# Molecular Biology in Plant Pathogenesis and Disease Management

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## Disease Management

Volume 3

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*of My Parents*
*for their Love and Affection*

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# Preface

Studies on various aspects of plant-pathogen interactions have the primary goal of providing information that may be useful for developing effective crop disease management systems. Molecular techniques have accelerated the pace of developing short- and long-term strategies of disease management. The strategies that do not depend on host genome modification are based on the principles of exclusion and eradication of pathogens. Molecular methods have played significant role in precise detection, identification, differentiation and quantification of pathogens in symptomatic and asymptomatic plant tissues, resulting in prevention by plant quarantines of introduction of exotic pathogens and elimination of destructive pathogens in infected plants or planting materials by certification programs. Development of cultivars with built-in resistance to microbial pathogens is considered as the most plausible disease management strategy. This approach involves genome modification by incorporation of resistance gene(s) by conventional breeding methods or transformation of plants by incorporation of desired genes from diverse sources.

Molecular techniques have greatly promoted the understanding of the mechanisms employed by plants to defend themselves against different kinds of microbial pathogens. Molecular studies on R proteins and downstream signal networks have focused the attention on the possibility of using *R* genes more effectively for containing the diseases. Marker-assisted selection (MAS) procedure has been extensively employed to select rapidly genotypes with resistance to disease(s). Post-transcriptional gene silencing (PTGS) in plants has been shown to be an effective basis for studying disease resistance mechanisms operating in some pathosystems. PTGS is a potential RNA-mediated defense response capable of protecting plants against viral pathogens. It has been possible to monitor the expression of thousands of host/pathogen genes simultaneously under different defense-related treatments. A better understanding of the role of various genes or gene clusters in infection and resistance phenomena would be possible by applying DNA microarray technology. Genetic engineering has helped to introduce novel resistance genes from diverse sources into crop plants to protect them against the economically important pathogens. Strategy depending on induction of natural defense mechanisms by employing biotic and abiotic inducers of resistance has been shown to be a practical possibility in certain crops. Although use of chemicals for containing crop diseases is followed frequently, emergence of pathogen strains resistant to the chemicals

has become a serious problem to be overcome. Molecular techniques have been employed to identify and monitor the pathogen strains exhibiting resistance to chemicals. With the possibility of sequencing of whole genomes of plants and pathogens of economic importance, a sound basis may be available for developing effective disease management systems, resulting in safe environment, food and feed for the humans and other organisms existing in this planet earth.

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 disease management systems is highlighted with suitable examples. Appendices containing protocols included in appropriate chapters will be useful for students, teachers 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

Various aspects of interactions between plants and microbial pathogens are studied with the primary aim of developing effective disease management systems based on the principles of exclusion, immunization and eradication, in order to reduce the qualitative and quantitative losses caused by microbial pathogens. The effectiveness of both short- and long-term strategies to contain the pathogen development in infected plants and to restrict the disease spread under field conditions has to be assessed. The usefulness of molecular methods for selection, adoption and integration of suitable disease management strategies to keep the pathogens at bay is discussed in six chapters included in the volume 3 of this treatise.

## 1.1 Strategies Not Depending on Genome Modification

The basic step in the development of an integrated disease management system is the use of seeds and planting materials certified to be free of designated pathogens and prevention of introduction of exotic pathogen(s) through imported plant materials that may or may not exhibit symptoms of infection. Domestic and international plant quarantines and certification programs need techniques that can provide reliable results rapidly. Several molecular techniques can be employed for detection, identification, differentiation and quantification of targeted microbial pathogen(s) to meet the stringent requirements of quarantines and certification programs. Different kinds of certification programs are in operation in various countries to suit their requirements, resulting in the elimination of infected plants and planting materials ensuring the supply of disease-free planting materials to the growers (Pallás et al. 2000; Narayanasamy 2001).

As an alternative strategy to chemical application for disease control, utilization of biocontrol agents (BCAs) holds promise because of its ecofriendly nature. Due to significant variations in the biocontrol potential of the fungal or bacterial species that can be employed as BCAs, precise identification of the strains/isolates, quantification and monitoring the population levels of the introduced BCA strains at different periods by using molecular techniques, become essential as in *Aureobasidium pullulans* (Schena et al. 1999, 2002). Molecular markers have been employed

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for identification and characterization of strains of *Bacillus subtilis* effective against soilborne pathogens such as *Rhizoctonia solani* and *Pythium ultimum* (Joshi and McSpadden Gardener 2006).

## 1.2 Strategies Depending on Genome Modification

Development of cultivars with built-in resistance to crop diseases is acknowledged to be the most desirable disease management strategy. It is ecofriendly and does not demand generally any additional effort other than normal cultivation practices adopted by the growers. Enhancement of host resistance to microbial infection may be achieved by (i) incorporating resistance (*R*) genes from cultivars or wild relatives through conventional breeding methods, (ii) transforming plants to express genes of choice from plants or other biological sources and (iii) inducing natural disease resistance of plants by applying biotic or abiotic inducers of resistance.

Understanding the mechanisms employed by plants to defend themselves against fungal, bacterial and viral pathogens may be useful to develop novel strategies to increase the level of resistance to diseases in susceptible cultivars. The *R* genes have been employed in resistance breeding programs with varying degrees of success. Cultivars with resistance to diseases can be developed much earlier by adopting marker-assisted selection (MAS) procedure compared to the traditional breeding methods. The molecular research on R proteins and downstream signal transduction networks has indicated the possibility of using *R* genes more effectively for disease control. Several signal transduction components in the defense networks have been characterized and they are being exploited as switches by which resistance can be activated against a range of pathogens (McDowell and Woffenden 2003). Evidence for allele-specific interaction between alleles of a particular R protein and corresponding pathogen-derived Avr protein has been obtained. In contrast, Avr proteins can function also as effectors promoting pathogen virulence in susceptible plant species incapable of recognizing the pathogen-associated molecular patterns (PAMPs).

Plant pathogens have evolved mechanisms independently to deliver effectors into plant cell cytoplasm. Cloning of *R* gene was achieved for the first time, by transposon tagging of *Hm1*, a gene in maize that governs resistance to race 1 strain of *Cochliobolus carbonum*. The gene encodes a reductase that inactivates the potent host-specific toxin (HST) elaborated by *H. carabonum* (Johal and Briggs 1992). Later successful cloning of the *Pto* gene that confers resistance to tomato against *Pseudomonas syringae* pv. *tomato* (*Pst*) was reported (Martin et al. 1993). This *avr*-induced resistance was shown to be due to a protein with similarity to serine-threonine protein kinases. In these cases, *R* genes appeared to function as receptors for *avr* gene products of pathogens. Detection of an effector by an R protein triggers rapid activation of very effective defense responses (Sequeira 2000; Dangl and McDowell 2006). The defense responses may be of two types namely non-host resistance effective against all races of the pathogen and host resistance effective against only some races of the pathogen. However, several components of

the signaling pathways appear to be common to both types of resistance (Thordal-Christensen 2003).

The emergence of *Arabidopsis thaliana* as a model plant has been responsible for accumulation of significant amount information in different branches of biological sciences in general. As the genome is comparatively small in size and entirely sequenced, *A. thaliana* is being used as a basic reference for all studies related to disease development and resistance. However, the need for verifying the relevance of the data obtained using *Arabidopsis* to understand the molecular basis of interaction of pathogens with economically important crops, has been well realized. Post-transcriptional gene silencing (PTGS) in plants, an RNA-degradation machinery, has been shown to be an effective basis for studying disease resistance mechanism in certain pathosystems. There is a complex relationship between PTGS and virus infection/ resistance. PTGS in plants inactivates some aberrant or highly expressed RNAs in a sequence-specific manner in the host cell cytoplasm and it is an innate antiviral defense in plants and animals (Soosar et al. 2005). As the ds-RNA is not synthesized naturally in plant cell cytoplasm, the plant's resistance mechanism reacts to the presence ds-RNA produced during virus replication. Virus-induced gene silencing (VIGS) is a characteristic manifestation of PTGS in which viruses are both triggers and targets of silencing. PTGS has the potential to be an RNA-mediated defense response to protect plants against plant viruses (Moissiard and Voinnet 2004; Vaucheret et al. 2001).

Endogenous small interfering RNAs (siRNAs) and microRNAs (miRNAs) have been shown to be important regulators of eukaryotic gene expression by guiding mRNA cleavage, translation inhibition or chromatin modification. The significant role of miRNA in basal defense against *Pseudomonas syringae* by regulating auxin signaling was demonstrated by Navarro et al. (2006). It has been possible to monitor the expression of thousands of genes simultaneously under different defense-related treatments and over different points of time, with the advent of large scale genomic sequencing, expressed sequence tagging and DNA microarray techniques. New pathogenesis-related genes, coregulated genes and associated regulatory system have been identified and characterized. DNA microarrays have been applied to study plant-pathogen interactions and downstream defense signaling providing a better understanding of the role of various genes or gene clusters in infection and resistance phenomena (Katiyar-Agarwal et al. 2006; Abramovitch et al. 2006).

The imperative need for alternative approaches to overcome the obstacles associated with conventional breeding methods was realized by researchers in time. The development of plant genetic transformation technology has provided a powerful tool to transfer desired genes from diverse sources to obtain plants with resistance to crop diseases. Genetic engineering methods enable the researchers to introduce novel resistance genes including genes from sexually incompatible species. Further, synthetic genes can also be designed to interfere with specific pathogens or virulence factors. *Agrobacterium tumefaciens*-mediated transformation protocols have provided significant success in transferring genes from diverse sources to confer resistance to diseases. Crops expressing the coat protein genes of viruses have shown encouraging results in terms of yield and quality of produce. Transgenic papaya

lines expressing the coat protein (CP) gene of *Papaya ringspot virus* (PRSV) have reached the stage for commercial exploitation (Souza Jr et al. 2005). The possibility of tackling the *Fusarium* wilt disease of tomato by developing transgenic plants expressing glucanase and chitinase genes was indicated by Ouyang et al. (2005). The usefulness of employing the genes expressing polygalacturonase-inhibiting proteins (PGIPs) for protecting tomato against *Botrytis cinerea* causing grey mold disease was indicated by Powell et al. (2000). A novel method of enhancing resistance of pears to the fire blight disease caused by *Erwinia amylovora* by transforming the pear plants with the elicitor gene $hrpN_{ea}$ was shown to be a feasible approach for reducing losses due to this disease (Malnoy et al. 2005).

## 1.3 Strategies Depending on Induction of Natural Defense Mechanisms

Two principal types of molecular mechanisms are known to be involved in the activation of natural disease resistance (NDR) systems existing in plants, when biotic or abiotic inducers are applied. Systemic acquired resistance (SAR) develops locally or systemically in response to pathogen infection or treatment with inducers of disease resistance. SAR is mediated by salicylic acid (SA)-dependent process, whereas induced systemic resistance (ISR) develops as a result of colonization of plant roots by plant growth-promoting rhizobacteria (PGPR) and it is mediated by jasmonate or ethylene-sensitive pathway (Pieterse et al. 1998). Development of resistance locally in treated tissues and systemically in tissues or organs far away from the site of application has been demonstrated. The effectiveness of SAR and ISR against fungal, bacterial and viral diseases to different degrees has been reported, suggesting the feasibility of adopting this approach for disease control in certain crops. The molecular mechanisms operating during induction of resistance in *A. thaliana*, form a window view of the interplay between microbial pathogens and other plant species treated with inducers (Wang et al. 2005). *Pythium oligandrum*, a biocontrol agent, or its elicitin oligandrin is able to induce the expression of defense-related genes involved in the production of lytic enzymes and consequently the level of resistance of grapevine plants to *B. cinerea* is significantly enhanced (Mohamed et al. 2007).

## 1.4 Strategies Based on Direct Effects of Chemicals on Pathogens

Various chemicals are applied on crops to restrict the incidence and spread of diseases. Although the chemicals are able to provide effective control of the target pathogen(s), the danger due to emergence of strains of pathogens showing resistance to chemicals that have specific sites of action on the pathogen, has been well realized. The changes in the nucleotide sequences of the β-tubulin gene of fungal pathogens have been revealed by molecular techniques. Application of molecular technique(s) to detect the fungicide resistant strains and subsequent development

of resistance management procedure has been shown to be an effective strategy for making right decisions in crop production (Reimann and Deising 2005).

The molecular techniques have the potential to be more precise, rapid, reliable and reproducible compared with the conventional techniques depending on pathogen isolation in cultures and microscopical observations. In addition, the molecular methods are amenable for automation making it possible to handle large amounts of experimental materials. With the possibility of genomics, proteomics and metabolomics techniques becoming available for many pathogens and major crop plant species, it would be possible to understand the interactions of plants with pathogens more comprehensively. Consequently a sound basis may be available for working out disease management systems for combating the pathogens at vulnerable stages in their life cycle, so that crops may be protected more effectively.

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# Chapter 2
# Exclusion and Elimination of Microbial Plant Pathogens

**Abstract** Following globalization, enormous increase in the movement of passengers and cargo shipments has become unavoidable. This situation has also increased significantly the possibility for introduction of new pathogens or new strains of the existing pathogens, necessitating application of diagnostic techniques that have the potential to provide reliable results rapidly. Identification of the pathogens precisely is essential to exclude the more virulent strains or pathogens into the given geographical location or country. For this purpose, the application of molecular techniques has been shown to be effective and advantageous. The detection of pathogens in seeds and propagative plant materials has helped eliminate infected consignments. The suitability and effectiveness of the molecular methods for detection of microbial pathogen is discussed with appropriate case studies.

Modern agricultural practices, globalization of trade and large scale movement of people and goods have created conditions favorable for introduction, incidence and spread of plant diseases caused by microbial pathogens. Crop management systems based on various principles aim (i) to reduce the introduction of the pathogen/disease; (ii) to suppress the initial amount of inoculum and (iii) to improve the level of resistance of crop cultivars to disease(s). Establishment of domestic and international plant quarantines and production of disease-free seeds and propagative materials have been significantly effective in preventing/reducing the disease incidence of various diseases caused by microbial pathogens.

With significant improvements made in passenger traffic and cargo transshipments via air and sea, the probability of unintentional introduction of pathogens has also increased by many folds. Natural introductions of invasive plant pathogens and insect pests have been estimated to be responsible for more than ten billion dollars annually in the United States alone (Pimentel et al. 2000). Regulatory methods have been formulated with the aim of preventing the import and spread of plant pathogens into the country, state or province. Legislative measures are formulated to regulate cultivation of crops and distribution of propagative materials between countries or states within the country. Regulatory control is enforced by establishment of quarantines and inspection of crops in field/greenhouses/warehouses for certification of produce to indicate the health status of the agricultural produce. Introduction of

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certain invasive pathogens has led to development of high-impact epidemics accounting for massive economic loss and sociological upheaval (Kingslover et al. 1983; Campbell et al. 1999). In addition, the perceived threat of intentional introduction with a potential to cause considerable damage to the agricultural and natural systems appears to be of great concern for some countries. Furthermore, the formation of new races and biotypes of indigenous pathogens adds another dimension to the problem of formulating effective systems to keep the pathogens at bay. A plant biosecurity system with the capability for early detection, accurate diagnosis and rapid response is required to prevent the establishment and dispersal of pathogens after introduction and to minimize the adverse effects of such introduced and newly evolved pathogens or races or biotypes (Stack et al. 2006).

## 2.1 Exclusion of Microbial Plant Pathogens

The plant quarantines, established with the primary objective of preventing the introduction and spread of diseases into new areas/countries, helps protect agriculture and the environment from avoidable damage to crops. The importance of establishing well-equipped quarantines has been recognized, after adoption of the General Agreement on Tariffs and Trade (GATT), as there is a dramatic increase in the movement of plant products, necessitating the enforcement of sanitary and phytosanitary measures at the global level. The International Plant Protection Convention (IPPC) was established in 1991 following the acceptance of GATT by the majority of countries. Basic principles required for formulating standards for plant quarantine procedures in relation to the international trade by an expert committee have been laid down (FAO 1991). The principles of establishing plant quarantines recognize the sovereignty of the country which has the right to implement the phytosanitary measures deemed fit by that country. An organism is considered to be of quarantine significance (QS), if its exclusion is perceived as important enough to agriculture and natural vegetation of the importing country.

### *2.1.1 Seeds and Propagative Plant Materials*

The infected seeds and asexually propagated plant materials such as tubers, bulbs and setts are the primary sources of infection. The populations of microbial pathogens – fungi, bacteria and viruses – present in the seeds and propagative planting materials have to be determined, based on the assessment of levels of infection using conventional and/or molecular detection and quantification methods. The advantages of employing molecular methods over conventional procedures have been discussed in Volume 1 Chapter 2. The tolerance limits for various pathogens have been prescribed by the International Seed Testing Association (ISTA). Most of the countries enforce zero tolerance to prevent the introduction of new pathogens into those countries. The possibility of introduction of fungal diseases such as celery leaf spot (*Septoria*

*apicola*), carrot leaf blight (*Alternaria dauci*) and onion neck rot (*Botrytis allii*), bacterial disease like bean halo blight (*Pseudomonas syringae* pv. *phaseolicola*) and virus diseases such as lettuce mosaic, soybean mosaic and bean common mosaic diseases through seeds has been recognized. Production of disease-free seeds to prevent the introduction of the causative agents into other countries has been strongly emphasized (Agarwal and Sinclair 1996; Maude 1996; Narayanasamy 2002). The International Seed Health Initiative (ISHI) founded in 1993 is an international consortium of seed industry and plant pathologists involved in seed health testing. Development of efficient, reliable seed health testing protocols in a timely manner is the primary objective of ISHI to assure that seed lots are sufficiently healthy for world-wide movement and to have a means of quickly testing new technologies for incorporation into seed health testing protocols (Maddox 1998).

Several techniques for the detection, identification, differentiation and quantification of microbial plant pathogens are available. The methods suitable for application in plant quarantines should have the following criteria: (i) results obtained should be reliable with high specificity; (ii) results should be available rapidly; (iii) it should be possible to assess pathogen population in question in relation to other pathogen(s); (iv) the technique capable of detecting two or more pathogens may be preferable; (v) the technique should be very sensitive, capable of detecting the pathogen(s) present in low concentration; (vi) it should be possible to detect latent/quiescent infections in plants, fruits or vegetables and (vii) the technique that can detect qualitatively and determine quantitatively the mycotoxins present in the seeds, fruits and vegetables may be preferable.

A serious threat to the export market for wheat from US to other countries was through seed infection by Karnal bunt disease. The available PCR assay could not differentiate *Tilletia indica* causing Karnal bunt disease from *T*. *walkeri* infecting rye grass. By employing five sets of PCR primers specific to *T*. *indica*, it could be precisely detected in wheat samples, enabling rapid identification and differentiation of the pathogen (Frederick et al. 2000). Carrot seeds are infected by *Alternaria alternata*, *A*. *radicina* and *A*. *dauci*, the former two species possessing high toxigenic potential. A PCR assay employing species-specific primers based on sequences of the ITS regions of the ribosomal repeat (rDNA) was useful for the differentiation of the three *Alternaria* species on carrot seeds and roots. The PCR assay can be used preferably, if results are required rapidly (Konstantinova et al. 2002). Use of disease-free seeds of crucifers is considered to be the effective management strategy for black spot disease of crucifers caused by *A*. *brassica*. A real-time PCR using primers designed on the basis of the sequence of two clustered genes potentially involved in pathogenicity. *A*. *brassicae* was specifically detected in the DNA extracted from seeds (Guillemette et al. 2004).

Detection of bacterial pathogens in seeds can be made more reliable by incorporating a biological or immunological step prior to conventional PCR. The bacteria present in the seeds are isolated in a general agar medium by plating the aqueous extract of the seeds and incubated for 45–48 h. The harvested bacterial cells are subjected to enzymatic amplification of DNA sequences of target bacteria. This technique BIO-PCR can detect *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) even if one bean seed

in a lot of 400–600 seeds, is infected (Mosqueda-Cano and Herrera-Estrella 1997). Immuno-magnetic separation (IMS) using specific antisera to concentrate *Acidovorax avenae* subsp. *citrulli* present in watermelon seeds, followed by PCR assay was shown to improve the sensitivity and specificity of the diagnostic test. The combination assay IMS-PCR has greater sensitivity (100-folds), compared to conventional PCR assay and as low as 0.1% seed infection (1 in 1000) can be determined by this procedure (Walcott and Gitaitis 2000).

Infected seeds form the most important sources of virus infection, since the viruses can easily spread to new areas or other countries through infected seeds. The incidence of *High plains virus* (HPV) infecting maize has been recently observed in the US and its occurrence has been reported from several other countries. Hence, a serious threat to the export of maize to other countries was evident. Sweet corn plants raised from seeds imported from the US were tested in a quarantine level 3 glasshouses in New Zealand. Application of ELISA test and RT-PCR assay confirmed the presence of HPV. These experiments confirmed the seed transmission of HPV in maize seeds and emphasized the need for indexing the seeds in post-entry quarantines (PEQs) to prevent the introduction of new viruses. A procedure for inspecting plants and testing cereal seedlings in quarantines using RT-PCR assay was also developed (Lebas et al. 2005). In the case of *Erwinia stewartii* (*Pantoea stewartii*) causing Stewart's wilt disease, maize seeds from the US are prohibited by many countries to prevent the introduction of this bacterial disease. The seed health test based on ELISA was prescribed by the National Seed Health System as the standard method for phytosanitary testing for the detection of *E. stewartii* (Pataky et al. 2004).

Immunoassays have been demonstrated to be useful for detection and quantification of microbial pathogens infecting propagative plant materials. The presence of *Spongospora subterranea* could be detected in potato tuber extract by using the polyclonal antibodies generated against the homogenate of spore balls (cystosori). The detection limit of ELISA was found to be as little as 0.08 sporeballs equivalent/ml (Harrison et al. 1993). Likewise, by using DPEM medium for anaerobic amplification of *Erwinia chrysanthemi*, ELISA test was used to detect the bacterial pathogen in seed potatoes. This procedure could be used for large scale application for detection of the pathogen in seed tubers and also for prediction of disease outbreaks in Switzerland (Cazelles et al. 1995). ELISA was shown to be as efficient as PCR assays in detecting *Clavibacter michiganensis* subsp. *michiganensis* (*Cms*), (causing potato brown rot disease) in symptomless potato tubers by efficient enrichment followed by DAS-ELISA test (Slack et al. 1996). Specific monoclonal antibodies that did not react with any of the 174 isolates of other pathogenic or unidentified bacteria isolated from potato tubers were used for this assay which had high level of specificity, with a detection limit of 1–10 CFU of *R. solanacearum* per ml (Caruso et al. 2002).

*Spongospora subterranea* could be detected in potato peel and tuber washings by employing specific primers (Sps1 and Sps2) based on sequences of the ITS region of rDNA of the target pathogen. These primers amplified a 391-bp product only from *S. subterranea*, but not from other fungi associated with potato tubers indicating the specificity of detection of the target pathogen. This procedure has the potential for application for disease risk assessment of seed potato stocks (Bell et al. 1999).

Potatoes are infected by more than 25 viruses causing serious losses (Salazar 1996). Among several diagnostic methods, PCR, RT-PCR and serological assays (DAS-ELISA) have been predominantly used for diagnosis of potato virus diseases. However, most of these techniques could detect only single virus. Multiplex RT-PCR assay has the potential for accommodating several primer pairs in one reaction, saving time and expense, in addition to its capacity for testing large number of samples. A multiplex RT-PCR system for simultaneous detection of five potato viruses using 18S rRNA as an internal control was developed. This new technique amplified cDNAs simultaneously from *Potato virus A* (PVA), *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV), *Potato virus S* (PVS) and *Potato virus X* (*PVX*), in addition to host 18S rRNA. This multiplex RT-PCR assay detected all viruses in different combinations and it was more sensitive (100-fold) for detection of PVX compared to commercially available DAS-ELISA protocol. PVX could be detected in some samples that DAS-ELISA failed to detect the virus (Du et al. 2006).

The infection of potato seed tubers by *Potato mop top virus* (PMTV) in seed tuber lots and ware potato was found to be significant in the US and Canada. The RT-PCR technique targeting CP gene in RNA3 of PMTV was highly efficient in detecting the virus (Xu et al. 2004). Diagnostic techniques that can provide results rapidly and reliably are needed for the detection of *Potato yellow vein virus* (PYVV), a quarantine pathogen to prevent its introduction or its subsequent spread in European and Mediterranean Plant Protection Organization (EPPO) region. Real-time RT-PCR assay based on TaqMan® chemistry or conventional PCR test was recommended for detection of PYVV reliably for enforcing quarantine regulations. In addition, these tests were also suggested for routine indexing of potato tubers for the presence of PYVV for production of virus-free seed tubers in South American countries where the incidence of this virus is quite high (López et al. 2006).

The imperative need to develop a reliable and sensitive technique providing results rapidly was found to be essential for the South African exporters to retain their competitive edge in the European market and access new markets like the United States. It is of quarantine importance to differentiate *Guignardia citricarpa* causing citrus black spot (CBS) disease from the harmless endophyte *G. mangiferae* which in not restricted by quarantine regulations. Timeliness and accuracy of pathogen detection and identification are critical factors for the export of citrus fruits, since the value of the consignment decreases rapidly with each additional day spent on holding. Hence, a same-day test that can provide results in one day was considered necessary for citrus fruit exports which were often rejected at harbor due to the presence of a single fruit spot suspected to be due to CBS disease. The one-day sensitive method involves the isolation of DNA directly from fruit lesions by means of the DNeasy Plant Minikit (Qiagen) and use of the primer set C1TR1C1 and CAMEL2 in conjunction with ITS4 primer to yield PCR amplicons of approximately 580-bp and 430-bp for *G. citricarpa* and *G. mangiferae* respectively. These two fungi could be distinguished unequivocally using this PCR protocol, eliminating the prior need for culturing these slow growing fungi, thereby shortening the time required to just one day to test for and verify the presence or absence of *G. citricarpa* in export consignments (Meyer et al. 2006).

### 2.1.2 Whole Plants

*Colletotrichum acutatum* can infect many crop plants including strawberry in which economic losses due to the pathogen are frequently high. As the incidence of the disease was absent in the Czech Republic, it was included in the List of Quarantine Pests to prevent its introduction. Three immunoassays namely plate-trapped antibody (PTA)-ELISA, immunoblot and immunofluorescence tests were employed for the detection of *C. acutatum* in extracts from petioles and roots of inoculated plants. Four polyclonal and two monoclonal antibodies were used. All antisera were genus-specific, but only one polyclonal antiserum IgG K91 showed high sensitivity. Using PTA-ELISA protocol and dot-blot, no cross-reaction with other fungi pathogenic to strawberry was observed. PTA-ELISA tests detected the pathogen in extracts of roots and crown of all cultivars at 7 dai, when no symptom of infection was visible. In petioles the infection was detected only in one cultivar, Elsanta. Dot-blot results were similar to that of PTA-ELISA test (Figs. 2.1 and 2.2). Latent infection of strawberry was also detected by these immunoassays. However, use of at least two of the tests is recommended for detecting latent infections in strawberry fruits (Krátká et al. 2002).

Infection of grapevine plants by *Xyllela fastidiosa* (*Xf*) has to be detected in the asymptomatic plants to prevent the spread of the disease. ELISA format was applied for the detection of *X. fastidiosa* in whole tissue samples and xylem fluid samples. Testing the xylem fluids by ELISA was more efficient than the tests on whole tissues from aymptomatic grapevine plants. There was no significant difference, when the

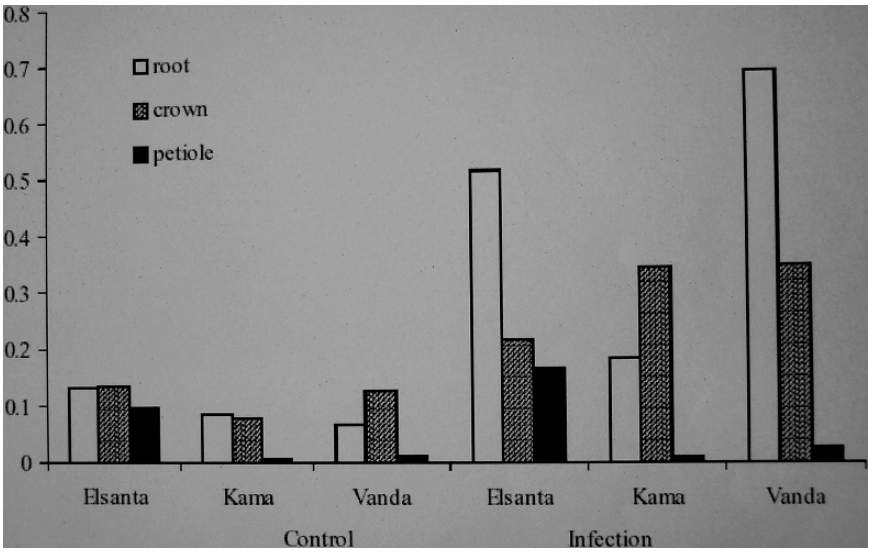


**Fig. 2.1** Detection of *Colletotrichum acutatum* (isolate 12A) using PTA-ELISA in strawberry plants cvs. Elsanta, Kama and Vanda at 7 days after inoculation (dai) (Courtesy of Krátká et al. 2002; Plant Protection Science Institute, Praha, Czech Republic)

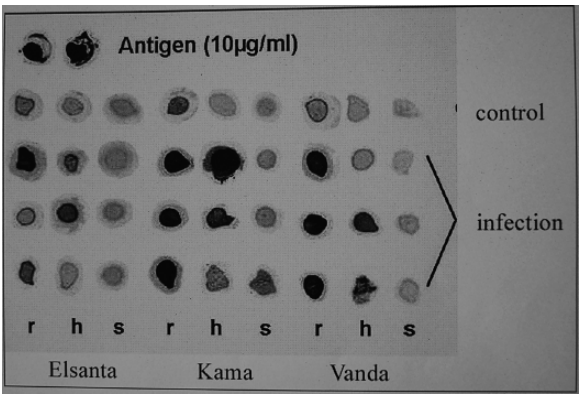


**Fig. 2.2** Detection of *Colletotrichum acutatum* (isolate 30A) using dot-blot in strawberry plants cvs. Elsanta, Kama and Vanda at 7 days after inoculation (dai) r: Root; h: Crown; s: Petiole. (Courtesy of Krátká et al. 2002; Plant Protection Science Institute, Praha, Czech Republic)

frequencies of detection of pathogen by ELISA and PCR in the case of symptomatic grapevine plants were compared (Bextine and Miller 2004).

A major limitation for large scale application of molecular techniques for detection, identification and differentiation of microbial plant pathogens are trained personnel, well-equipped laboratories and cost-effectiveness of tests chosen. In addition, quarantine restrictions on carrying living organisms across the borders, prevent the use of equipped laboratories in other countries by developing nations. Nevertheless, it is possible to undertake pathogen isolation and purification in the countries that lack facilities for testing. The DNA of the pathogen to be investigated, can be sent to laboratories in other countries for analysis. Thus the bio-risks associated with moving the living organisms across the borders can be avoided. The lack of an easy DNA extraction procedure without using toxic organic compounds such as phenol and chloroform necessitated the development of a method for DNA of high quality and purity that is suitable for restriction digestion and PCR-based analysis. A protocol involving inactivation of proteins by using SDS/proteinase K and precipitating polysaccharides in the presence of high salt was developed for extracting plant, fungal and bacterial DNA of high quality. As many as 100 samples can be processed per day. The DNA isolated was entirely digested with five restriction enzymes: *Eco*RI, *Rsa*1, *Taq*1. *Eco*RV and *Hind*III. PCR analysis could be performed using enterobacterial repetitive intergenic consensus (ERIC) sequence, sequence characterized amplified region (SCAR) and random amplified microsatellite primers. The fungal pathogens such as *Colletotrichum lindemuthianum* and *Phaeoisariopsis griseola* and the bacterial pathogen *Xanthomonas campestris* pv. *phaseoli* infecting bean were isolated and their DNAs were subjected to PCR analysis for characterizing them. This newly developed procedure has the potential for application in quarantine services and marker-assisted selection (MAS) breeding (Mahuku 2004).

Strawberry plants are infected by several viruses which are transmitted by diverse types of vectors such as aphids, whiteflies, nematodes and fungi. Nucleic acid-based RT-PCR assay has been developed for the detection of most of the strawberry viruses. RT-PCR and real-time RT-PCR assays have been found to be effective for the detection of *Strawberry crinkle virus* (SCV) (Posthuma et al. 2002; Mumford et al. 2004).

Application of RT-PCR and ELISA tests for the detection of *Strawberry mild yellow edge virus* (SMYEV) was reported to be effective. These assays could detect SMYEV not only in strawberry, but also in all other sources of the virus characterized by symptoms on indicator plants (Thompson et al. 2003). *Strawberry mottle virus* (StMoV) was efficiently detected by employing primers based on conserved nucleotide sequence in the 3′ noncoding region. Sixteen isolates of StMoV were detected using a single primary pair in RT-PCR format (Thompson and Jelkmann 2003). The incidence of a new virus infecting strawberry designated *Strawberry chlorotic fleck virus* (StCFV) was detected by RT-PCR assay in commercial fields (Martin and Tzanetakis 2006).

The rapidity with which the diagnostic procedure provides the results is a critical factor for its application, even if the test has other advantages. For example, direct tissue blot immunoassay (DTBIA) has been shown to be a reliable and sensitive test for detection of *Citrus tristeza virus* (CTV), its sensitivity being comparable to RT-PCR assay (Lin et al. 2002). But this procedure required longer time (3–7 h) to give results. Hence, an improved DTBIA protocol that could provide results much earlier (within 1 h) was developed. Prints of fresh young stems of citrus plants (infected by CTV and healthy) were prepared by gently and evenly pressing the freshly cut surface of the stems onto nitrocellulose membrane. The blots of samples were incubated with prereaction solution of CTV-specific antibodies and labeled secondary antibodies [Appendix]. All samples from greenhouse plants infected by CTV (isolate T-36) were positive to CTV-specific PABs and MABs, whereas healthy plants were negative to all of the antibodies tested. The improved DTBIA was as reliable as the other immunoassays and almost as reliable as PCR in detecting CTV in field samples. The prereaction step introduced in the DTBIA protocol was responsible for the drastic reduction in the time required for obtaining the results (Lin et al. 2006).

Plant viruses, except a few are disseminated from infected plants to healthy plants by insects, nematodes and fungi that act as vectors. The viruses that have biological relationship with the vector species, are able to multiply in the insects and pass onto next generation through eggs. The vector insects are considered as important sources of infection for these propagative type of viruses. *Frankliniella occidentalis*, a thrip species is involved in the transmission of *Tomato spotted wilt virus* (TSWV) belonging to the genus *Tospovirus*, family *Bunyaviridae*. The Western flower thrips is a major pest of several agricultural and horticultural crops and it is a quarantine pest in Taiwan. For the efficient and reliable detection of *F. occidentalis*, a species-specific one-tube nested PCR-RFLP technique was developed. This method consisted of amplification of the rDNA region by a common primer pair CS 249/CS 250, followed by a second PCR with species-specific pair FO1/FO2 for *F. occidentalis*. The limit of detection was 1 pg DNA of *F. occidentalis* for this assay which is rapid and simple for the identification of the insect which is a major pest as well as a vector of an economically important virus that has a wide host range (Liu 2004).

Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) and bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) are the diseases that infect tomato. The symptoms induced by these bacterial pathogens are quite similar and likely to be confused with each other. In order to detect and identify these using crude DNA extracts and primer sets COR 1/2 (bacterial speck) and BSX 1/2 (bacterial

spot) was developed. All 29 pathogenic strains of *Pst* produced a 689-bp amplicon with COR 1/2, whereas the 37 geographically diverse *Xav* strains generated the 579-bp BSX 1/2 amplicon. The detection limit of the assays was 30–50 CFU/ reaction. Latent infections of apparently healthy and greenhouse-grown seedlings or young field plants may function as important sources of infection of the bacterial speck and bacterial spot diseases. The PCR protocol was modified to one where freeze-boil DNA extraction was applied to bacteria collected by centrifugation from the wash water from 10-g samples of symptomless young seedlings. The population of bacteria required for detection was $10^5$ CFU of *Pst* (Cupples et al. 2006).

The choice of detection technique may be critically important in determining the success or failure of regulatory systems involved in preventing the introduction and spread of pathogen(s). A 5′ fluorogenic exonuclease (TaqMan) assay was developed to detect and quantify the fungal pathogen *Phytophthora ramorum* in plant materials. This method is sensitive being able to detect as little as 15 fg of target DNA, when used in nested design or 50 fg, when used in a single round of PCR. None of the other *Phytophthora* species (17) DNA was amplified by the primers employed, indicating high specificity of the test (Hayden et al. 2006).

## 2.2 Use of Disease-Free Planting Materials

Certification is a procedure that facilitates building up nursery stocks and also commercial production by subjecting them to controls for securing trueness-to-type and ensuring freedom from specified plant pathogens as directed by official regulations or endorsed by competent governmental agencies (Martelli and Walter 1998). The practical application of such conceptually simple measures can be expected to be the most powerful means for sanitary upgrading of the commercial production agencies involved in production of horticultural produce/plants. Nevertheless, little attention has been bestowed to promote internationally recognized certification schemes that following application, would enhance free trading of high quality nursery materials among the participating countries. Various political, commercial and technical impediments hamper the acceptance of international agreement on certification protocols (Rowhani et al. 2005).

The primary objective of certification schemes worldwide is to identify healthy sources for propagation through application of time-tested indexing procedures as well as modern molecular methods. The actual technique(s) employed may vary depending on the specific pathogen(s) targeted, the endemic disease(s) in the geographical location (country), availability of techniques, cost of testing and the requirements of the industries served. The first basic step is the establishment of foundation or nucellar source plants which are free from all known harmful pathogens and professionally identified for true-to-type phenotype. Various countries have established an authority to monitor the operations connected with certification of plant propagative materials. Foundation Plant Services (FPS) in the United States of America and the Interprofessional Technical Center for Fruits and Vegetables (CTIFL) in France have

been entrusted with the responsibility of overseeing various operations carried out by nurseries and licensed propagators. The French National Certification Scheme of Citrus has been functioning since 1977 (Verniere 2000).

All plants for plantings in the case of deciduous fruit trees are produced by vegetative propagation. Once diseased plants are established in commercial orchards, the most effective control option is the removal of infected plants. Hence, use of disease-free seeds and propagative planting materials is the next effective disease management strategy in order to restrict disease incidence and spread. Certification programs are in operation in several countries for the production of disease-free nuclear stocks. Establishment of disease diagnostic centers (DDCs) is the basic requirement of the certification programs. Though conventional methods may be useful, adoption of modern molecular techniques is considered to be responsible for the dramatic enhancement in the levels of sensitivity, reliability and rapidity of disease diagnosis, increasing the credibility of the agency offering diagnostic service. For example, a multiplex PCR protocol using primers based on the sequences of *hrpF* gene could efficiently detect pathovars of *Xanthomonas campestris* involved in black rot disease of crucifers. This technique detected one infected seed present in seed lots of 10,000 healthy seeds (Berg et al. 2005). By applying a real-time PCR assay using specific primers based on the 16S–23S rDNA ITS sequences of different isolates, *Burkholderia glumae* was detected in rice seed lots and whole plants rapidly (Sayler et al. 2006). Another distinct advantage of employing molecular diagnostic methods is that they are amenable for automation facilitating testing of large number of samples and provision of conclusive results much earlier compared with the time required for traditional techniques. Furthermore, diagnostic kits have been commercially produced enabling the growers to use the tests right in their fields to determine the health status of their crops/planting materials.

There is practically no possibility of eliminating viruses/viroids from seeds/planting materials by applying chemicals. The feasible approach to prevent or reduce the disease incidence would be the use of seeds and planting materials that have been certified free of these pathogens. This approach has practical utility for horticultural crops that are propagated by stem cuttings, grafting or budding. The mother plants have to be indexed for the presence of all viruses infecting the particular crop. Stone fruit trees are affected by a large number of viruses belonging to different genera such as *Ilarvirus*, *Nepovirus*, *Trichovirus*, *Tombusvirus* and *Potyvirus*. In addition, two viroids *Hop stunt viroid* (HSVd) and *Peach latent mosaic viroid* (PLMVd) have also been reported to infect stone fruit trees. Both the viruses and viroids can be transmitted through planting materials. Diganostic methods for plant viruses based on nucleic acid sequences are being continuously improved. The stone fruit certification programs appear to be a compromise between simplicity of automation and sensitivity. The certifiable material may be assayed by serological or nonradioactive molecular hybridization methods. More sensitive techniques, however, are expensive as in the case of real-time PCR or microarray technology. These methods may be applied to test the primary sources or pre-basic materials as well as for imported dormant budwood during postentry quarantine or sanitation purposes (Pallás et al. 2000).

The French National Certification Scheme of Citrus functions at the International Technical Center for Fruit and Vegetables (CTIFL) under the authority of the Ministry