mating type (US-8) and A1 mating type (US-11) existing in the USA and Canada were resistant to the fungicide metalaxyl (Daayf et al. 2000; Gavino et al. 2000). The US-8 was commonly present on potatoes, while US-11 caused severe infection on tomatoes. As late blight of tomatoes suddenly reemerged as a major concern in several tomato production areas, destroying 80–90% seedlings in seed beds, investigations were taken up to differentiate strains of *P. infestans* found on potato, tomato and other nonpotato solanaceous weed hosts. Isolates of *P. infestans* from four solanaceous hosts, black night shade, hairy night shade, petunia and tomato were characterized for mitochondrial DNA haplotype, mating type, metalaxyl resistance, allozymes of glucose 6-phosphate isomerase and peptidase and DNA fingerprint with the RG57 probe. Analysis of the isolates showed close similarity of the petunia, hairy and black nightshade isolates to potato isolates. *P. infestans* isolates from tomato fields in New Jersey belonged to A2 mating type, metalxyl resistant and mtDNA haplotype Ia. These isolates were homozygous at the loci coding for both glucose-6-phosphate isomerase and peptidase, having *Gpi122/122*, *Pep* 100/100. RG57 analysis revealed that all the tomato isolates from New Jersey had unique and fingerprint unreported

earlier. Likewise, the isolates from Pennsylvania also were found to be form a new genotype with quite different fingerprints from *P. infestans* isolates recovered from potatoes (Deahl et al. 2006)

Differentiating *Fusarium* spp. in the *Gibberella fujikuroi* (teleomorph) complex based on morphological characters, without relying on DNA-based techniques, has been very difficult. Since the mycotoxins are produced only by some species, it is essential to identify *Fusarium* spp. with certainty to reduce the risks associated with contaminated food grains. Five strains each from *Fusarium andiyazi*, *F. nygamai*, *F. pseudonygamai*, *F. thapsinum* and *F. verticillioides* were analyzed by AFLP technique. The strains of each species were $\geq$73% similar in AFLP profile. The strains sharing $\geq$65% of other AFLP bands were considered as conspecific. Two species *F. verticillioides* and *F. nygamai* produced high levels of fumonisins, but little or no moniliformin. On the other hand, *F. pseudonygamai* and *F. thapsinum* produced high levels of moniliformin, but little or no fumonisin. All the four species were toxigenic. These five species which would have all been called *F. moniliforme*, differ considerably in terms of pathogenicity and toxin production profile. Their incorrect identification probably account for inconsistencies and differences reported earlier in literature (Leslie et al. 2005). Several species of *Fusarium* associated with cassava root rot disease in Cameroon were subject to AFLP groups analysis. At least 13 distinct AFLP groups of *Fusarium* could be distinguished, each one probably a distinct species. Two largest of the AFLP groups corresponded to *F. oxysporum* and *F. solani* species complex (Bandyopadhyay et al. 2006).

The extent of genetic diversity among *Verticillium dahliae* isolates within vegetative compatibility groups (VCGs) was investigated using 53 artichoke isolates, 96 isolates from cotton seven from cotton soil and 45 from olive trees and AFLP and PCR techniques. The isolates of *V. dahliae* within a VCG subgroup are molecularly similar to the extent that clustering of isolates correlated with VCG subgroups irrespective of host plant species and geographical locations. VCGs differed in molecular variability, with the variability being highest in VCG2B and VCG2A. Further, VCG2B isolates from artichoke were grouped into two distinct clusters that have a bearing on PCR markers of 334-bp or 824-bp. The VCG2B isolates exhibited a correlation between molecular differences and their virulence to artichoke and cotton cultivars (Collado-Romero et al. 2006).

The knowledge of the genetic structure of pathogen populations has important implications for breeding for disease resistance and fungicide screening programs. Based on the type of symptoms induced, two forms of *Pyrenophora teres* f. *teres* (PTT; net form) and *P. teres* f. *maculata* (PTM; spot form) were differentiated by Smedegard-Petersen (1971). Both spot and net forms of *P. teres* consist of a large number of pathotypes and resistance to the two forms is inherited independently (Ho et al. 1996; Arabi et al. 2003). The isolates of PTT and PTM could be distinguished more reliably by AFLP method than leaf symptoms. Cluster analysis following the application of AFLP procedure, divided the isolates of *P. teres* into two strongly divergent groups, corresponding to the net and spot forms of *P. teres* (Williams et al. 2001; Rau et al. 2002). In a later investigation, the Czech isolates of *P. teres* were screened by AFLP technique for their genetic diversity. The isolates of

P. graminea, *P. tritici-repentis* and *Helminthosporium sativum* were also included in the study. Each species had distinct AFLP profiles. Using 19 primer combinations, 948 polymorphic bands were scored. The PTT and PTM forms clustered into two distinct groups. The diagnostic markers-83 specific for PTT and 134 specific for PTM forms-were identified (Leišova et al. 2005).

The usefulness of AFLP procedure to study the genetic variation below the species level in a variety of taxa has been indicated. The genetic diversity of 114 isolates of *Pyricularia oryzae* (*Magnaporthe grisea*) collected from rice in the Red River Delta in Vietnam during 2001 and nine additional Vietnamese *P. oryzae* isolates was analyzed based on 160 polymorphic AFLP markers. Twelve different AFLP genetic groups among 123 field isolates were recognized based DNA similarity and cluster analysis. Isolates from *japonica* and *indica* hosts clustered into two separate groups with at least 60% dissimilarity with little evidence for gene flow between the two populations. In isolates from *indica* hosts, three groups representing 99% were predominantly encountered. Significant genotype flow occurred between the *indica* population south of Red river and the *indica* population north of Red river. Two avirulent isolates and 23 pathotypes were identified by inoculating 25 isolates selected from the 12 AFLP groups. The results suggested that the genetic structure of the *P. oryzae* populations of the Red River Delta in North Vietnam may be highly diverse (Thuan et al. 2006).

3.1.8 DNA Fingerprinting

The DNA fingerprinting procedure has been useful in bringing out similarities between races, biotypes, strains or related species of microbial pathogens. Based on the pathogenicity, the strains (41) of *Fusarium oxysporum* f.sp. *melonis* causing wilt disease of melons were divided into three groups that corresponded to Risser's races 0, 2 and 1-2y. Genetic variation among strains was analyzed by DNA fingerprinting with four repetitive DNA sequences-FOLR1 to FOLR4. Fingerprinting with four probes resulted in the recognition of 36 fingerprint types. The genetic groups formed after cluster analysis were correlated with races identified based on pathogenicity. The races 2 and 1-2y produced fingerprint types that formed a single cluster, while two distinct genetic groups were present in race O. The races 0 and 2 could not be differentiated using the parameters of fingerprinting suggesting the need for employing appropriate nuclear markers for differentiating the strains of fungal pathogens (Namiki et al. 1998).

3.1.9 Microsatellite Amplification

Microsatellites or simple sequence repeats (SSRs) are molecular markers that consist of tandem repeats of one to six DNA base pairs. The SSRs are highly versatile. PCR-based markers, generally associated with a high frequency of length

polymorphism. They are present in both coding and non-coding DNA sequences of all eukaryotes including plants analyzed (Gupta et al. 1996). Specific microsatellite population genetic markers have been shown to be useful in providing novel insight into many biological aspects of oomycetes pathogen *Plasmopara viticola* (Gobbin et al. 2003). PCR amplification of the four *P. viticola* specific microsatellite (SSR) loci, ISA, GOB, CES and BER and sequence-based fragment analysis were performed for high throughput analyses. *P. viticola* genotype were defined as a combination of eight microsatellite alleles, providing a multilocus genotype for each individual. Individuals with the same multilocus satellite genotype were considered to represent individual members of the same clone. Four multiallelic microsatellite markers were used to genotype the pathogen, *P. viticola*. All populations had high levels of gene and genotypic diversity. Significant low to moderate genetic differentiation was detected even between geographically close populations of European countries such as France, Germany, Greece, Italy and Switzerland. The high levels of genetic diversity in *P. viticola* were found to be in contrast to *P. infestans* and *P. cinnamomi*. GOB was the most polymorphic SSR and hence it proved to be useful in sharpening the distance among *P. viticola* populations. Significant isolation by distance could be discernible in central Europe populations of *P. viticola*. In contrast, no significant isolation by distance was noted in Greek populations possibly due to national geographic barriers like mountain and sea. It is likely that successful infection of grapevines in different European countries during the last 125 years, since its introduction from America, could be due to the high variability of *P. viticola* isolates (Gobbin et al. 2006).

A marker system that links expressed sequence tags (ESTs) and SSR has been applied to determine genetic variation. The frequency and distribution of SSRs in transcript sequences from oomycetes *Phytophthora infestans*, *P. sojae* and *P. ramorum* were investigated. The SSR lengths were found to be very restricted in the coding regions of oomycete genomes and approximately 99% of all the SSRs analyzed were shorter than 30-bp. There were no quantitative differences in the distributions of SSR motifs between *P. sojae* and *P. ramorum.* However, marked differences were detected based on comparison with *P. infestans.* The frequency distribution of SSRs was found to be strongly species-dependent. The results showed that glutamine was encoded by most highly abundant repeat in *P. sojae* and *P. ramorum*: (CAG) n. In contrast, the most common repeat in *P. infestans* encoded glutamic acid: (AAG) n. SSRs have been shown to be valuable molecular markers that can be used in fungal and fungus-like population genetics, genetic mapping and strain fingerprinting studies (Garńica et al. 2006).

3.1.10 Single-Strand Conformation Polymorphism Analysis

Single-strand conformation polymorphism (SSCP) analysis is useful to detect small nucleotide changes in the target fungal DNA fragments by electrophoresis. Different species of a fungal genus may be efficiently distinguished using SSCP analysis. The

DNA polymorphism in the ITS1 region of DNA of nine species of *Melampsora*, causing the willow rusts diseases in Japan, was assessed. The SSCP patterns in the amplified ITS1 region of *M. capracearum*, *M. epiphylla*, *M. larici-urbaniana, M. microsora* and *M. yezoensis* were shown to be species–specific. In the case of *M. chleidonii–pierotii* and *M. coleosporioides, M. epitea* and *M. humilis*, the SSCP patterns did not vary significantly, indicating their close relationship. The PCR-SSCP analysis to detect genetic variations in *Melampsora* spp. was more sensitive than PCR-RFLP analysis (Nakamura et al. 1998). SSCP analysis of the ITS2 region of the ribosomal DNA was used to rapidly and objectively identify different species of *Fusarium* associated with asparagus decline disease in UK and Spain. Over 360 fusarial field isolates were subjected to SSCP analysis. They were differentiated into four principal species viz., *F. oxysproum* f.sp. *asparagi* (*Foa*), *F. proliferatum, F. redolens* and *F. solani*. *Foa* was most frequently isolated from UK site, whereas *Foa* and *F. proliferatum* were present more frequently in Spanish site. *F. culmorum* was of minor importance. Two population of *Foa* were distinguished by a single ITS2 base transition. *F. proliferatum* was more abundant in Spain than in the UK (Wong and Jeffries 2006).

3.2 Molecular Basis of Variability of Bacterial Pathogens

Differentiation of bacterial plant pathogens based on conventional methods-biochemical and pathogenicity tests-are time-consuming and cumbersome. Often results are inconclusive. The molecular methods, in contrast, not depending on the cultural and environmental conditions provide results rapidly and reliably.

3.2.1 Immunoassays

Among the immunoassays, enzyme-linked immunosorbent assay (ELISA) has been widely employed for the detection and differentiation plant pathogenic bacteria. Benedict et al. (1989) demonstrated that 178 strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) could be differentiated and classified them into groups I–IV based on their reaction with four monoclonal antibodies (MABs). Two of these MABs cross-reacted with *X. campestris* pv. *oryzicola*, but no reciprocal reaction between the MAB specific for *X. campestris* pv. *oryzicola* and any of the four groups of *Xoo* strains could be seen. In a later investigation, 63 strains of *Xoo* were grouped into nine reaction types, consisting of four serovars and seven subserovars using MABs and polyclonal antibodies (PABs) (Huang et al. 1993). Three serovars, were differentiated among 215 strains of *Xanthomons albilineans* causing sugarcane leaf scald disease by immunofluorescence assay. Serovar I was the largest including strains from Australia, United States, Guadeloupe, India, Mauritius, South Africa. Serovar II contained strains from Africa-Burkino Faso, Cameroon, Kenya and Ivory Coast,

whereas serovar III, the smallest included strains from Caribbean Islands, Oceania (Fiji) and Asia (Sri Lanka) (Rott et al. 1994).

Xanthomonas campestris vesicatoria (*Xcv*) causes bacterial spot disease of tomato and pepper (chilli). *Xcv* strains were grouped into two races based on their ability to infect resistant tomato breeding line Hawaii 7998 (Wang et al. 1990). Later based on the ability to hydrolyze starch (Amy^+ or Amy^-) and the presence of an α or β band on silver stained SDS-PAGE, *Xcv* strains were divided into two groups. Group A consisted of Amy^- strains that had β-band (Bouzar et al. 1994). According to the new classification of phytopathogenic bacteria based on total DNA similarities, the A and B group strains of *Xcv* were named *X. axonopodis* pv. *vesicatoria* (*Xav*) and *X. vesicatoria* respectively (Vauterin et al. 1995). The ability of MABs to differentiate strains of *Xav* was investigated by producing three MABs 7AH10, 5HB3 and 4AD2. These MBs were specific to and could distinguish *Xav* strains that were able or unable to hydrolyze starch. The MAB 7AH10 generated against the Amy^- strains reacted positively with all the Amy^- strains and one of 11 Amy^+ strains in ELISA tests. The MAB 5HB3 recognized 97% of worldwide collection of *Xav* strains made in this investigation. By assaying on Japanese collection of strains against three MABs, the Amy^+ strains could be distinguished from Amy^- strains. All MABs reacted with the bacterial lipopoly-saccharide fraction of cell wall during immunoblotting (Tsuchiya et al. 2003).

3.2.2 Restriction Fragment Length Polymorphism

Analysis based on restriction fragment length polymorphism (RFLP) involves the use of specific restriction enzymes that react with specific sites on the test bacterial DNA, generating distinct band patterns. RFLP analysis has been useful for detection and differentiation of bacterial pathogens and their strains or pathovars. The RFLP patterns generated by digestion with *Pst* restriction enzyme from *X. oryzae* pv. *oryzae* (*Xoo*) strains of race 2 commonly prevalent in the Philippines could be used as basis for differentiation. The strains of *Xoo* occurring in the United States were considered to be not closely related to the Asian strains based on the differences in RFLP pattern, suggesting that the bacterium inciting bacterial blight in the US is likely to be a different pathovar of *X. oryzae* (Leach and White 1991). The investigation on the genetic diversity of *Xoo* strains in India indicated that all the strains collected in 16 of 18 locations sampled belonged to pathotype 1b with a single lineage (Yashitola et al. 1997). The strains of *X. campestris* were grouped into three RFLP groups 1, 2 and 3, based on band patterns generated. The strains belonging to RFLP group 1 and 3 had highly conserved RFLP patterns, while RFLP group 2 could be further subdivided. The RFLP groups corresponded to the groups recognized based on the results of biochemical and physiological tests and host range, indicating the usefulness of RFLP analysis to distinguish strains *X. campestris* causing bacterial leaf streak disease of cereals (Alizadeh et al. 1999).

Likewise, *Burkholderia glumae, B. gladioli, B. glumae* and *B. vandii* infecting rice could be differentiated by applying RFLP analysis (Ura et al. 1998).

The strains of *Xanthomonas axonopodis* pv. *citri* (*Xac*) (21) and *X. campestris* (*Xc*) (14) and five other pathovars of *X. campestris* were subjected to RFLP analysis. The strains of *Xac* and *Xc* showed distinct differences in the patterns generated, confirming the occurrence of *Xc* also in citrus nurseries. The cosmid clone PXCF 13–38 that includes almost the entire *hrp* genes cluster in *Xac* was employed as a probe for RFLP analysis of xanthomonads. Various species of xanthomonads could be identified by using the dendrogram developed during the investigation (Hartung and Civerolo 1989; Kanamori et al. 1999).

Erwinia amylovora (*Ea*) causing fire blight disease in apple, elicits a rapid and localized collapse of leaf tissue in nonhosts such as tobacco. This reaction known as hypersensitivity reaction is governed by a gene cluster of 30 kb designated *hrp* which is also necessary for pathogenicity of the bacterial pathogens. Close to the *Ea hrp* gene cluster, separated by only 4 kb is the *dsp* region coding for disease-specific function (Barny et al. 1990; Bauer and Beer 1991). The *dsp* region contains the *dspAIE* and *dspB/F* genes (Gaudriault et al. 1997; Bogdanove et al. 1998). RFLP analysis was applied to determine possible genetic variability of *hrpN* and *dspA/E* genes of *Ea*. The strains of *Ea* (73) collected from 13 Maloideae host species and from *Rubus* spp. and isolated from different geographic areas were examined by using RFLP analysis of the 3′*hrpN* gene and/or of a fragment of 1341 bp of the dsp A/E region. Restriction analysis of a fragment of 613 bp of the dspA/E region with *Cfo* I revealed an RFLP pattern that could be used to differentiate the *Ea* strains from *Rubus* spp. and *Amelanchier* sp. from all other strains (Giorgi and Scortichini 2005).

3.2.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) has been one of the frequently used molecular techniques for the detection of microbial plant pathogens. In a few cases, the PCR has been successfully applied for the differentiation of strains of bacterial pathogens. By employing tRNA consensus primers for amplification of DNA of different strains of *Xylella fastidiosa* (*Xf*), it was possible to recognize three different fingerprint groups. The strains causing citrus variegated chlorosis (CVC) and mulberry leaf scorch (MLS) were found to be different from other strains (Beretta et al. 1997). Primer sets of *X. axonopodis* pv. *citri* (*Xac*) that could distinguish pathotype A from *X. aurantifolii* pathotypes B and C were developed. These primers were designed based on sequence differences in the ITS region and the *pth* (pathogenicity) gene. The primer sets designed based on ribosomal sequences exhibited high levels of specificity for *Xac*. On the other hand, primers based on the sequences of the *pth* gene were universal for all types of citrus bacterial canker.

The emergence of copper-resistant bacterial pathogens has resulted in substantial reduction in the use and efficacy of copper-based bactericides. Copper resistance

genes in *X. campestris* pv. *vesicatoria* (*Xcv*) infecting pepper were located in plasmids in strains from California, Florida and Oklahoma (Bender et al. 1990; Cooksey et al. 1990). A strain of *Xcv*, XvP26 occurring in Taiwan had chromosome-encoded copper resistance (Canteros et al. 1995; Basin et al. 1999). The copper-resistant *Xcv* strains on pepper (51) and tomato (34) collected from several regions in Taiwan from 1987 to 2000 and nine copper-resistant strains from US and South America were investigated. The determinants for copper resistance on a 7652 bp *Xba* I/*Eco* RI chromosomal fragment in *Xcv* strain XvP26 were identified. The size of the 5.5 kb region that was functional in copper resistance in XvP26 was found to be similar to *Pseudomonas syringae* pv. *tomato* and *X. arboricola* pv. *juglandis.* No PCR amplification product was amplified using primers JB8 and JB18 from all strains from Taiwan, USA or South Africa whether they were resistant to copper or not, except the strain XvP26. This strain, XvP26 contained a uniquely oriented copper gene cluster, indicating that the introduction of such a strain into Taiwan was a rare event or chromosomal transfer from another organism might have occurred (Basin et al. 2005).

The PCR-based techniques involve the use of universal primers that generate an array of DNA amplicons known as genomic fingerprints. Generally these procedures including repetitive sequence-based rep-PCR have been frequently applied to fingerprint plant pathogenic bacteria. They generate a collection of genomic fragment via PCR, which are resolved as banding patterns that help to have high level of taxonomic resolution. Rep-PCR analysis examines the specific conserved repetitive sequences [repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences and BOX elements] distributed in the genomes of bacterial species of diverse origin. The primer sets based on the sequences corresponding to REP-ERIC and BOX sequences are employed. Rep-PCR has been widely employed to identify microbial pathogens, to differentiate strains and to assess their genetic diversity.

The field isolates of *Xanthomonas fragariae* were identified by generating the genomic DNA fingerprints using PCR amplification. The repetitive (rep)-PCR fingerprints of isolates were well correlated with pathogenicity tests (Opgenorth et al. 1996). Likewise, the patterns of DNA fragments generated by rep-PCR, after gel electrophoresis, could form the basis for differentiation of five subspecies of *Clavibacter michiganensis* subsp. *michiganensis*, *sepedonicus, nebraskensis, tessellarius* and *insidiosum*. The five subspecies recognized by rep-PCR fingerprinting technique were identical with current classification. The DNA primers (REP, ERIC and BOX) corresponding to conserved repetitive element motifs in the genomes of subspecies of *C. michiganensis*, viz., *michiganensis, sepedonicus, nebraskensis, tessellarius and insidiosum* were used to generate their genomic fingerprints. The patterns of DNA fragments obtained after agarose gel electrophoresis support the division of *C. michiganensis* into five subspecies (mentioned above). In addition, the rep-PCR fingerprints indicated the presence of at least four types viz., A, B, C and D within *C. michiganensis* subsp *michiganensis* (*Cmm*) based on limited DNA polymorphisms. Furthermore, relatively large number of naturally occurring avirulent *Cmm* strains with rep-PCR fingerprints identical to those of virulent *Cmm* strains (Louws et al. 1998).

The primers based on the sequences of the repetitive element *IS1112* commonly found in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) were used to generate PCR amplicons. The bands (13–35) amplified from *Xoo* genome provided a basis to assess the diversity within *Xoo* populations sampled (George et al. 1997). In a later investigation, the utility of *IS112*-based PCR in generating specific genomic fingerprints and their usefulness in determining genetic variation in Indian isolates of *Xoo*. Primers PJEL1 and PJEL2 used in insertion sequence *IS1112*-based PCR produced specific and reproducible fingerprint patterns for *Xoo* isolates tested. The isolates (16) were grouped into five different clusters (Gupta et al. 2001). The geographic origin of strains introduced into Florida, United States, was established by employing rep-PCR using ERIC and BOX primers (Cubero and Graham 2002). The simplicity, robustness and relatively high-resolution power offered, have made rep-PCR genomic fingerprints technique as the preferred one for characterization of phytobacterial populations.

The bacterial strains *X. axonopodis* pv. *phaseoli* (*Xap*) and *X. axonopodis* pv. *phaseoli* var. *fuscans* infect common bean inducing identical symptoms. The interrelatedness, genetic diversity and geographical distribution of the common bean bacterial blight (CBB) pathogens were determined by applying RFLP analysis of PCR amplified 16S ribosomal gene including the 16S–23S intergenic spacer region and repetitive element PCR (rep-PCR). The RFLP of *Xba* I digested genomic DNA using *Hrp* and pectate lyase genes as probes revealed that *Xap* and *Xap* var. *fuscans* were distinct (Chan and Goodwin 1999). The PCR-RFLP analysis, after digestion with *Hae* III of the pathogen DNAs, yielded five fragments in *Xap* var. *fuscans* and six fragments in *Xap* isolates. Two fragments, approximately 300 and 400 bp were detected only in *Xap* isolates. On the other hand, a 700 bp fragment was present in *Xap* var. *fuscans* isolates. Furthermore, *Mbo*I generated polymorphic bands and fragments characteristic of the two strains. By using rep-PCR analysis, *Xap* and *Xap* var. *fuscans* isolates could be precisely differentiated. Of 63 polymorphic fragments evaluated, 95% were polymorphic in *Xap* isolates compared to 72% in *Xap* var. *fuscans* isolates. Cluster analysis showed that two strains were genetically different (Fig. 3.3). The genetic diversity in *Xap* was greater than in *Xap* var. *fuscans* (Mahuku et al. 2006). The isolates of *Burkholderia glumae*, infecting rice seeds and causing panicle blight disease were divided into two major groups based on rep-PCR genomic finger printing and 16S–23S ribosomal DNA ITS sequence analysis. The genetic diversity of the isolates was relatively low as indicated by 16S–23S rDNA ITS sequence analysis (Sayler et al. 2006).

Xanthomonas campestris pv. *campestris* (*Xcc*) infecting *Brassica* spp. was differentiated into six pathogenic races based on their pathogenicity. The pulsed-field gel electrophoresis (PFGE) was applied to *Xcc* to generate physical maps of pathovars and to determine 16S rRNA copy number (Lin and Tseng 1997; Tseng et al. 1999). Later this technique was used to assess the genetic diversity among different isolates of *X. gardneri* infecting processing tomatoes (Quezado-Duval et al. 2004). Three molecular typing methods (rep-PCR, PFGE and AFLP) were employed to determine genetic diversity among isolates of *Xcc* existing in Israel and other geographical locations. A total of 22 isolates were divided into 11, 12 and 13 differential genotypes based on PFGE, AFLP and repetitive sequence based (rep-PCR) analyses

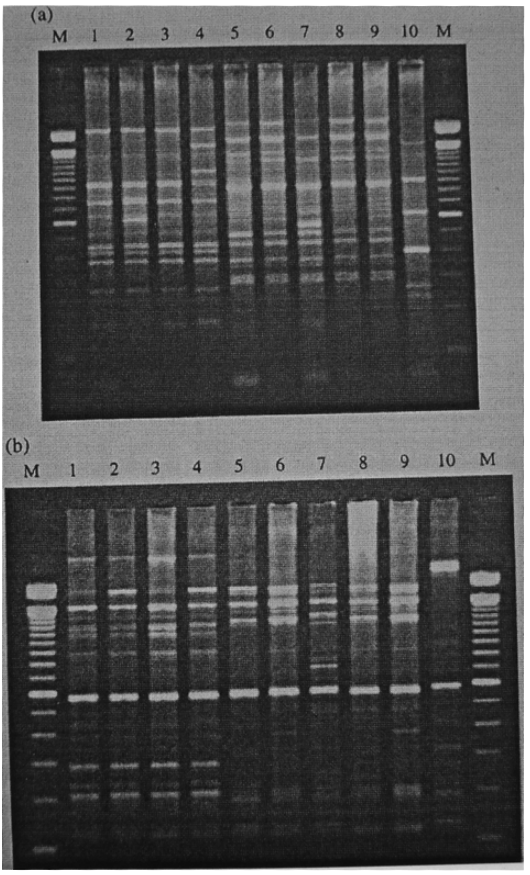

Fig. 3.3 Differentiation of isolates of *Xanthomonas axonopodis* pv. *phaseoli* var *fuscans* (*Xapf*) based on rep-PCR profiles generated by amplifying genomic DNA with ERIC and REP primers on agarose gel (**a**) ERIC primers; (**b**) REP primers. Lane M: 100-bp molecular size markers (standard); Lane 1–4: Isolates of *Xapf*; Lane 5–9: Isolates of *X. axonopodis* pv. *phaseoli*; Lane 10: Non-pathogenic *Xanthomonas* strain. (Courtesy of Mahuku et al. 2006; Blackwell Verlag, Berlin, Germany)

respectively. All collections of isolates yielded different genotypes and the findings indicated the high heterogeneity within *Xcc* than that was estimated earlier. The two race-3 isolates, HRI 5212 and HRI 6412 clustered in different PFGE, AFLP and rep-PCR types, indicating race affiliation cannot be inferred by DNA fingerprinting. No correlation between the genotype and pathogenicity was evident in this investigation (Valverde et al. 2007).

Xylella fastidiosa (*Xf*), a xylem-limited pathogen exists as strains with different pathogenic potential and variations in host range. The OLS strains (causing oleander leaf scorch) do not infect grape or almond, whereas PD strains (causing grapevine Pierce's disease) have a broader host range capable of infecting grape, alfalfa, almond and some weed species, but they do not infect oleander, peach or citrus. Almond strains (ALS) are included in two subspecies *X. fastidiosa* subsp. *multiplex* (*Xfm*) and *X. fastidiosa* subsp. *fastidiosa* (*Xff*). A relatively simple PCR based procedure was developed to distinguish the PD, ALS and OLS strains. PCR using primers XF 1968-L and XF 1968-R amplified a 638-bp fragment from OLS strains but not from PD strains or ALS strains that are included in *Xff*. PCR with

primers XF2542-L and XF 2542-R amplified a 412-bp fragment from PD strains, but not from OLS strains. PCR with primers ALM1 and ALM2 produced fragment of 521 bp from strains isolated from almond that belong to *Xfm*. The combination of the three primers set allowed the distinction of the two ALS genotypes of *Xfm*. Similar results were obtained from analysis of sequences of 16S–23Sr DNA ITS (Hernandez-Martinez et al. 2006).

3.2.4 Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD)-technique has been applied to identify, differentiate and establish the relationship between bacterial pathogens and their strains/pathovars. The combination of RAPD and PCR procedure has the potential to detect polymorphism throughout the entire genome as compared with other nucleic acid-based techniques. This technique is able to generate a spectrum of amplified products characteristic of the template DNA due to arbitrary priming at a relatively low annealing temperature at multiple locations. RAPD markers may be useful for strain identification and differentiation and other genetic analyses of microbial plant pathogens.

The association of *Erwinia* spp. and pectolytic pesudomonads with soft rot diseases pointed to the need for reliable and rapid identification of the causative agent(s). The RAPD-PCR technique was applied to differentiate *Erwinia carotovora* subsp. *atroseptica* and *carotovora*. Two randomly chosen primers were used to distinguish *E. carotovora* from pectolytic, fluorescent *Pseudomonas* spp. In addition, RAPD analysis was also useful in differentiating the subsp. *carotovora* and *atroseptica*. The results of RAPD technique in identifying 49 soft rot bacteria correlated well with those of biochemical tests (Mäki-Valkama and Karjalainen 1994; Parent et al. 1996). The RAPD banding profiles were generated by PCR amplification using six different 10-mer primers for differentiating strains of *E. amylovora.* The number of RAPD fragments shared between strains was used as the basis of cluster analyses. The strains infecting the Pomoideae and two strains infecting *Rubus* sp. (subfamily Rosoideae) formed two distinct groups, whereas two strains isolated from Asian pear in Hokkaido, Japan, constituted the third group (Momol et al. 1997).

Erwinia carotovora subsp. *carotovora* (*Ecc*) causing bacterial soft rot of mulberry (*Morus* spp.) was differentiated into two types (types 1 and 2). Type 1 strains were similar to *Ecc*, whereas type 2 strains were distinct from *Ecc* and other *E. carotovora* strains. The fragment containing the *pel* gene after digestion with *Sau3* AI restriction enzyme, produced three RFLP patterns (patterns 1, 2 and 3) (Seo et al. 2003). Later seven more mulberry strain of type 2 and reference strains were analyzed by SDS-PAGE and RAPD techniques. On the basis of SDS-PAGE profiles of whole-cell proteins, type 2 strains had high similarity with one another. In addition, the presence of one unique peptide band (28 kDa) was also seen. RAPD analysis of *Ecc* strains using primer OPB-07 was efficient in identifying and

differentiating the strains. Type 2 strains were clearly differentiated from type 1 strains as well as other *E. carotovora* strains, indicating the homogeneous nature of the strains tested. In contrast, other *E. carotovora* strains, including type 1 strains revealed genetic variability. Furthermore, the RAPD primer OPB-07 yielded a potential strain specific marker for type 2 strains (Fig. 3.4) (Seo et al. 2004).

Strains of different species of *Xanthomonas* and *Pseudomonas* have been identified and differentiated by applying RAPD analysis. It was possible to identify and differentiate strains of *X. albilineans* and to monitor the appearance of any new strain (s) in a geographic location by RAPD fingerprinting method (Peramul et al. 1996). Likewise, the races of *Pseudomonas syringae* pv. *pisi* were detected and identified based on the amplicons generated by the primers designed on the sequences of two unique fragments. Two pairs of oligonucleotide primers based on the sequences of the cloned DNA fragments of race 7 and 2 and the isolates were classified into groups I and II (Arnold et al. 1996). The strains (25) of *Xanthomonas axonopodis* pv. *dieffenbachiae* isolated from different host plant species and geographical locations were fingerprinted by using RAPD-PCR technique. RAPD markers (289) were generated in eight individual DNA profiles. There was a correlation between serotypes and the RAPD profiles for some groups of isolates. The RAPD profiles appeared to have some relationship with the host range of isolates. The study showed that RAPD markers are valuable tools for the study of the genetic relatedness among *X. axonopodis* pv. *dieffenbachiae* strains (Khoodoo and Jaufeerally-Fakin 2004).

Genetic variability of bacterial pathogens may be due to recombination and/or mutation. The study on molecular characterization of the strains of *E. amylovora* (*Ea*) and the sequence analysis of the *hrpN* gene revealed that the PCR product of the *hrpN* gene from strains of *Ea* from *Rubus* spp. and *Amelanchier* sp. following digestion with *Rsa*I enzyme, was smaller than the other strains. Sequence analysis of the gene revealed that this was due to the absence of a 60 bp fragment in

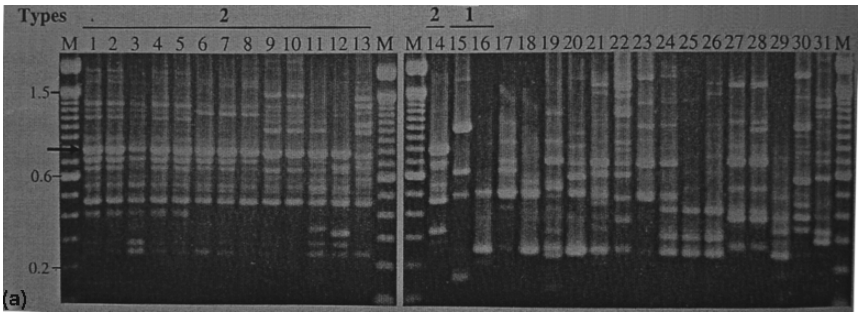

Fig. 3.4 Differentiation of strains of *Erwinia carotovora* using RAPD analysis employing primer OPB-07 on agarose gel

Arrow indicates the strain-specific marker for type 2 strains. Lane M: 100-bp molecular size marker (standard); Lane 1–14: Type 2 strains of *E. carotovora*; Lane 15 and 16: Type 1 strains of *E. carotovora*; Lane 17–31: Other strains of *Erwinia*. (Courtesy of Seo et al. 2004; The Phytopathological Society of Japan and Springer-Verlag, Tokyo)

the noncoding region downstream of the gene. The strain PD2915 isolated from *Amelanchier* sp. showed five same-sense substitutions and one missense subsitution at position 868 of the *hrpN* gene, converting aspartic acid to asparagine. In the *dspA/E* coding region, the four strains showed 13–14 missense point mutations, in some cases yielding drastic amino acid substitutions (Giorgi and Scortichini 2005).

Xylella fastidiosa, causing citrus variegated chlorosis (CVC) also is known to infect several economically important plants. Based on RAPD analysis and variable number tandem repeat (VNTR) of the strains from sweet orange cultivars, it was concluded that the population structure of *X. fastidiosa* (*Xf*) was not significantly affected by different sweet orange cultivars (Coletta-Filho and Machado 2003). In a later investigation, strains (360) of *Xf* isolated from sweet orange cv. Pera plants in five geographic regions of Brazil were examined for their genetic variation by using RAPD and VNTR markers. The VNTR markers were found to be more efficient for discrimination of *Xf* strains isolated from citrus compared with markers based on the H values (mean of genetic diversity) and the number of polymorphic alleles. The high values of genetic differentiation among *Xf* strains from different regions suggest a genetic structure according to region of host origin (Coletta-Filho and Machado 2003). The isolates of *Xf* cause grapevine Pierce's disease (PD), phony peach (PP) disease, plum leaf scald (PLS) and leaf scorch in almond (ALS), oak (OAK) and oleander (OLS) were studied. A high-resolution DNA sequence approach was adopted to examine the evolutionary relationships, geographic variation, and divergence lines among the isolates of *Xf*. Three genetically divergent phylogenetic clades PRC, MULT and OLS that differ by 2.6–3.3% at synomymous sites. Two of these clades corresponded to *X. fastidiosa* subsp. *piercei* (PD and some ALS isolates) and *X. fastidiosa* subsp. *multiplex* (OAK, PP, PLS and some ALS isolates). The third clade included all of the OLS isolates into genetically distinct group designated *X. fastidiosa* subsp. *sandyi*. These clearly defined clades indicate that *X. fastidiosa* has been a clonal organism (Schuenzel et al. 2005).

3.2.5 DNA–DNA Hybridization

The similarities between genomic fingerprints have been demonstrated to reflect DNA–DNA hybridization content similarities among strains within the bacterial genus. Such high levels of agreement between DNA fingerprint profiles and DNA–DNA homology values appear to indicate that rapid genomic fingerprint procedures may preclude, complement or even replace DNA–DNA experiments to clearly define *Xanthomonas* and other bacterial species under the present nomenclature scheme. Four phenotypic xanthomonad groups are pathogenic to pepper, tomato or both hosts. The groups A and C are found in *X. axonopodis* pv. *vesicatoria* (*Xav*), group B in *X. vesicatoria* and group C in *X. gardneri*. The DNA–DNA hybridization data indicated that *Xav* group A and C strains have less than 70% DNA relatedness with each other, with the type strain of *X. axonopodis* and with other classified

species within the genus *Xanthomonas* and hence they are given species status. It was proposed that the A strains of *Xav* be named as *X. euvesicatoria* and the C group strains be included in a new species *X. perforans* sp.nov. *X. gardneri* was proposed for the strains that have less than 70% relatedness with any of the *Xanthomonas* species (Jones et al. 2004).

The taxonomic relatedness among strains of *X. axonopodis* pv. *citri* (*Xac*), inducing five forms ("A", "B", "C", "D" and "E") of bacterial canker disease was determined by conducting DNA–DNA relatedness assays, sequencing the 16S–23S ITS regions and performing AFLP analysis. Three distinct genotypes of citrus pathogens (*Xac*, pathovars *aurantifolii* and *citrumelo*) were recognized: taxon I included all "A" strains; taxon II had "B", "C" and "D" strains and taxon III contained all "E" strains. It was proposed that taxa I,II and III citrus strains be named respectively *X. smithii* subsp. *citri, X. fuscans* subsp. *aurantifolii* and *X. alfalfae* subsp. *citrumelo* (Schaad et al. 2005).

Ralstonia solanacearum (*Rs*) is a heterogeneous species capable of causing vascular wilt diseases in many hundred plant species (Hayward 1991). By applying RFLP typing, *Rs* was divided into 46 multilocus genotypes (MLGs) which clustered into two major groups of strains designated division 1 and 2 (Cook and Sequeira 1994). The analyses of sequences of the 16S–23S rRNA gene ITS region, the polygalacturonase and endoglucanase gene also provided support for the earlier conclusions in addition to the identification of the third group of strains originating in Indonesia (Fegan et al. 1998). The fourth group of strains occurring in Africa was identified by employing PCR-RFLP analysis of *hrp* gene and sequencing of the endoglucanase gene of *Rs* (Poussier and Luisetti 2000; Poussier et al. 2000). These studies revealed that *Rs* species complex included four broad genetic group roughly corresponding to the geographic origin of strains. The four genetic groups were named as phylotypes. The strains that cause Moko and Bugtok diseases belong to phylotype II, while strain causing blood disease was placed in phylotype IV, based on the analysis of the sequences of the 16S–23S rRNA gene intergenic spacer region and the endoglucanase gene (Fegan and Prior 2005). Further dissection of phylotypes showed that *Rs* Moko disease-causing strains are polyphyletic forming four related, but distinct cluster of strains. Futher, strains causing Bugtok disease are indistinguishable from strains causing Moko disease in the Philippines. The strains causing blood disease were found to be closely related based on analysis of partial endoglucanase gene sequences (Fegan and Prior 2006).

In another investigation, the genetic diversity and relationships of *R. solanacearum* species complex strains from Asia and other continents were determined based on 16S rDNA endoglucanase and *hrpB* gene sequences. Various levels of polymorphisms were observed in each of these DNA regions. The highest polymorphism (about 25%) was found in the endoglucanase gene sequence. The *hrpB* sequence had about 22% polymorphism. The phylogenetic analysis consistently divided the strains into four clusters. The cluster 1 included all strains from Asia, which belong to biovars 3, 4, 5 and N2. Cluster 2 comprised the Asian strains of *R. solanacearum* (as biovars N2 and 1) isolated from potato and clove as well as the banana blood disease bacterium (BDB) and *Pseudomonas syzygii*. Cluster 3 contained strains from potato, race 2 biovar 1

strains from banana and race 1 biovar/strains isolated from America, Asia and other parts of the world. Cluster 4 was exclusively composed of African strains. This study revealed the distribution and genetic diversity of Asian strains and the similarity of Asian strains to those in other regions (Villa et al. 2005).

Potato common scab caused by gram-positive soil bacteria in the genus *Streptomyces* is responsible for heavy losses both in quality and quantity of marketable potatoes. A new strain isolated from scabby potatoes contained the characteristic pathogenicity island (PAI) and genes encoding the synthetase for the pathogenicity determinant thaxtomin and for a second pathogenicity factor, tomatinase. But the new strain lacked a third gene *nec1*, characteristic of the *Streptomyces* PAI. Instead, it contained a unique 16s rDNA gene sequence closely related to those of other pathogenic *Streptomyces* spp. This new strain was more virulent on potato and infected radish also. The results suggested that the new strain could have arisen by horizontal transfer of a PAI into a saprophytic streptomycete (Wanner 2007).

3.2.6 Amplified Fragment Length Polymorphism Technique

The population genetics of *Xanthomonas arboricola* pv. *pruni* (*Xap*) causing bacterial spot on stone fruits were studied. The bacterial populations in the United States, Itatly and France were compared by applying fluorescent amplified fragment length polymorphism (FAFLP) analysis, using sequences of four housekeeping genes (*atpD*, *dnaK, efp* and *gln* A) and the intergenic transcribed spacer regions. Among the 23 strains analyzed, only low diversity was discernible, since no sequence polymorphism could be noted. Population diversity as determined by FAFLP was lower for the West European population than for the American population. The same bacterial genotype was detected on the populations of *Xap* that existed in five countries on three continents, suggesting that this bacterial pathogen could have been aided by human activities for long distance spread and that it originated possibly in the United States (Boudon et al. 2006). The genetic diversity of *X. campestris* pv.*campestris* (*Xcc*) infecting commercial crop plant species has been studied in some detail. However, the variations among the strains of *Xcc* infecting weeds growing in cultivated and waste lands have not been assessed. By using AFLP technique, fingerprinting of over 68 strains was performed. The sequence analysis indicated the existence of seven genotypes of *Xcc* that were confined to either coastal or inland sites or both sites in California, USA. The strains of *Xcc* present in noncoastal weeds were unique and genetically distinct from strains present in weed populations growing in cultivated lands (Ignatov et al. 2007).

3.2.7 PCR-Based Suppression Subtractive Hybridization

Suppression subtractive hybridization (SSH) is a PCR-based method that can be applied to identify differences between prokaryotic genomes with different phenotypes

including those of pathogenic and nonpathogenic strains of the same species and between different, closely related species. In addition, SSH has been effective for analyzing genetic differences between plant pathogenic strains varying in host specificity (Harakava and Gabriel 2003; Zhang et al. 2005). The strains of *Erwinia amylovora* (*Ea*) infecting raspberry and other brambles (*Rubus* spp.) are unable to infect apple and pear, but they are closely related to the strains capable of infecting apple and pear as evidenced by similar AFLP and PCR fingerprints. PCR-based SSH was used to isolate sequences from *E. amylovora* strain Ea 110 pathogenic on apples and pear, that were present in three closely related strains with differing host specificities. SSH was used to generate six subtractive libraries to compare the genomes of fruit-infecting *Ea* strains with those of *E. pyrifoliae*, a Japanese *Erwinia* sp. strain and a Rubus-infecting strain of *Ea*. Using the SSH libraries, the strain-specific sequences including genes encoding a putative type III secretion system (TTSS) effector, a TTSS apparatus component and several putative membrane proteins were identified. In addition, an *Ea* 110-specific sequence homology to a TTSS secretion apparatus component of the insect endosymbiont *Sodalis glossinidus*, as well as an Ep1/96-specific sequence with homology to the *Yersinia pestis* effector protein tyrosine phosphatase YopH were also identified (Triplett et al. 2006).

3.3 Molecular Basis of Variability of Viral Pathogens

Plant viruses have much simpler structure compared with fungal and bacterial pathogens. Consequently the variations in the structural features and genomic constitution have to be studied to differentiate one from the other closely related ones. The plant viruses have a coat protein (CP) that encloses the viral nucleic acid. The coat protein (CP) plays a crucial role in vector transmission. The CP may be specially adapted for transmission by one or more species of the vector(s). The information on amino acid sequences of CP and nucleotide sequences of the viral genomes have provided the basis for differentiation of serotypes, pathotypes and strains of viruses. The immunological and nucleic acid-based techniques have been demonstrated to be useful for differentiation of plant viruses and their strains.

3.3.1 Immunological Techniques

Among the immunological techniques, the enzyme-linked immunosorbent assay (ELISA) has been frequently used for differentiation of virus strains based on the specific reaction with polyclonal or monoclonal antibodies. The hybridoma technology for generating monoclonal antibodies has enhanced the specificity and sensitivity of immunodiagnostic techniques (Khan and Dijkstra 2002; Narayanasamy 2001, 2005).

The monoclonal antibodies (MABs) react with specific epitopes present on CP or other structural or nonstructural virus proteins. The MAB reacted with

the nucleocapsid proteins of *Tomato spotted wilt virus* (TSWV), but not with envelope-associated proteins in the ELISA format. The isolates (20) of TSWV were grouped into three serotypes depending on their reactions to the antisera against virus nucleocapsid protein and glycoprotein (de Avila et al. 1990, 1992). The *Groundnut bud necrosis virus* (GBNV) was considered to be distinct from TSWV, since GBNV did not react with the antisera to TSWV obtained from different sources and this serotype seemed to be confined to Asia (Reddy et al. 1992). Based on nucleotide sequences of "N" gene and amino acid composition of the N proteins, TSWV, *Groundnut ringspot virus* (GRSV). *Tomato chlorotic spot virus* (TCSV) and *Impatiens necrotic spot virus* (INSV) were considered as four different species under *Tospovirus* within Bunyaviridae (de Avila et al. 1993). In a later investigation, the isolate M316, recovered from a naturally infected tomato in Argentina was analyzed using antisera against the tospovirus species TSWV, *Tomato chlorotic spot virus* (TCSV), GRSV and INSV. The reactivity of M316 was maximum with GRSV. The results of DAS-ELISA and Western blot analysis confirmed that M316 isolate belonged to serogroup II. The M316 isolate was almost identical on its nucleotide sequence of *N* gene with that of GRSV. The results indicated that the distinction of tospoviruses on the basis of N protein sequence divergence and serology appear to be most reliable paramaeters to establish species within *Tospovirus* genus (Dewey et al. 1995). Two strains of *Cowpea mild mottle virus* (CMMV) naturally infecting groundnut (*Arachis hypogaea*) inducing mild mottle (MM) and severe mottle (SM) symptoms were investigated using both biological and molecular properties. Both isolates reacted similarly, in ELISA and gel diffusion tests, with antisera to the carlaviruses *Cowpea mild mottle virus, Cassia mild mosaic virus* and *Potato M virus.* The MM isolates alone were transmissible by *Bemisia tabaci*, in addition to the differences in the electrophoretic mobilities of virus particles and amino acid composition of the coat proteins. Hence, the isolates were considered to be distinct strains of CMMV (Sivaprasad and Srinivasulu 1996).

Potato virus Y is the type species of the genus *Potyvirus* in the family *Potyviridae* which includes other genera *Rymovirus*, *Bymovirus* and *Ipomovirus. Potato virus Y* (PVY) isolates are divided into three main strain groups designated PVY^{O}, PVY^{N} and PVY^{C} according to symptoms induced in tobacco cv Samsun and potato. PVY^{C} isolates are nonaphid-transmissible (Blanco-Urgoiti et al. 1998). Potyvirus includes a large number of viruses showing relatedness to different degrees based on serological properties. Both PABs and MABs have been used to establish relationship between viruses. The MABs and PABs raised against the helper component-protease (HC-Pro) purified from plants infected with a nonaphid-transmissible strain of PVY were used to differentiate strains of PVY (Canto et al. 1995). A strain of *Potato Virus M* (PVM) were used to differentiate strains of PVM (Canto et al. 1995). A strain of *Potato virus M* (PVM) differing in the coat protein sequences in the amino terminals region was differentiated from other strains of PVM by applying ELISA format (Cavileer et al. 1998). Two serotypes of *Sweet potato feathery mottle virus* (SPFMV) occurring in Uganda could be differentiated by a specific MAB raised against the CP of SPFMV. These two serotypes differed significantly in their geographical distribution and ability to infect some cultivars of sweet potato (Kareyeija et al. 2000).

A new virus infecting *Ranunculus asiaticus* plants was detected and differentiated from three other viruses by applying DAS-ELISA antigen-coated plate (ACP)-ELISA and Western blot analysis. This virus was identified as a new member of the genus *Maclura virus* (Turino et al. 2006).

Fruit crops are infected by two or more viruses or their strains simultaneously posing considerable difficulty in resolving the components of mixed infections. The MABs raised against the CP of *Grapevine leaf roll-associated virus –1* (GLRaV-1) differed in their levels of specificity of reaction with CP as assessed by DAS-ELISA format and Western blotting. Two MABs (IC4 and IB7) that detected 25 and 32 isolates of GLRaV-1 respectively were used for the specific diagnosis of this virus. On the other hand, another MAB IG10 reacted with both GLRaV-1 and GLRaV-3 CPs (Seddas et al. 2000). Likewise, detection of the *Grapevine virus D* (GVD) was possible, when the specific MAB was employed in ELISA technique (Boscia et al. 2001). The *Plum pox virus* (PPV) serotypes PPV-D and PPV-M were differentiated by employing specific MABs in DAS-ELISA format. The results of the assay were correlated well with that of PCR assays or RFLP analysis of PCR products (Candresse et al. 1998). The strain-specific MAB raised against the sweet cherry isolate of *Plum pox virus* (PPV-SC) did not react in ELISA test with any of the additional PPV isolates indicating the ability of the MABs to differentiate the PPV-SC isolate from others (Myrta et al. 2000). The comparison of reactivity of MABs raised against isolates of *Prunus necrotic ringspot virus* (PNRV) infecting roses revealed that the most common PNRV serotype in rose was different from the most prevalent serotype in *Prunus* spp. While all rose isolates (27) could infect *Prunus persica* seedlings, three of the four *Prunus* isolates had poor pathogenic potential on *Rosa indica* (Moury et al. 2001).

Apple chlorotic spot virus strains could be identified and differentiated by employing different MABs. For routine screening of strains by ELISA, MAB5 was suitable, whereas MAB1, MAB2, and MAB9 were used for detection and differentiation of nontypical strain of the virus (Malinowski et al. 1997). Two serogroups of Israeli *Citrus tristeza virus* (CTV) strains were differentiated by using MABs. These groups were correlated with groups differentiated by sequencing of their coat protein genes (Shalitin et al. 1994). The isolates of CTV that induced stem pitting in sweet orange could be differentiated from other isolates that did not cause stem pitting by employing different combinations of PABs and MABs for trapping and as intermediate detecting antibodies in indirect DAS-ELISA. This differentiation has a practical utility, since the sweet oranges are seriously affected due to infection by stem pitting-inducing strains of CTV (Nikolaeva et al. 1998). The variability of CP gene of *Citrus psorosis virus* (CPsV) was determined by analyzing 53 psorosis field sources in Italy using 23 MABs. The analysis revealed nine serogroups and at least 10 different epitopes. The reaction patterns in triple antigen sandwich (TAS)-ELISA grouped the sources into nine patterns designated A to I, the most frequent pattern being A with 39.6% of the sources. Different species and cultivars growing in the same plot generally exhibited distinct reaction patterns. Analysis of 40 isolates from different countries using 24 MABs showed 14 reaction patterns and at least 17 different epitopes (Alioto et al. 2003).

Differentiation of 12 well-characterized strains of *Cucumber mosaic virus* (CMV) was possible by preparing MABs by using a mixture of CMV isolates belonging to subgroups I and II. In this study, the presence of virus-and subgroup specific epitopes on CMV-CP was also demonstrated (Hsu et al. 2000). Likewise, 51 isolates and strains of CMV viruses in Bulgaria were biologically characterized and serologically differentiated into subgroups I and II using different variants of ELISA with both PABs and MABs and immunodiffusion tests. The results of these tests correlated with PCR and RFLP data reported earlier (Hristova et al. 2002). The incidence of *Tomato mosaic virus* (ToMV) and *Tobacco mosaic virus* (TMV) together under field conditions was observed. By employing the specific MAB (10.H1) in plate-trapped antigen (PTA)-ELISA ToMV was efficiently detected in infected tomato plants. No cross-reaction with TMV was noted, since this MAB recognized only the band corresponding to the ToMV CP (17.5 kDa) indicating the reliability of the assay employed (Duarte et al. 2002).

Virus strains/serogroups have been differentiated more efficiently by using specific MABs raised against the target virus(es). The MABs raised against *Chinese wheat mosaic virus* (CWMV) were used to identify and differentiate the wheat and oat furoviruses, CWMV, *Soilborne wheat mosaic virus*-Oklahoma isolate ((SBWMV-Okl), *Oat golden stripe virus* (OGSV) and *European wheat mosaic virus* (EWMV) (Ye et al. 2000). By employing specific MABs, the incidence and distribution of the serotypes of *Barley yellow dwarf virus* was studied. The extent of incidence of PAV serotype transmitted by *Rhopalosiphum padi avenae* and MAV serotype transmitted by *Macrosiphum avenae* was demonstrated to be directly related to their respective aphid vector populations (Quillec et al. 2000). By using a specific MAB, the isolates of *Wheat yellow mosaic virus* from France and Japan could be differentiated in antigen-coated plate (ACP)-ELISA and indirect DAS-ELISA formats (Hariri et al. 1996). A new virus infecting Angelonia plants was assigned to the genus *Carmovirus* based on the results of ELISA and immunoblotting tests using PABs produced against the new virus which was designated *Angelonia flower break virus* (AnFBV) (Adkins et al. 2006).

The geminiviruses are characterized by the bisegmented (geminate) shape, the circular single-stranded DNA genome and transmission in a persistent manner by the insect vectors. The geographical distribution of the whitefly (*Bemisia tabaci*) – transmitted geminiviruses designated begomoviruses, roughly parallels that of the vector. The extent of serological relationships among different geminiviral species has been assessed by different immunological techniques. By employing monoclonal antibodies raised against *African cassava mosaic virus* (ACMV), *Indian Cassava mosaic virus* (ICMV) and a West African isolate of *Okra leaf curl virus*, it was demonstrated that the some epitopes detected by MABs SCR20 and SCR23 were conserved, whereas SCR32 and SCR58 detected smaller range of epitopes. Comparison of the epitope profiles of about 50 distinct begomoviruses showed that many of these profiles were specific for the target virus from other begomoviruses (Harrison and Robinson 1999). The begomovirus CP plays a crucial role in vector transmission. The whitefly-transmitted begomovirus have antigenically related particles, whereas geminiviruses transmitted by leafhoppers are antigenically unrelated

and have different vector species, revealing a parallelism between vector specificity and antigenic affinity (Harrison and Robinson 1999).

3.3.2 Nucleic Acid-Based Techniques

Detection, identification and differentiation of plant viruses and their strains have been done more precisely by applying nucleic acid-based techniques, more frequently by polymerase chain rection-based protocols. Crops like potato, tomato and other horticultural plants are infected by several viruses and their strains individually and in combination resulting in huge losses. The viruses/strains that are highly virulent have to be identified and differentiated to estimate the losses caused and to apply corrective/preventive measures to contain them.

The cucurbit-infecting Tobamoviruses have been distinguished based on the responses of *Chenopodium amaranticolor* and *Datura stramonium.* The International Committee on the Taxonomy of Viruses (ICTV) suggested, sequence similarity, host range and antigenic relationships as the criteria for species demarcation in the genus *Tobamovirus* (Lewandowski 2000). A new virus causing severe mosaic on cucumber fruits in greenhouses was identified as *Cucumber fruit mottle mosaic virus* (CFMMV). Complete sequencing of the viral genome indicated its genome organization was typical of *Tobamovirus.* But its sequence was distinct from other viruses described earlier on cucurbits. The riboprobe generated from the CP gene of *Cucumber green mottle mosaic virus* – Israel (CGMMV-Is) reacted strongly with total RNA extracted plants infected with (CGMMV-Is) and with its homologous virion RNA. However, this probe did not react with total RNA extracted from plants infected with CFMMV or with virion RNA of this virus. The reciprocal hybridization test also indicated similar differential reaction by the riboprobes (Fig. 3.5). Based on sequence data, phylogenetic analysis and biological properties, the separation of cucurbit-infecting viruses into two subgroups was proposed: subgroup I comprising of strains and isolates referred to as CV3, CV4, CGMMV-W, CGMMV-SH and CGMMV-Is and subgroup II consisting of CGMMV, CGMMV-Y and CFMMV (Antignus et al. 2001).

The presence of a protein covalently linked at the 5′-end of the genome and of a polyadenylated tail at the 3′ end, the genes for proteins putatively involved in the replication complex organized as a polyprotein precursor and their encoded amino acid sequences with conserved motifs, are the conserved features of potyvirus RNA genomes (Glais et al. 2002). The strain of *Potato virus Y* inducing potato tuber necrotic ringspot disease (PTNRD) was designated PVY^{NTN}. This strain causing enormous loss, was differentiated from another strain PVY^{N} by employing strain-specific endonucleases that cleave their respective PCR products. Single cleavages of PCR products derived from the 5′ end of PVY^{NTN} genome by *NcoI* and that of the PVY^{N} by *BglII* were effected and this was followed by PAGE analysis of digests. The restriction patterns formed the reliable basis for differentiation of these two strains of PVY. The components of mixed infections of potatoes could be resolved by PCR based techniques (Rosner and Maslenin 1999; Lorenzen et al. 2006).

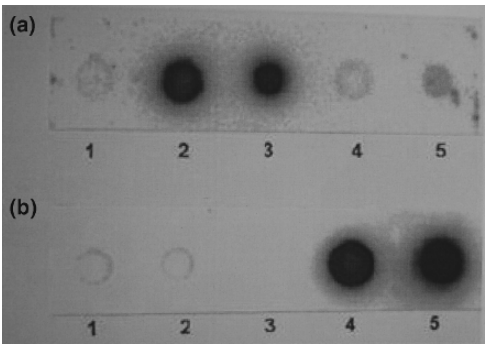

Fig. 3.5 Identification of *Cucumber green mottle mosaic virus* (CGMMV-Is) and *Cucumber fruit mottle virus* (CFMMV) by dot-spot hybridization of RNA preparations of the viruses using virus-specific riboprobes from cloned coat protein (CP) genes of respective viruses
(**a**) Lane 1: Total RNA from healthy cucumber; Lane 2: Total RNA from leaves infected by CGMMV-Is; Lane 3: CGMMV-Is virion RNA; Lane 4: Total RNA from leaves infected by CFMMV; Lane 5: CFMMV virion RNA; (**b**) Lane 1: Total RNA from healthy cucumber; Lane 2: Total RNA from leaves infected by CGMMV-Is; Lane 3: CGMMV-Is virion RNA; Lane 4: Total RNA from leaves infected by CFMMV; Lane 5: CFMMV virion RNA. Note the positive reaction of the riboprobes only with the respective viruses. (Courtesy of Antignus et al. 2001; The American Phytopathological Society, St. Paul, MN, USA)

The ability to induce veinal necrosis symptoms on indicator plants is considered as one of the chief characteristics used in classifying *Potato virus Y* isolates. Though several biological and molecular techniques have been tested, the results could not be linked to this biological property (inducing necrosis). A one-step fluorescent RT-PCR assay on a single nucleotide polymorphism (SNP) linked to the necrosis-inducing property of PVY isolates was developed. By using this protocol, 42 PVY isolates were assigned to their respective groups, in addition to achieving codetection of mixed samples containing close to equivalent PVY^N and PVY^O quantities. This assay is simple, rapid and sensitive. It is possible to conduct 96 tests in less than 3 h (from sampling to diagnostic results) with a detection limit of 10^5 PVY copies. This procedure has the potential for reliable classification of PVY isolates and being a substitute for biological assays performed on tobacco cv. Xanthi (Jacquot et al. 2005).

The potyviruses *Maize dwarf mosaic virus* (MDMV), *Sugarcane mosaic virus* (SCMV), *Johnsongrass mosaic virus* (JGMV) and *Sorghum mosaic virus* (SrMV) could be differentiated by extracting the total RNA followed by RT-PCR steps that generated a 327-nucleotide fragment of capsid protein gene. This fragment was subjected to nucleases *AluI* and *DdeI* and virus-specific restriction patterns were generated. Strains that have been characterized earlier and field-collected isolates of potyviruses infecting maize were efficiently differentiated by this technique (Marie-Jeanne et al. 2000). The necrotic strains of *Peanut stripe virus* (PStV-T) was differentiated from nonnecrotic isolates based on the nucleotide polymorphism existing in the CP sequence genes. The strain-differentiating oligonucleotides were deisgned

by cloning and sequencing the 3′ region of the PStV-T strain, including a part of the NIb region, the complete CP gene and the 3′-untranslated region. Nucleotide sequence differences unique to PStV-T were identified by comparing the sequences of nonnecrotic isolates of PStV. The 3′ end mismatch in nucleotides was considered as the basis for differentiating PStV strains. Such variations could be used for rapid, sensitive and reliable detection and differtiation of PStV strains (Pappu et al. 1998).

The molecular variability of *Lettuce mosaic virus* (LMV) isolates was assessed using immunocapture (IC)-RT-PCR technique coupled with direct sequencing. The isolates groups appeared to correlate with the geographical origins of the isolates rather than with their virulence/pathogenicity. The Californian isolates of LMV and the Western European isolates exhibited significant sequence similarities (Revers et al. 1997). Simultaneous detection and typing of two serotypes D and M of *Plum pox virus* (PPV) could be more efficiently performed by PCR-ELISA than by applying IC-PCR protocol. By employing strain-specific capture probes, two major serotypes could be differentiated without RFLP analysis of PCR products (Olmos et al. 1997; Pollini et al. 1999). *Bean common mosaic virus* (BCMNV) and their pathogroups (PGs) were detected and differentiated by applying a RT-PCR protocol in combination with restriction endonuclease analysis based on nucleotide sequences. Two virus-specific primer pairs that generated a PCR product specific for each virus were designed. Four BCMV–PG-V isolates were differentiated from isolates of BCMV pathogroups PG I, II, IV and VII by applying a RT-PCR protocol. Digestion of BCMNV-PCR products with restriction enzyme *Xba*I followed by electrophoretic separation helped differentiation of two BCMNV pathogroups (Xu and Hampton 1996). Four different viral species infecting *Ranunculus asiaticus* were detected and identified and a new viral species belonging to the genus *Potyvirus* was also differentiated. The viral genome fragments were amplified through RT-PCR and the amplicons were cloned and sequenced. Sequence and phylogenetic analysis suggested that three of the isolates belonged to the genus *Potyvirus* (Turino et al. 2006).

Virus isolates associated with sugarcane plants showing mosaic symptoms collected from 12 locations in Louisiana were identified by employing RT-PCR technique to distinguish *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV). None of the leaf samples tested showed the infection by SCMV during 2001–2003. RT-PCR based RFLP analysis revealed that SrMV strains I, H and M were prevalent in these locations. During the earlier survey (1978–1995) the predominance of strain H was noted. No RT-PCR product could be detected by either the SCMV or the SrMV-specific RT-PCR primer set for 8% of the plants showing mosaic symptoms suggesting the possible involvement of another virus capable of inducing mosaic symptoms in sugarcane besides SCMV and SrMV (Grisham and Pan 2007).

The isolates of *Pea seedborne mosaic virus* (PSbMV) from Australia (14), Pakistan (13) and one reference isolate from USA were examined using a RT-PCR assay based on an amplicon from the variable HC-Pro coding region of potyviruses in order to distinguish PSbMV isolates from seven other legume-infecting potyviruses. RFLPs generated from the HC-Pro RT-PCR products of all 28

PSbMV isolates classified them into eight groups and into three clusters based on a phylogenetic tree construction. There was no distinct association of the groups and clusters with either pathotype or geographical locations. The results indicated that the RT-PCR-RFLP combination may be applied to specific identification of PSbMV and has the potential for use as a simple, qualitative and rapid means of placing PSbMV isolates into groups and for mapping and tracking isolates of viruses in space and time (Torok and Randles 2007).

The relationship between the molecular variability of the isolates of *Prunus necrotic ringspot virus* (PNRSV) and their biological diversity was examined. Variations in the electrophoretic mobility of the RNA4 transcripts of PNRSV synthesized from the corresponding PCR products from six isolates of PNRSV were noted (Rosner et al. 1998). In another investigation the isolates (25) of PNRSV differing in the type of symptoms induced in six different *Prunus* spp. were used. The PCR amplicons from sequences of these strains were subjected to RFLP analysis, using *Eco*RI, *Taq*1 or *Rsa*I and most of the isolates could be differentiated by the restriction patterns. All isolates clustered into three groups based on the sequence comparison and phylogenetic analyses of RNA4 and coat proteins. No definite relationship between the type of symptoms induced or host specificity and the molecular variability could be established (Aparicio et al. 1999). Genetic diversity in 20 isolates of *Grapevine fan leaf virus* (GFLV) occurring naturally on grapevine was assessed. A 605 bp fragment containing a part of viral CP was amplified by RT-PCR procedure. Sequence variation among isolates was characterized by RFLP analysis and confirmed by sequencing. The infected grapevine plants had a complex mixture of closely related genomes. RFLP analysis after digestion with *Alu*I restriction enzyme revealed that GFLV populations in Tunisian vineyards consisted of two restriction types corresponding to distinct sub-populations SP1 and SP2. Relatively SP2 was more abundant. The sequences of isolates showed variation as much as 11% from each other (Fattouch et al. 2005).

Rice tungro disease (RTD) is caused by a combination of two viruses viz., *Rice tungro spherical virus* (RTSV) with an RNA genome and *Rice tungro bacilliform virus* (RTBV) with a DNA genome. Most of the symptoms of RTD are induced by RTBV, while RTSV helps the transmission of RTBV by the leafhopper vector. The RFLP analysis of the DNA of RTBV could form the basis for differentiating four strains G1, G2, Ic and L. Digestion with endonucleases *Pst*I, *Bams* HI, *Eco* RI and *Eco*RV of the viral DNA, produced identical restriction patterns for strains G1 and Ic. However, these strains could be differentiated from G2 and L strains, by digestion with *Eco*RI and *Eco*RV. These two enzymes generated patterns that could be used to differentiate strain G2 from strain L. The study indicated the possibility of using the RFLP analysis to determine the variability of a large number of field samples (Cabauatan et al. 1998). The potato leafroll virus (PLRV) and other viruses *Beet Western Yellows virus* (BWYV), and *New York barley yellow dwarf virus* (BYDV) were efficiently distinguished by restriction enzyme analysis of the PCR amplicons. In addition, all the five BYDV serotypes could be simultaneously detected and differentiated (Robertson et al. 1991). The RFLP profiles of the isolates of *Barley yellow dwarf virus* (BYDV) could provide a basis for their differentiation. The isolate BYDV-PAV-DK1 which

occurred rarely, could be differentiated based on the unique restriction enzyme pattern generated after digestion of the PCR products with *Hae*III from coat protein region. This unique RFLP profile of BYDV-PAV DK1 may be used for tracking the incidence of this isolate in epidemiological studies (Moon et al. 2000).

Sequence analysis of the 19 isolates of *Citrus tristeza virus* (CTV) indicated limited diversity of the CP gene in the populations. Diversity was slightly more in region "V" than in region "C" of the CP sequences. Phylogenetic analysis of the "V" and "C" regions of the CP showed that CPs V of Campania (in Italy) were clearly separated from the CPs V-4 isolate from Florida (in USA). The CTV isolates formed a cluster for C region, whereas two clusters for V region were identified (Alioto et al. 2003). CTV exists in the form of distinct strains with different biological properties, based on which the strains could be divided into two groups. The major coat protein (CP) gene was amplified by IC-RT-PCR technique to generate an amplicon of 672 bp from all isolates. Analysis of the nucleotide and the deduced-amino acid sequences of the CP genes revealed only minimal genetic variation among the isolates tested. However, the isolates of CTV from the eastern and western parts of Iran formed into two groups, suggesting that they might represent two independent introductions of the virus into Iran (Alavi et al. 2005).

The genome RNA (gRNA) of *Citrus tristeza virus* is a single stranded, positive sense, about 20 kb in size and organized into 12 ORFs, potentially encoding 19 protein products. Deletion mutagenesis of an infection cDNA clone of the genome showed that none of the proteins encoded by ORFs 2–11 was necessary for virus replication. Variation in pathogenicity characteristics of CTV isolates has been assessed indicating genetic diversity of the isolates. But the molecular determinant of symptom expressoin are yet to be studied. Serological reactivity determined using monoclonal antibodies, ds-RNA analysis or molecular hybridization experiments have revealed changes in CTV isolates after host passage or aphid transmission. These changes have been considered as basis of differentiation of CTV isolates. Genetic variation in two groups of CTV isolates, one from a Spanish source by successive host passages (T385, T317, T318 and T305) and other isolates (T388 and T390) obtained after aphid transmission of a Japanese source was investigated. Single-strand conformation polymorphism (SSCP) analysis of genes p18 and p20 was used for characterizing the population structure of the CTV isolates. The estimation of genetic diversity within and between isolates and evaluation of genetic differentiaton between population was performed based on nucleotide sequences of representative haplotypes of each isolates and gene. In some isolates within-isolate diversity was greater than diversity with other isolates because their population contained distinctly related sequence variants. Genetic variation of different genes, repeated inoculations in the field, homologous RNA recombination between sequence variants and the presence of different defective RNAs are some of the factors that may contribute to biological variability of CTV isolates (Ayllón et al. 2006).

Citrus tristeza virus (CTV) has a major capsid protein (CP) with molecular mass of 24 kDa. A panel of 12 CTV isolates of different origins with different biological properties were compared for polymorphism in CP gene of by cleavase fragment length

polymorphism (CFLP) and single-stranded conformation polymorphism (SSCP) analyses. Infected plant material from biologically characterized sources was analyzed by immunocapture RT-PCR followed by CFLP analysis targeted to CP gene. All isolates, except one, appeared in different clusters, frequently grouped with isolates or standards with which they shared some kind of geographic or biological relationship. By SSCP analysis, in contrast, most of the clones were not clustered in the same way. To assess the ability of CFLP to analyze biological samples, which may consist of a mixture of genomic variants, the CP gene of 12 CTV isolates was obtained directly from infected plants by immunocapture RT-PCR technique and analysed. Except a few, the isolates were correctly clustered according to the sequences of the variants composing the isolates. In artificial mixed infections of mild and severe isolates, the patterns obtained were more closely related to the severe isolates. This CFLP technique has potential for precise identification and differentiation of CTV isolates occurring under natural conditions (Marques et al. 2006).

Papaya is seriously affected by *Papaya ringspot virus* biotype P (PRSV-P) and *Papaya leaf distortion mosaic virus* (PLDMV), both belonging to the genus *Potyvirus*. PRSV strains are grouped into two biotypes which differ in their ability to infect papaya and cannot be differentiated by immunological assays. PRSV-W infecting Cucurbitaceae cannot infect papaya like PRSV-P (Purcifull et al. 1984). The biotype PLDMV-C isolated from a cucurbitaceous weed was unable to infect papaya, but it is able to react with a PLDMV-P antiserum, indicating the serological relationship between the two biotypes PLDMV-P and PLDMV-C. The CP sequence of PLMDV shares only 55–59% amino acid identity with that of PRSV, supporting the finding that they are serologically unrelated (Maoka et al. 1996). The molecular relationships among four strains of PLDMV were analyzed based on the amino acid sequences of CP. No potyvirus sequences identical to those of PLDMV-P(M) – P(YM) and-(CT) were found using a BLAST search. The phylogenetic analysis based on the CP amino acid sequences grouped PLDMV strains in one cluster, distinct from clusters of other potyviruses. In the PLDMV cluster P biotype strains (LDM, YM and M) are closely related to each other but slightly separated from strain T of biotype C. The CP sequences of three strains of PLDMV-P shared high identities of 95–97% whereas they shared lower identities of 88–89% with that of PLDMV-C. The C biotype differed from the P biotypes in its host range and CP amino acid sequence (Maoka and Hataya 2005).

Pepper (chilli) is infected by five *Potyvirus* spp. *Potato virus Y* (with worldwide distribution), *Tobacco etch virus* (TEV) and *Pepper mottle virus* (prevalent in America), *Pepper veinal mottle virus* (PVMV) and *Chilli veinal mottle virus* (ChiVMV) (present commonly in Africa and Asia respectively) (Brunt et al. 1978). To assess the genetic diversity within PVMV and the relationships with ChiVMV and other *Potyvirus* spp., the nucleotide sequences of the 3′–proximal part of the NIb gene, the entire CP gene and the 3′ nontranslated region (NTR) of PVMV isolates and potyvirus E, were determined. By applying RT-PCR, the isolates were differentiated into two species based on large indel in the CP gene. On the other hand, the isolates of PVMV and ChiVMV were grouped into three and two pathotypes respectively (Moury et al. 2005).

A novel carmovirus infecting Angelonia designated *Angelonia flower break virus* (An FBV) was characterized by determining the complete nucleotide sequence of a Florida isolate of AnFBV and CP sequences of Israeli and Maryland isolates. The AnFBV-CP was found to be most closely related to *Pelargonium flower break virus* (PFBV), whereas the AnFBV replicase shared higher identify with PFBV, *Carnation mosaic virus* (CarMV) and *Saguaro cactus virus* (SgCV). In addition, phylogenetic analysis also indicated the inclusion of AnFBV in the genus *Carmovirus* (Adkins et al. 2006).

Tomato spotted wilt virus (TSWV) with a wide host range, has quasi-spherical and enveloped particles enclosing a tripartite single-stranded genome composed of L, M and S RNA, while L RNA is of negative sense and M and S RNAs have an ambisense coding strategy (Goldbach and Peters 1996; Moyer 1999). The S RNA encodes the nonstructural protein NSs in a viral sense and the nucleocapsid protein N in a viral complementary sense. The nucleotide sequence identity in the N gene among TSWV isolates has been of high order (94–100%). They tend to group according to geographical location phylogenetically (de Avila et al. 1993; Pappu et al. 1998). TSWV, is able to adopt rapidly by reassortment of genome segments to infect new plant species (Moyer, 1999). The RT-PCR protocol was applied to differentiate four tospoviruses viz., TSWV, *Impatiens necrotic spot virus* (INSV), *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV) (Mumford et al. 1996; Dewey et al. 1996). Australian isolates (29) of TSWV were amplified by RT-PCR and directly sequenced. Nucleotide sequences of a 587 base pair region of the N gene (S RNA) were determined and compared. The nucleotide sequences of the Australian isolates were 95.7–100% identical in pair-wise comparison. The close relationship among isolates was confirmed also by phylogenetic analysis. On the other hand, population diversity within single TSWV isolates could also be detected when sequences of independent RT-PCR reactions were compared. No significant difference was seen in the N gene region of the TSWV isolates that break TSWV resistance genes in tomato or pepper when compared with other isolates. This indicates that a different region of the viral genome may possibly be involved in the breakdown of resistance (Dietzgen et al. 2005).

Carnation etched ring virus (CERV) with a double-stranded DNA genome belongs to the genus *Caulimovirus*. The genes of caulimoviruses are conserved. The ORFs I, II and III encode the movement protein (MP), aphid transmission (AT) protein and the DNA-binding protein respectively. The ORF IV encodes the coat protein (CP) which is processed by an aspartic protease included in the poly-functional protein encoded by ORF V. Formation of inclusion body (IB) matrix protein is governed by ORF VI which has a role in host range and symptom expression. The CERV-Indian isolate showed 99% similarity to other CERV isolates in respect of ORFI. The phylogenetic analysis based on MP indicated that *Cauliflower mosaic virus* (CaMV) and *Figwort mosaic virus* (FMV) formed one cluster, whereas *Mirabilis mosaic virus* and *Dahlia mosaic virus* (DMV) clustered into another group. Other caulimoviruses formed into a larger cluster. CERV-In showed 92% sequence similarity with the Dutch isolate in the case of genes of ORF II. The amino acid sequence similarity to CaMV and FMV was 51% and 37% respectively. High percentage (97%) of homology for DNA

binding protein (ORF III) between Indian and other CERV isolates was observed. This study revealed that true relatedness within the genus of *Caulimovirus* may be established more accurately by comparing the entire polyprotein sequence instead of its segments. The multiplex PCR protocol employed may be preferable because of economic aspects and time-saving properties (Raikhy et al. 2006).

The whitefily-transmitted begomoviruses are mostly bipartite and both components (DNA-A and DNA-B) have a size range of 2500–2800 nucleotides (nt). The viruses with monopartite genomes lack DNA-B. Protein-coding sequences in the virus strand are present in both DNA components. The genes located in the DNA-A and DNA-B encode various proteins with specific functions (Table 3.1) DNA-A contains *AV1* in the virus strand and the complementary strand has *AC1, AC2* and *A3* genes. *AV1* encodes the virus CP, while *AC1* controls the production of Rep proteins required for viral DNA replication. *AC2* produces a protein functioning as a transcriptional activator for the virus-sense genes in both DNA-A and DNA-B. The protein product of *AC3*, though not essential for infectivity, enhances the replication of the virus. The gene *BV1* in the virus and *BC1* in the complementary strands of DNA-B generate products involved in virus movement within the host plant and act cooperatively. The transport of viral DNA between the nucleus and cytoplasm is potentiated by the BV1 protein, whereas BC1 protein is involved in the viral cell-to-cell movement. In the DNA-A, an intergenic region (IR) is located in between the initiation codons of *AV2* and *AC1*. Similarly, an equivalent intergenic region is present in DNA-B also in between the initiation codons of *BV1* and *BC1*. The IR in DNA-A and DNA-B has very similar or identical sequences. The IR includes several regulatory elements (Hill et al. 1998).

The most conspicuous and important variation among the genomes of begomoviruses is the absence of a DNA-B component in a few viruses such as *Tomato yellow leaf curl virus* (TYLCV) (Navot et al. 1991). The sequences of DNA-B have been reported to be more diverse than those of DNA-A. This may facilitate hybridization of nucleic acid probes specific for DNA-A to a greater or lesster extent with the DNA of heterologous begomoviruses (Swanson et al. 1992). Among the DNA segments, IR exhibits the maximum variation, showing little similarity to one another (Zhou et al. 1998). The most conserved region among begomovirus genomes is the *AV1* (CP) gene. The relationships among begomoviruses based on

Table 3.1 Structure of begomovirus genomes

Genome component	Gene functions	References
DNA-A virus strand	*AV1* – Production of CP	Laufs et al. 1995
Complementary strand	*AC1* – Replication protein	
	AC2 – Transcriptional activation	Sunter and Bisaro 1991, 1992
	AC3 – Enhancement of viral replication	Sunter et al. 1990
	AC4 – Symptom production	Jupin et al. 1994
DNA-B virus strand	*BV1* – Virus movement between nucleus and cytoplasm	Sanderfoot and Lazarowitz 1996
Complemetnary strand	*BC1* – Cell-to-cell movement	Noueiry et al. 1994.

the amino acid sequences of CP show the close relatedness of the viruses occurring in a geographical location (Hong and Harrison 1995).

The cassava mosaic disease (CMD) is caused by several distinct whitefly- transmitted Geminiviruses and their strains. *African cassava mosaic virus* (ACMV) infects cassava in all regions.*East Africa cassava mosaic virus* (EACMV), *East Africa cassava mosaic Cameroon virus* (EACMCV). *East Africa cassava mosaic Zanzibar virus* (EACMZV) and *South African Cassava mosaic virus* have been reported to show variations. Virus identities were vertified by sequence analysis of DNA-A IR sequences. In addition, the sequences of PCR product comprising the DNA-A IR were analysed. EACMCV isolates from Nigeria were more closely related to EACMCV from Cameroon (EACMCV-CM) than to EACMCV-CI an isolate from Ivory coast (Pita et al. 2001). Comparative analysis of complete DNA-A genome sequences revealed 98% sequence identities of the EACMCV isolate from Nigeria. The IR sequences of ACMV isolates from East Africa were closely related to the IR sequences from ACMV from Nigeria. Analysis of EACMV isolates sequenced, did not show any considerable variations also indicating a tight sequence relationship with EACMCV-CM (Ariyo et al. 2005).

According to the guidelines of International Committee of Taxonomy of viruses (ICTV), to define an isolate of the begomovirus as distinct species, a rule of-thumb value of $<89\%$ nucleotide sequence identity threshold for DNA-A has been suggested. The nucleotide sequence identity of DNA-B is not to be considered as a reliable indicator of species demarcation due to the possibility of component reassortment or recombination in bipartite begomoviruses (Fauquet et al. 2003; Idris and Brown 2004). The nucleotide sequence identity among *Pepper gold mosaic virus* (PepGMV) components ranged from 91 to 96% DNA-A and from 84 to 99% for DNA-B, with each pepGMV component most closely related to the corresponding component of *Cabbage leaf curl virus* (CaLCV). The nucleotide sequence identity of the isolates of PepGMV is above the range suggested by ICTV for species demarcation threshold (89%). The three virus isolates were considered as distinct strains of PepGMV with capacity to exchange genetic material (Brown et al. 2005).

3.4 Molecular Basis of Variability of Viroid Pathogens

Single-strand conformation polymorphism (SSCP) technique was suggested as a the reliable alternative for the detection of differences in the genomic DNA. The strands (+ and −) of the double-stranded DNA, when separated, become metastable sequence-specific folded structures with distinct electrophoretic mobilities in non-denaturating polyacrylamide gels. It is possible to identify even single nucleotide exchanges, under these conditions (Orita et al. 1989). The SSCP analyses require less time compared to RFLP technique and its usefulness may be realized, when serologically indistinguishable strains of viroids have to be differentiated. SSCP analysis was applied to assess the variations in the sequences of field isolates of *Citrus exocortis viroid* (CEVd). Seven different groups of variants exhibiting one to six changes that did not reflect the overall variability among the CEVd clones were recognized. Additional

single nucleotide variations among clones that initially clustered together could be recognized by using SSCP analysis (Palacio and Duran-Vila 1999).

A single-tube reverse transcription-(RT)-PCR protocol was developed for the simultaneous detection of *Apple dimplefruit viroid* (ADFVd), *Apple scar skin viroid* (ASSVd) and *Pear blister canker viroid* (PBCVd) using a common pair of primers. However, the almost similar sizes of the resulting cDNAs did not show clear separation after electrophoresis, limiting the applicability of this protocol for detection and differentiation of the viroids. Later, an RT-PCR method with fluorescent primers that allow the simultaneous detection and differentiation of ASSVd and ADFVd in infected apples was employed. The sequence variability of two ADFVd field isolates from two commercial apple cultivars was studied. Sequencing of 18 full-length cDNA clones revealed five new sequence variants. Comparison of sequences revealed nine polymorphic positions distributed in different regions of the AFDVd molecule. By employing two viroid-specific primers, each labeled with a different fluorescent dye in a multiplex fluorescent RT-PCR amplification, the viroids ADFVd and ASSVd could be detected and differentiated. This technique simplifies and facilitates distinction of the amplified products. In addition, it avoids the use of mutagenic and cancer-inducing agent ethidium bromide for staining the gels (Di Serio et al. 2002).

Appendix 1: Microsatellite-Primed (MP) Polymerase Chain Reaction for DNA Fingerprinting (Ma and Michailides 2005)

A. Preparation of DNA

i) Cultivate the fungal pathogen (*Botrytis cinerea*) isolate in petridish containing potato dextrose agar (20 ml) for 4 days at 25°C; harvest the mycelia and wash in sterile water.
ii) Snap freeze in liquid N and lyophilize
iii) Extract the genomic DNA using the Fast DNA Kit (Qbiogene Inc., Carlsbad, CA); suspend the final genomic DNA from each isolate in DNA Elution Solution ultra-pure water (DES) and standardize the DNA concentration to 10 ng/μl using the Hoefer DyNA Quant 200 Flurometer (Hoefer Pharmacia Biotech. Inc. San Francisco, CA).
iv) Confirm the purity of DNA from each isolate for suitability for PCR amplification by generation of a single band with universal primers ITS 1 and ITS4.

B. Microsatellite- Primed (MP) – PCR Amplification

i. Perform PCR amplifications in a 50 μl volume containing 100 ng of template DNA, 1.0 μM microsatellite primer, 0.2 mM of each dNTP 2.5 mM $MgCl_2$, Ix Promega *Taq* polymerase buffer and 2U of Promega *Taq* polymerase buffer.

ii. Follow the parameters for amplification: an initial preheat at 95°C for 3 min; 40 cycles at 94°C for 1 min; 50°C for 1 min, 72°C for 1.5 min and terminated with a final extension at 72°C for 10 min.
iii. Perform the PCR twice for each isolate
iv. Separate the PCR amplicons using 1.5% agarose gels in Tris-acetate (TAE) buffer; stain the gels with ethidium bromide and photograph.

Appendix 2: Amplified Fragment Length Polymorphism (AFLP) Analysis of *Pythium* spp. (Garzón et al. 2005)

A. Purification of DNA of Test Fungal Pathogen

i. Cultivate the pathogen (*Pythium* spp.) in 20% Vg broth shake cultures for 3–10 days at 110 rpm; collect the mycelium on filter paper using a Buchner funnel; rinse with water and macerate using liquid N.
ii. Extract the DNA using DNAzol ES plant genomic DNA isolation reagent (MRC, Inc., Cincinnati, OH) as per manufacturer's instructions.
iii. Assess the quality of the DNA by applying the agarose gel electrophoresis technique by comparing the samples with a High DNA Mass Ladder (Invitrogen Life Technologies, Carlsbad, CA) and by measuring the nucleic acid/protein absorbance ratio using spectrophotometer.

B. AFLP Analysis

i. Double digest genomic DNA and ligate the DNA fragments to adapters, using *Eco* RI and *Mse* I digestion enzymes and T4 ligase in a single step restriction – ligation reaction at 37°C for 8 h in a vol of 10 μl [T4 DNA ligase buffer 1.1 μl; 0.5 M NaCl 1.1 μl; *Mse* I 0.05 μl; *Eco* RI 0.5 μl; T4 DNA ligase 0.03 μl; BSA (1 mg/ml) 0.55 μl *Mse* I adapter solution (5 μM of each primer) 1 μl; DNA template ($\sim$100 ng/μl) 1.0 μl; polymerase chain reaction water 4.1 μl]
ii. Dilute restriction/ligation products (R/L) upto 200 μl in TE-0.1 (20 mM Tris–HCl, 0.1 mM-EDTA, pH 8.0)
iii. Perform preselective amplification using primers *Eco* RI+A and *Mse* I+C primers in 20 μl PCR reactions [R/L 4.0 μl; *Eco* RI preselective primer (10 μM) – 0.5 μl; *Mse* I preselective primer (10 μM) 0.5 μl; *Taq* DNA polymerase (5 U/ml) 0.08 μl; 10 $\times$ Taq buffer 2 μl; 10 $\times$ dNTPs (2 mM each) 2 μl; PCR water 10.92 μl].
iv. Follow the parameters: 2 min at 72°C followed by 25 cycles of a 20 s DNA denaturation step at 94°C, a 30-s annealing step at 56°C and a 2 min extension step at 72°C.
v. Dilute the PCR products upto 200 μl with TE-0.1

vi. Perform selective amplificaiton using the same *Mse* I preselective primer and fluorescently labeled *Eco* RI + AG primer with the following cycling profile: 2 min at 94°C followed by nine cycles of a 20-s denaturing step at 94°C, a 30-s annealing stepwise step at 65°C reducing 1°C every cycle down to 57°C and a 2-min extension step at 72°C, followed by 20 cycles of a 20-s denaturitng step at 94°C, a 30-s annealing step at 56°C and a 2-min extension step at 72°C.
vii. Perform electrophoresis with aliquots and 0.8 μl of the final products inpolyacrylamide gels and read using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) and analyze using the Gene Scan analysis software ver.3.7 (Applied Biosystems) and visual evaluation of gel images and spectrograms to produce one AFLP band profile (AFLP fingerprint) per isolate.

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Glossary

ABC transporters A large family of proteins embedded in plasma membrane characterized by a highly conserved ATP-binding domain; they constitute a class of membrane transporter proteins capable of transferring sugar molecules, inorganic ions, polypeptides, certain anticancer drugs and antibiotics

Acquired resistance Enhancement of resistance of plants following infection or treatment with biotic or abiotic resistance inducers

Actin Structural proteins are present abundantly in eukaryotic cells that can interact with many other proteins; these proteins polymerize to form cytoskeletal filaments

Activator Transcription factor that stimulates transcription

Active site The region of an enzyme that binds substrate molecules and catalyzes an enzymatic reaction

Active transport Export of an ion or small molecule across a membrane against its concentration gradient driven by the hydrolysis of ATP

Alarm signal In response to pathogenic infection, chemical(s) produced, may send signal to host cell proteins/genes leading to synthesis of compounds inhibitory to the invading pathogen

Algorithm A logical description of the manner in which a problem can be solved and that can be used as a specification for how to write a computer program; a computational procedure that employs a combination of simple operations to process, analyze and present pictorially data about sequences of DNA, RNA, proteins or other molecules

Alignment This is an arrangement of two or more molecular sequences one below the other in such a way that regions that are identical or similar are placed immediately below one another

Allele It is an alternate form of a gene occupying a given locus on the same chromosome which controls expression (of product) in different ways

Allozyme An allele of the gene may be involved in the production of an enzyme with properties slightly different from that of the enzyme produced by the gene concerned

Amino acid profile Quantitative delineation of how much of each amino acid is present in a particular protein

Aminoacyl-tRNA synthetase Enzyme that catalyzes the linkage of an amino acid to a specific tRNA to be used for protein synthesis

Amplicon A specific sequence of DNA that is produced from the template fragment by reactions such as polymerase chain reaction assay

Amplification Synthesis of additional copies of a DNA sequence found either as chromosomal or extrachromosomal DNA

Analog A compound or molecule that is a structural derivative of a "parent" compound; this term also refers to a molecule that may be structurally similar, but not identical to another, and may exhibit many or some of the biological functions of the other

Ankyrin A protein capable of binding spectrin and links the actin cytoskeleton to the plasma membrane

Annealing The process by which the complementary base pairs in the strands of DNA combine

Annotated gene A gene is said to be annotated when it has been recognized from a large segment of genome sequence and if its cellular role is known to some extent

Antibody A protein (immunoglobulin) formed in an animal circulatory system in response to introduction of an antigen (usually a protein or nucleoprotein)

Anticodon The sequence of three bases on tRNA that combines with the complementary triplet codon on mRNA

Antigen A protein or live or inactivated microbe capable of inducing the production of a specific antibody in an animal system and capable of reacting with that antibody in a specific manner

Antisense technology A molecular technique that uses a nucleic acid sequence complementary to mRNA so that the two bind and the mRNA is effectively neutralized

Apoplast Space outside of the plasma membrane of cells constituted by cell walls and conducting cells of the xylem in which aqueous phase of intercellular solutes is present

Apoptosis It is a natural phenomenon encountered in animal cells for the elimination of cells involving a highly regulated, energy-dependent process by which a cell brings about its own death (programmed cell death in plants)

Attacins These proteins constitute a class of antimicrobial peptide produced in the insect hemolymph; synthesis of outer membrane proteins of Gram negative bacteria is inhibited by attacins

Avidin A protein found in egg white and bacteria like *Streptomyces* that binds with very high affinity to the vitamin biotin (vitamin H)

Avirulence Opposite of virulence indicating the inability of a pathogen to infect a particular species or cultivar of a plant, presumably because of the presence of avirulence gene(s) whose product(s) alerts the host resistance mechanism

Avirulence conferring enzyme (ACE) The avirulence gene of the fungal pathogen coding for the enzyme that is recognized by the resistant cultivar carrying corresponding resistance gene. The rice resistance gene *Pi33* recognizes *Magnaporthe grisea* isolates carrying *ACE 1* gene

***Avr* gene** Pathogen gene that is responsible for the inability to infect a given plant species or cultivar

Avr protein The protein coded for by the *avr* gene, capable of functioning as an elicitor of defense responses in plants

Bacterial artificial chromosome (BAC) DNA molecules that are used as cloning vectors, derived from plasmids. This type of vector can be employed for cloning large inserts (of about 150,000 bp) of DNA in bacteria

Base pair (bp) Association of two complementary nucleotides in a DNA or RNA molecule stabilized by hydrogen bonding between their base components: adenine pairing with thymine or uracil and guanine pairing with cytosine

Biofilm Bacteria or fungi may be embedded in polysaccharide matrix that plays a role in attachment to host surface, colonization and invasion

Bioinformatics Computer based analysis of data on biological sequencing of the genome of an organism to predict gene function, protein and RNA structure, genome organization and molecular bases in relation to responses of plants to microbial pathogens

Biolistic This term has been coined from the words “biologic” and “ballistic”; refers to process involving the use of pellets coated with the desired genes that are fired from a gun into seeds or plant tissues in order to get plants expressing these transgenes

Bioluminescence Emission of light from a living organism

BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) This program is used widely for searching sequence databases for entries that are similar to a specified sequence in question

Bootstrapping A statistics protocol used to estimate the reliability of a result (such as construction of phylogenetic tree) that involves sampling data with replacement from the original set of data

Calmodulin A small cytosolic regulatory protein that binds four Ca^{2+} ions; the Ca^{2+}/calmodulin complex can interact with many proteins resulting in inhibition or activation of those proteins

Capsid Protein coat of viruses that encloses the viral genome

***Cauliflower mosaic virus* 35S promoter (CaMV 35S)** This promoter consisting of DNA sequences of the virus is employed very frequently in genetic engineering to control expression of an inserted gene (synthesis of desired protein in a plant)

cDNA A complementary DNA strand to an RNA strand and synthesized from RNA strand using reverse transcriptase enzyme. It is also known as copy DNA.

cDNA clone A DNA molecule synthesized from a mRNA sequence via sequential use of reverse transcription and DNA polymerase.

cDNA library A collection of cDNA clones which represent all the genetic information expressed by a given cell or by a given tissue type is known as a cDNA library

Cecropins Antimicrobial proteins isolated from insects capable of forming pores in and causing lysis of bacterial cell membrane (see humoral immunity)

Chaperon protein A protein molecule attached to and to facilitate the export of an effector protein secreted through a type III secretion system of bacteria. It may protect the effector protein from coming in contact with other proteins. The chaperones may also assist with correct folding as the protein molecule emerges from the cell's ribosome.

Chemotaxis Capacity of a cell or organism to move toward or away from certain chemicals

Chromosome walking A technique employed for determining the location of and sequencing a given gene within the genome of the organism, by sequencing specific DNA fragments that overlap and span collectively

cis-acting protein This protein has the exceptional property of acting only on the molecule of DNA from which it was expressed.

Cistron Viral sequences of nucleotides of DNA/RNA that code for particular protein and are considered to be analogous to genes of other organisms

Clade A clade consists of all the species descending from an internal node of genealogical tree

Clone Genetically identical individuals produced asexually from one individual

Cloning of DNA Introduction of a segment of DNA from one species into the DNA of another species leading to the formation of many copies of the hybrid DNA by replication

Codon A sequence of three nucleotide (triplet) bases on mRNA that interacts with the anticodon on tRNA and specifies the incorporation of a specific amino acid into a polypeptide during translation process

Codon usage The frequency of occurrence of each codon in a gene or genome, especially the relative frequencies with which synonymous codons are used

Colony hybridization A technique used in situ hybridization to identify bacterial colonies carrying inserted DNA that is homologous with some particular sequence (used as probe)

Complementary sequences Two nucleic acid sequences that may form an exactly matching double strand as a result of A-T and G-C pairing. Complementary sequences run in opposite directions

Constitutive genes These genes code for certain protein products that are required at all times for general cell maintenance and metabolism. These genes are expressed as a function of the interaction of RNA polymerase with the promoter without additional regulation. They are also known as household genes

Contigs Overlapping DNA segments that as a collection, form a longer and gapless segment of DNA

Cosmid It is a larger insert cloning vector, useful for analysis of highly complex genomes; forms an important component of many genome mapping projects; cosmid vectors are constructed by incorporating the *cos* sites from the phage λ

Cross-protection Enhancement of resistance in plants following infection by one virus or its strain(s) to reinfection by the same virus or its strains/related viruses

Cytoplasmic resistance Resistance attributed to the genetic material present in the cell cytoplasm

Cytoskeleton A three-dimensional network of fibrous elements comprising primarily of microtubules, actin microfilaments and intermediate filaments found in the cytoplasm of eukaryotic cells; the cytoskeleton offers structural support for the cell and permits directed movement of organelles, chromosomes and even the cell

Cytosol Unstructured aqueous phase of cytoplasm excluding organelles, membranes and insoluble cytoskeletal components

Dalton A mass unit used to indicate the size of a biomolecule; one dalton is equivalent to molecular mass of a hydrogen atom

Data mining A computerized program used to search for relationships between and overall patterns among the data available within a database.

Defensins Defense-related, cysteine-rich, antimicrobial peptides present in the plasma membrane of host plant species; they constitute a group designated defensins capable of providing resistance against microbial plant pathogens

Denaturation The conformation of a protein or nucleic acid may be drastically altered because of disruption of various bonds due to heating or exposure to chemicals; this may result in loss of biological function(s)

Dendrogram A branching diagram that shows the relative sequence similarity between many different proteins or genes to indicate the phylogenetic relationships; typically horizontal lines indicate the degree of differences in sequences, while vertical lines are used for clarity to separate branches

DNA fingerprinting/DNA profiling This technique involves the use of restriction enzymes and electrophoresis to determine the differences and similarities in the DNA of individual organisms

DNA library This represents a collection of cloned DNA molecules/fragments of entire genome or DNA copies of all mRNAs produced by a cell type (cDNA library) inserted into suitable cloning vector

DNA ligase The enzyme that seals breaks in DNA strand and also catalyzes the formation of the final phosphoester bond in DNA replication

DNA microarray Oligonucleotides or fragments of cDNAs are printed on a glass slide or membrane filter at a high density, permitting simultaneous analysis of thousands of genes by hybridization of the microarray with fluorescent probes

Domain A portion of polypeptide chain that folds into a compact globular units of the protein forming the basic unit of the tertiary structure and remains distinct even within the tertiary structure of the protein; a discrete part of a protein with its own distinct function

Downstream A relative direction of DNA sequence, as the DNA is usually written with 5′ end to the left; downstream would be to the right of a reference point – eg. the start codon is downstream of the protmoter

Ectopic A gene inserted in an unnatural location

Effector proteins Bacterial virulence determinants injected into host cells via type III secretion system (TTSS) of the bacterial pathogen

Effectors These molecules can manipulate host cell structure and function, thereby facilitating infection (virulence factors/toxins) and/or triggering defense responses (avirulence factors/elicitors)

Elongation factor A group of nonribosomal proteins required for continued translation of mRNA (protein synthesis) following initiation; guiding the elongation phase of transcription or translation, during protein synthesis

Endoplasmic reticulum (ER) An extensive network of membrane-enclosed tubules and sacs involved in protein sorting and processing as well as in lipid synthesis

Episome An independent genetic element (DNA) that is present inside the bacterial species in addition to the normal bacterial cell genome. The episome can replicate either as an autonomous unit or as one integrated into host genome.

Epitope A specific group of atoms on an antigen molecule that is recognized by the specific antibody produced against the antigen concerned; it is also called as antigenic determinant

Exon A segment of an eukaryotic gene that contains a coding sequence and this part of gene sequence is transcribed into an mRNA and translated to give rise to a specific domain of the protein

Expressed sequence tags (ESTs) These are partial sequences of cDNA clones in an expressed cDNA library; useful for identifying all unique sequences (genes) to determine their functions; a profile of mRNAs allowing cloning of a large number of genes being expressed in a cell population

Expression vector A modified plasmid or virus that carries a gene or cDNA into suitable host cell in which it directs synthesis of the encoded protein; expression vector can be used to screen DNA libraries for a gene of interest or to produce large amounts of a protein from its cloned gene

Expressivity The intensity of realization of the effect of a gene in a phenotype; the degree to which a particular effect is expressed by individuals.

Extracellular matrix Secreted proteins and polysaccharides that fill the spaces between cells and bind cells and tissues together

Flagellin These proteins associated with flagella of bacteria, are capable of functioning as a receptor system for general elicitors

Flanking sequence A segment of DNA molecule that either precedes or follows the region of interest on the molecule

Flux The flow of intermediates in metabolism; the rate at which substrates enter and exit a pathway

Footprinting This is a technique employed for identifying protein-binding regions of DNA or RNA based on the ability of a protein bound to a region of DNA or RNA to protect that region from digestion

Free radical A molecule that has one or more unpaired electrons

Functional genomics Functions and interactions of genes or groups of genes belonging to host plants, pathogens or both are genetically examined

Gene cloning Characterization of gene functions by isolating and multiplying individual gene or sequences by insertion into bacteria (*Escherichia coli*); the sequences are duplicated as the bacteria multiplies by fission

Gene expression Conversion of genetic information within a gene into an actual protein or cell process; an overall process applied to assess the information encoded in a gene that can be converted into an observable phenotype (production of a protein)

Gene expression profiling Identification of specific genes that are "switched on" in a cell at particular point of time or process, enabling the precise definition of the phenotypic condition of that cell

Gene flow Movement of genes (under examination) through specific process, from one population to another population geographically separated apart

Gene knockouts Genetically modified individuals containing either a defective gene or lacking a gene

Gene silencing Interrupting or suppressing the activity of desired gene(s), resulting in the loss of coordination for production of specific proteins

Gene targeting Insertion of antisense DNA molecules into selected cells of the organism in order to block the activity of undesirable genes such as oncogenes

Genetic code The correspondence between nucleotide triplets and amino acids in proteins; the sequence of bases in mRNA specify the amino acid sequence of a polypeptide, read in triplets (codons), based on a set of rules

Genome sequencing Reading of all nucleotides present in the entire genomic DNA of the organism in an orderly way

Genomic library Contains a collection of recombinant DNA clones that collectively constitute the genome of the organism (see DNA library)

G protein A family of membrane–bound cell-signaling proteins regulated by guanine nucleotide binding

Green fluorescent protein (GFP) A protein from jellyfish possessing the property of fluorescing; useful as a marker in fluorescence microscopy

GUS gene Production of β-glucuronidase (GUS protein) in certain organisms such as *Escherichia coli* is controlled by this gene; it is commonly used as a marker gene for genetically engineered plants

Haplotype A collection of alleles in an individual that are located on one chromosome; alleles within a haplotype are, often, inherited as a single unit from one generation to another; it also refers to a group of genomic variations found repeatedly in many individuals within a population

Harpins Proteins coded by *hrp* genes present in the type III secretion system (TTSS) of phytobacterial pathogens; these proteins can induce resistance in susceptible plants

Heterotrimeric G protein A guanine nucleotide-binding consisting of three subunits

High-throughput methods Large number of genes or gene products can be studied using these partially automated protocols

Homologs The individuals considered to have sequences that are evolutionarily related by descent from a common ancestor; proteins or genes exhibiting similarity (homology)

Homology A sequence of amino acid in two or more proteins that are identical to each other; nucleic acid homology indicates the presence of complementary strands that can hybridize with each other.

Horizontal gene transfer Incorporation of a "foreign" gene acquired from an unrelated species into the genome of another organism

Host-specific toxins (HSTs) Some pathogens elaborate toxins that can induce all primary symptoms as the pathogen itself; production of HSTs is governed by specific genes which are expressed in susceptible plants

Hot spots Events such as mutations with unusual high frequency may occur in certain sites in genes, termed as hot spots

Humoral immune response In response to infection, rapid production and secretion of soluble blood serum components occurs in the animal body

Humoral immunity By injecting viable nonpathogenic or inactivated phytopathogenic bacteria into insects, formation of new proteins in the hemolymph of these insects is induced leading to the development of humoral immunity; these proteins have antibacterial properties; cecropins and attacins are formed in the hemolymph of *Hyalophora cecropia*

Hybridization probes DNA or RNA molecules that are complementary to a region in DNA; useful for detecting specific gene(s) in fingerprinting

Hybridoma A hybrid cell line produced by fusing a myeloma (capable of multiplying indefinitely) with a lymphocyte (capable of producing antibodies); the hybridoma provides continuous supply of specific immunoglobulins (antibodies)

Hypersensitive response (HR) A protective or defense response of plants to infection or inoculation with pathogen in which the initially infected cells and the adjacent ones express the phenomenon of programmed cell death (PCD) resulting in self destruction to cordon off the infected area and to restrict the spread of pathogen to other cells/tissues (see programmed cell death)

Idiotype The specific site of antibody molecule capable of combining with the specific site in the antigen (epitope) is said to have an idiotype (for that epitope), serving as an identifying characteristic of an epitope

Indel An insertion or deletion occurring in a protein or nucleic acid sequence; frequently it may be difficult to find out whether a change in a sequence is due to a deletion in that sequence or an insertion of a related sequence

Indexing Testing the plants or seeds or propagative plant materials for the presence of microbial pathogens by biological and/ molecular techniques

Induced systemic resistance (ISR) Colonization of roots of plants by plant growth promoting rhizobacteria (PGPRs) induces systemic resistance to pathogens infecting tissues/organs far away from the roots of plants; this type of resistance to disease is referred to as induced systemic resistance

Inducible promoter The promoter in which start or increase of promotion occurs usually in the presence of a pathogen or toxin or toxic metabolites of the pathogen leading to initiation of defense-related activity

In silico Experimental process performed on a computer and not by bench research

Intergenic sequence DNA sequence between two genes; sometimes called as "junk DNA"

Intron The sequence for protein synthesis is absent in the intron; this noncoding sequence of the gene interrupting exons is cut out of the mRNA (splicing) prior to translation

Isoforms Different forms of the same protein whose amino acid sequences differ slightly and whose general activities are similar; isoforms may be encoded by different genes or by a single gene whose primary transcript undergoes alternative splicing

Isozymes Different forms of an enzyme involved in the same or similar reactions, but need different optima for their activity

Karyopherin A family of nuclear transport proteins that function as an importin, exportin or occasionally both; each karyopherin binds to a specific signal sequence in a protein (cargoprotein) to be imported or exported

Killer toxin (KT) A proteinaceous toxin isolated from microorganisms like yeast has antimicrobial properties

Knockout gene Selective inactivation of a desired gene by replacing it with a non-functional (disrupted) allele in an otherwise normal organism

Lectins Plant proteins capable of binding to specific carbohydrates; they can be used to detect the carbohydrates in situ

Leucine-rich repeats (LRR) Segments of amino acids containing multiple copies of leucine present repeatedly in a protein; these proteins are known as LRR proteins

Ligand A molecule other than an enzyme substrate that binds tightly and specifically to a macromolecule, usually a protein forming a macromolecule-ligand complex

Linkage On the same chromosome two different loci governing two different traits may be inherited together; closer the loci greater are the chances of linkage

Linker A short segment of ds-DNA that can be ligated onto a second fragment of DNA to facilitate the cloning of that fragment. Linkers contain a restriction site so that they can be ligated to produce the desired sticky ends for ligation

Locus The locatable position of a gene on a chromosome; all alleles of a particular gene occupy the same locus

Marker-assisted selection (MAS) A known resistance gene or chromosomal sequence closely linked to a gene that is used as a genetic marker to select the progenies of crosses or genotypes containing the selected marker by DNA testing; this procedure is very useful in hastening the development of cultivars with built-in resistance to crop diseases

Messenger RNA (mRNA) An RNA molecule serving as a template for protein synthesis

Metabolome It represents the entire metabolic content of a cell or organism; the complete set/complement of all metabolites and other molecules involved in or produced during a cell's metabolism

Microarray A glass slide or silicon chip onto which several DNA probes are deposited for simultaneous determination of gene expression levels of many genes in the same tissue sample

MicroRNA (miRNA) A naturally occurring short non-coding RNA that can act to regulate gene expression

MicRNAs (messenger-RNA-interfering complementary RNAs) These complementary RNA molecules bind to the RNA transcripts of specific genes resulting in blockage of their translation; they are also called antisense RNAs

Molecular cloning Insertion of a desired DNA fragment into a DNA molecule (vector) that can replicate independently in a host cell

Molecular markers DNA sequence(s) that is characteristic of the DNA segment/gene is used for comparing or detecting the similarities of related organisms or genotypes (see marker assisted selection)

Monocistronic Messenger RNAs encoding a single polypeptide chain

Monoclonal antibody An antibody secreted by a clonal line of B lymphocytes

Monophyletic A group of species on a phylogenetic tree sharing a common ancestor that is not shared by species outside the group; a clade is a monophyletic group

Motif A sequence of amino acids or nucleotides that perform a particular role and is often conserved in other species or molecules

Movement proteins Virus coded-specific proteins involved in the movement of plant viruses in the host plants

Mutagen A chemical capable of inducing a high frequency of mutations

Nonhost resistance Resistance exhibited by a plant species on which the pathogen in question is unable to establish infection; the plant species is termed as a nonhost and the interaction is called as incompatible

Ontogenic resistance The level of resistance of plants may vary with the growth stages of host plant species; plants are highly susceptible to viruses in the early growth stages (seedling), but they develop resistance progressively as the plants become older

Open reading frame (ORF) Region of a gene which contains a series of triplet coding for amino acids without any termination codon is known as ORF; the sequences contained in the ORF may be potentially translatable into a protein

Operon One or more genes may be present in this gene unit; they specify a polypeptide and an operator regulates the structural gene

Orthologs Sequences from different organisms (species) that are evolutionarily related by descent from a common ancestral sequence and later diverged from one another as a result of speciation

Pathogenesis-related (PR) proteins PR proteins are coded for by the host plant, but induced only in pathological or related conditions. They are produced postinfectionally during plant-pathogen interactions; they have different roles in the development of resistance to the diseases caused by pathogens; these proteins are classified into 14 families; PR genes/PR proteins are widely used as marker genes/proteins to study the defense mechanisms of plants

Pathogenicity factors The factors (genes) of pathogen origin, are essential for initiation of infection and further colonization of plants

Peptide mapping Following partial hydrolysis of a protein, a characteristic two dimensional pattern may be recognized (on paper or agar gel) due to the separation of a mixture of peptides

Phytoalexins These are low-molecular antimicrobial compounds that accumulate at the site of infection by incompatible pathogens. Several biosynthetic enzymes are involved in the production of phytoalexins and hence highly coordinated signal transduction events are required

Phytoanticipins Toxic compounds naturally present in the plants that can affect the development of pathogens adversely

Plasmid Represents an independent, stable, self-replicating piece of extrachromosomal DNA in bacterial cells; it does form a component of normal cell genome, but does not get integrated into the host chromosome

Point mutation A mutation resulting in a change in only one nucleotide in a DNA molecule

Polycistronic Coding region representing more than one gene may be present in the mRNA; it codes for two or more polypeptide chains; RNA genomes of plant viruses (such as *Tobacco mosaic virus*) are polycistronic

Posttranscriptional modification A set of reactions that occur to change the structure of newly synthesized polypeptides

Primer A short nucleic acid sequence containing free 3′-hydroxyl group that forms base pair with a complementary template strand and functions as the starting point for addition of nucleotides to copy the template strand

Probe Defined RNA or DNA fragment radioactively or chemically labeled that is used to detect specific nucleic acid sequences by hybridization

Programmed cell death (PCD) Death of cells at the site of initiation of infection by the pathogen as the early response of the host; these cells have a predetermined function amounting to suicide to prevent further spread of the pathogen to other cells/tisssues (see hypersensitive response)

Promoter A region of DNA upstream of a gene that can act as a binding site for a transcription factor and ensure the transcription of the gene concerned

Proteasome A large protease complex that degrades proteins tagged by ubiquitin

Proteome The entire range of proteins expressed in a specified cell

Proteomics Comprehensive analysis of all cell proteins

Pseudogene It is the nonfunctional copy of the gene

Quorum sensing The ability of bacterial or fungal propagules to sense the concentration of certain signal molecules in their environment prior to activation of infection process

Reactive oxygen species (ROS) Intermediate and radical species generated from oxygen such as superoxide or hydrogen peroxide as resistance response in plants

Recognition factors Specific receptor molecules or structures on the host plant surface useful for recognizing the presence of a pathogen

Recombinant DNA A hybrid DNA formed by incorporation of DNA segment from one species into another species

Recombinant protein A polypeptide synthesized by transcription of the hybrid DNA and translation of the mRNA

Regulatory proteins These proteins can bind to DNA and influence the action of RNA polymerase thereby acquire the ability to control the rate of protein synthesis

Reporter gene It is a gene inserted into the DNA of a cell capable of reporting the occurrence of signal transduction or gene expression

Repression of gene function Inhibition of transcription or translation by binding of a product of a gene (repressor protein) to a specific site in the DNA or RNA molecule.

Restriction endonucleases Hydrolytic enzymes capable of catalyzing the cleavage of phosphoester bonds at specific nucleotide sequences in DNA

Restriction site This is a specific nucleotide segment of (base pairs) in a DNA molecule that can be recognized and cleaved by the restriction endonuclease enzyme employed

Reverse genetics A method of analysis of gene function by introducing mutations into a cloned gene

Reverse transcription Synthesis of DNA from an RNA template using appropriate reverse transcriptase

RNA-induced silencing complex (RISC) Large multiprotein complex associated with a short ss-RNA that mediates degradation or translational repression of a complementary or near complementary mRNA

RNA interference (RNAi) Degradation of mRNAs by short complementary double-stranded RNA molecules

RNA processing Refers to various modifications that are made in RNAs within the nucleus

RNA splicing A process that results in removal of introns and joining exons in pre-mRNA

Sequencing The process used to determine the sequential arrangement of amino acids/nucleotides in protein/nucleic acid molecule

Serotypes Groups of an organism differentiated based on the variations in the serological reactions with different antibodies; monoclonal antibodies are frequently used for characterization of serotypes of plant pathogens

Signaling Communication established between and within cells of an organism

Signal molecules Host factors (molecules) that respond to the presence or initiation of infection by a pathogen and transmit the signal to and activate proteins or genes in the tissues away from the site of pathogen entry leading to restriction of pathogen spread/disease development

Signal transduction Reception, conversion and transmission of "chemical message" by a cell

Single nucleotide polymorphism (SNP) Variation detected in individual nucleotides within a DNA molecule; SNPs usually occur in the same genomic location in different individuals

siRNAs Specific short sequences of dsRNA of less than 30-bp in length that can trigger degradation of mRNA containing the same sequence (present in siRNA) within the cell as part of process known as RNA interference (RNAi)

Site-directed mutagenesis A laboratory protocol employed to modify the amino acid sequence of a protein

Somaclonal variation Variability detected in different calli for various characteristics, including disease resistance; the calli exhibiting resistance to disease(s) may be regenerated into whole plants that are tested for the level of resistance to disease(s)

Sticky ends Exposed complementary single strands of DNA can bind (stick) to complementary single strands in another DNA molecule, producing a hybrid piece of DNA

Synteny Two genetic loci are presumed to be linked to the same chromosome, whether or not linkage has been demonstrated, as against the asyntenic loci that are linked to different chromosomes

Systemic acquired resistance (SAR) Resistance to diseases caused by microbial pathogens can be induced by biotic and abiotic inducers of resistance in treated plants; a set of genes referred to as "SAR genes" is activated by the inducers resulting in the systemic resistance in various organs/parts of plants

TATA box A conserved sequence in the promoter of several eukaryotic protein-coding genes where the tranascription initiation complex assembles

Tat pathway The twin-arginine translocation (Tat) system is involved in physiological functions of bacterial pathogens; the Tat pathway operates in the inner membrane of Gram negative bacteria

Transactivating protein A protein that "switches on" a cascade of genes/ gene regulation

Transactivation Activation of transcription by binding a transcription factor to the DNA regulatory sequence in question

Transcription The genetic information contained in one strand of DNA is used as a template and transcribed through the enzyme-catalyzed process to specify and produce a complementary mRNA strand; the genetic information in the DNA is rewritten into mRNA

Transcription factor A protein that binds to a regulatory region of DNA, often upstream of the coding region and influences the rate of gene transcription

Transcriptome The entire set of all gene transcripts (mRNA segments derived from transcription process) in a specified organism; provides the knowledge of their roles in the growth, structure, health and disease of the organism concerned

Transcripts During transcription of a gene, various segments of mRNA, known as transcripts, are formed

Transduction Bacterial genes may be transferred from one bacterium to another by means of bacterial virus (bacteriophage)

Transformation This a process by which free DNA may be transferred directly into a competent recipient cell; plant cells may be transformed with genes from different kinds of organism to enhance the level of resistance to microbial pathogen(s)

Transgenic An organism carrying in its genome one or more DNA sequences (transgene) from another organism

Translation Synthesis of a polypeptide chain from an mRNA template

Transposon A DNA sequence (segment or molecule) capable of replicating and inserting one copy (of itself) at a new location in the genome

Trypsin inhibitor (TI) protein This protein is constitutively expressed in mature maize kernels of resistant maize genotype at higher levels compared to susceptible leaves

Tubulin A family of globular cytoskeletal proteins that polymerize to form microtubules

Two-hybrid system The yeast or bacterial system that is employed for detecting specific protein-protein interaction; the protein of interest is used as a "bait" to "fishout" proteins that may bind to it (referred to as "prey")

Ubiquitin A small protein present in all eukaryotic cells that has an important role in "tagging" other protein molecules; the tagged protein molecules are said to be "ubiquitinated".

Upstream A relative direction of nucleic acids often used to describe the location of a protmoter relative to the start transcription site; the start codon is upstream of the stop codon.

Vector An agent used to carry foreign DNA in recombinant DNA technology; natural carrier of microbial pathogens, commonly viruses from infected plant to healthy plant resulting in the spread of the pathogen/disease under natural conditions

Virulence factors Genes or factors essential for and contribute to the virulence of the pathogen; may not be required for the growth and reproduction of the pathogen

Yeast two-hybrid system An experimental technique of detecting the protein-protein interactions in yeast cells

Zinc finger domain A kind of DNA-binding domain consisting of loops containing cysteine and histidine residues that bind zinc ions

Index