P. Narayanasamy

Microbial Plant Pathogens-Detection and Disease Diagnosis:

Fungal Pathogens
Vol. 1

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Fungal Pathogens, Volume 1

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Cover illustration:

Banana Sigatoka leaf spot (Volume 1)
The infected leaf shows brown spots with gray central areas. Intensive spotting leads to extensive drying of leaves. The disease is caused by Mycospharella musicola and M. fijiensi.
(Courtesy of P. Narayanasamy, Tamil Nadu Agricultural University, Coimbatore, India)

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Dedicated to the Memory of my Parents
for their Love and Affection

Preface

Incidence of diseases on cereals and other crops is known from time immemorial. However, the causes of these diseases could not be correctly identified, because of the prevailing superstitious belief and religious dogmas that suggested that diseases were God-sent curses as punishment for the sins committed by the people of the region/country. Probes to ascertain the real causes of plant diseases were blunted and discouraged. But analytical thoughts began to percolate in the brave and discriminating minds. As the scientific reasoning and discernment pervaded the human mind, the ignorance covering the nature of the causes of plant diseases began to steadily disappear. With development of compound microscopes and other tools, it was possible to visualize the presence of fungi in the infected plant tissues. Concerted and enduring efforts of early researchers like Anton de Bary (1883–1898), proved unequivocally that potato late blight and other diseases were caused by fungi. Evidences provided by experimentation for establishing the microorganisms as the causative agents of crop diseases began to accumulate from nineteenth century. As different kinds of microbes were discovered to be responsible for various diseases infecting the same crop plant species and one pathogen to be able to infect many crops, wild and weed plant species, the need for developing efficient methods of detection of microbial pathogens rapidly and reliably was realized. Techniques had to be developed to detect, differentiate and quantify the fungal pathogens present in different ecosystems. Furthermore, rapid detection and precise identification of the pathogens form the basic requirement for the development of short- and long-term strategies for the effective management of crop diseases.

Fungi constitute a group that was first recognized as the disease-causing agents in plants. They are comparatively well developed and have several morphological, cultural, biological, physiological and biochemical characteristics that are used as the basis for the tests employed for detection of fungal pathogens and diagnosis of the diseases caused by them. The inadequacy of morphological and biochemical characteristics alone to detect and identify the fungal pathogens and to differentiate the varieties, races and biotypes within the same morphological species was indicated by several studies. Immunoassays and nucleic acid-based techniques have been demonstrated to have an edge over the conventional isolation-based methods. Hence, modern methods that are more sensitive, specific, rapid and reliable have been

developed by the intensive research efforts of scientists working in various countries around the world.

This volume presents exhaustive information based on extensive literature search on various methods of detection of fungal pathogens and diagnosis of diseases caused by them. Comparative effectiveness of different techniques is discussed critically to enable the researchers, teachers, extension specialists and graduate students to choose the suitable methods for their investigations. In addition, several protocols have been presented as appendix in appropriate chapters to meet this requirement. This volume is expected to provide the necessary platform for planning more critical studies that may improve the efficiency and reliability of the existing methods that have been employed for simultaneous detection of two or more kinds of pathogens, without compromising the sensitivity and specificity of detection of fungal pathogens and diagnosis of the diseases caused by them.

Coimbatore, India P. Narayanasamy

Acknowledgement

With a deep sense of humility, gratitude and reverence, I bow to my Alma Mater that continues to remain a source of inspiration for me for over five decades. I wish to place on record my appreciation to my colleagues and graduate students of the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India for their suggestions and critical comments. I am thankful to Dr. T. Ganapathy, Professor of Plant Pathology, for providing technical assistance requiring enormous patience and skill. Permission granted by different copyright holders to reproduce the figures published in various journals is gratefully acknowledged.

It is with great pleasure, I thank profusely my wife Mrs. N. Rajakumari who showers her love and kindness, enabling me to devote my attention exclusively for the preparation of this book. Expression of abundant affection and endless encouragement of my family members Mr. N. Kumar Perumal, Mrs. Nirmala Suresh, Mr. T. R. Suresh and Mr. S. Varun Karthik has been the source of support for all my academic efforts during my career.

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Chapter 1
Introduction

Abstract Among the microbial plant pathogens, the fungal pathogens have more elaborate structural features and they were the first to be demonstrated to be the causative agents of plant diseases. This volume aims to provide required information on various techniques for the detection, differentiation and quantification of fungal plant pathogens in infected plants, planting materials, environment, as well as accurate diagnosis of the diseases even in plants and plant materials that may not exhibit any recognizable symptoms of infection. The efficacies of different techniques are compared; their limitations are indicated and the suitability of the techniques for large scale application is highlighted. The important role played by the disease diagnostic centers, plant quarantine and certification programs in providing advice to the growers, prevention of introduction of new diseases and establishment of disease-free nuclear stocks has to be realized and necessary facilities should be provided for their efficient functioning.

1.1 Microbial Plant Pathogens as a Major Limiting Factor of Crop Production

Microbial plant pathogens including fungal pathogens have the ability to infect a few or a wide range of plant species causing varying magnitude of quantitative and qualitative losses in crops cultivated in different ecosystems. Global losses caused by crop diseases have been estimated to range from 9% to 14.2% of potential yield (Orke et al. 1994). The estimates of losses made later indicated that about 14.1% of produce may be lost due to crop diseases with a monetary value of $220 billions per annum, the developing countries suffering more losses compared with developed countries (Agrios 2005). The loss assessments have been made for different types of diseases with different levels of accuracy. However, irrespective of the levels of accuracy, the estimates of losses emphasize the imperative need for measures to be taken urgently to avoid the losses to the extent possible. To achieve this aim, three principles of crop disease management viz., exclusion, eradication and immunization are adopted to formulate short- and long-term disease management strategies (Narayanasamy 2002). The effectiveness of crop disease management systems

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depends heavily on the rapid, reliable and sensitive detection of microbial plant pathogens and accurate diagnosis of the diseases caused by the putative pathogen(s) detected in infected plants.

1.2 Discovery of Fungi as Plant Pathogens

Various microorganisms have been demonstrated as the causative agents of plant diseases. Fungal pathogens including oomycetes have been responsible for several destructive diseases such as potato late blight, wheat stem rust, rice blast and grapevine downy mildew that have ruined the economy of several countries resulting in famine and migration of millions of humans to other countries to escape starvation and ultimate death. Occurrence of diseases affecting cereals and wine crops has been mentioned in ancient scriptures. But they were attributed to supernatural elements, because of the prevailing superstitious beliefs, religious dogmas and faiths. Theophrastus (ca. 300 BC) and other Greek philosophers expressed grave concern over the drastic reduction in crop yields and speculated on the cause of the crop diseases (Orlob 1964). Plant diseases were considered as God-sent curses as punishment for sins committed, because of lack of scientific and analytical observations. However, scientific thoughts took roots based on the contributions of early researchers like Micheli (1729), Tillet (1755) and Prévost (1807) who provided strong evidence for the role of fungi as the primary causes of plant diseases. Anton de Bary (1831–1888) established in 1861 beyond doubt that potato late blight disease was caused by a fungus. He led a distinctive group of students from different countries striving for explorative excellence which unfolded unquestionable conclusions on several diseases like smuts, rusts and downy mildews affecting various crops based on experimentation. His remarkable contributions formed the cornerstone for the future development of modern investigations on fungal, bacterial and viral plant pathogens causing a wide range of diseases affecting crops all over the world. Hence, he is deservingly regarded as the father of Plant Pathology (Horsfall and Cowling 1977).

1.3 Detection of Fungal Plant Pathogens and Disease Diagnosis

Crops are infected by one or more fungal pathogens at different stages of growth. The pathogen(s) may gain access to the susceptible host plant species through infected seeds or vegetatively propagated planting materials such as tubers, corms, suckers, setts etc. Furthermore, the pathogen propagules may be present in the soil, water, air, natural vectors and alternative host plant species providing inoculum for newly planted crops. Incidence of diseases not known to occur earlier may be observed from time to time. The nature of the cause of the newly observed disease has to be determined immediately to minimize its further spread.

Conventional methods of detection of fungal pathogens depend on isolation of the putative fungal pathogen or a suitable nutrient medium, microscopic examination of spores and spore-bearing structures and identification based on the taxonomic characteristics. These procedures are time-consuming, labor intensive and often provide inconsistent results and require considerable knowledge of fungal taxonomy. Biochemical and physiological characteristics of the fungus isolated from infected plants have been used for the identification of some fungal pathogens with more certainty compared with isolation-based methods. Antibody-based immunological techniques have been employed for detection, differentiation and quantification of fungal pathogens rapidly. However, the problems associated with production of specific antisera that may be effective for the detection of fungal pathogens at different stages of their life cycle still remain formidable. Nucleic acid-based techniques, especially based on polymerase chain reaction (PCR) have been demonstrated to be more precise, sensitive, rapid and reliable for the detection, differentiation and quantification of fungal pathogens present in symptomatic, as well as in aymptomatic plants and plant materials (Chapter 2).

Fungal pathogens can survive in the environment in the absence of susceptible host plant species, as they produce spore forms resistant to adverse conditions. Further many of them, except the obligate pathogens that cause rusts, powdery mildews and downy mildews, are capable leading a saprophytic life using organic substrates present in the soil. Special methods have to be applied for their isolation from soil samples. The techniques based on the biological, immunological and nucleic acid-based characteristics have been employed for the detection and quantification of fungal pathogens in the soil. Presence of fungal pathogens in irrigation/and rain water has been detected using various techniques, including a step for concentrating the pathogen population to enhance the sensitivity of detection. Airborne fungal spores play a vital role in the spread of the diseases affecting aerial parts of the plants. Alternative host plants including weed and wild plant species have been shown to be important sources of inoculum and they play an important role in the survival and perpetuation of the fungal pathogens in the absence of crop plants. It is essential to detect and quantify the pathogen populations in the alternative hosts that may be near the cropped areas by applying efficient methods. Comparative efficacies of different detection methods are discussed in Chapter 3.

Fungal pathogens are known to exist as different *formae speciales*, varieties, strains, biotypes or races that differ in their virulence (pathogenic potential). Suitable efficient techniques have to be employed for characterization of different strains within a morphologic species for the development of effective management systems. The pathogen may produce a new strain that can overcome resistance of newly introduced resistant crop cultivar. Further, emergence of fungicide resistant strains has become a problem of concern for both the grower and the administrators. Constant and consistent monitoring of production of new strains with greater infection potential or higher levels of resistance to chemicals of fungal isolates present in a geographical location is very important to prevent the imminent destruction due to the new strain of the pathogen. Identification of fungicide resistant strains is essentially required for making decisions for the continuation or withdrawal of the fungicide that

is being used in the location concerned. Likewise, useful information may be obtained from these studies for the development of varieties that have to be resistant to all strains of the fungal pathogen existing in a geographical location. The information available in this aspect is critically discussed in Chapter 4.

Accurate diagnosis of crop diseases represents the culmination of all efforts to detect, differentiate and identify the fungal pathogen isolated from the newly observed disease in a region. Efficient isolation of the putative pathogen has to be achieved using appropriate general or selective nutrient media, followed by inoculation on the natural host plant species and fulfilling all steps of Koch's postulates to prove the pathogenicity of the fungi isolated from the infected tissues. The role of disease diagnostic centers (DDCs), plant quarantines and certification programs in disease diagnosis is well recognized. Exclusion of fungal pathogens by proper testing by quarantine personnel and provision of disease-free planting materials to the growers by the personnel of certification programs can be expected to have a significant impact on profitable cultivation of crops (Chapter 5).

The information reflecting extensive literature search is presented in an easily understandable style. It is expected that the various aspects of detection, differentiation, identification and quantification of the fungal pathogens in the plants and environment, as well as the diagnosis of the diseases caused by them presented in this volume, will be highly useful to the researchers, teachers and graduate students in the Departments of Plant Pathology, Microbiology, Plant Protection, Molecular Biology and Plant Breeding. In addition, the extension plant pathologists in disease diagnostic centers and personnel of plant quarantine and certification programs will find the information to have practical utility. Presentation of several protocols appended as appendices in appropriate chapters will assist in selecting the right procedures for reaching their research targets.

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Chapter 2
Detection of Fungal Pathogens in Plants

Abstract Among the microbial plant pathogens, fungus-like and fungal pathogens have well developed thallus consisting of hyphae, asexual and sexual reproductive structures. The morphological characteristics of these structures and various kinds of spores produced by them have been the basis of identification up to genus/species level and classification of these pathogens into family, order and class. However, the *formae speciales*, strains, varieties or biotypes within a morphologic species have to be identified using other characteristics such as pathogenicity, biochemical and immunological properties or nucleotide sequences of the genomic DNA. Isozyme analysis, vegetative compatability group (VCG) analysis and electrophoretic mobility of cell wall proteins have been shown to be useful for the detection of strains of some fungal pathogens. The usefulness of immunoassays for early detection and precise identification has been significantly enhanced following the development of enzyme-linked immunosorbent assay (ELISA) and monoclonal antibodies which exhibit greater sensitivity and specificity compared with isolation based methods which are laborious and time-consuming. Nucleic acid-based diagnostic techniques depending on the variations in the nucleotide sequences of the pathogen DNA have become the preferred ones, because of their greater speed, specificity, sensitivity, reliability, and reproducibility of the results obtained, following the development of polymerase chain reaction (PCR). Several variants of PCR and commercial kits for on-site adoption under field conditions, away from the laboratory, are now available, providing the results in a short time. The possibility of detecting two or more pathogens simultaneously has become bright after the development of DNA array technology. A wide range of diagnostic techniques can be applied for detection, identification and quantification of fungal pathogens present in the infected plants, propagative plant materials and postharvest produce. Speed, specificity, sensitivity and cost-effectiveness are the primary factors that may determine the suitability and choice of the diagnostic tests.

Plants are infected by different kinds of microbial pathogens and the required inoculum for infection may be present in the soil, water and/air, in addition to plant host. In many cases, the pathogens may be carried by seeds or propagative planting

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materials such as tubers, corms, suckers and setts. Whatever may be the source of inoculum, the susceptible plant species or crop varieties may exhibit clear visible local symptoms in or on the tissues where infection is initiated. If the pathogen is able to find favorable conditions for further development, systemic symptoms are induced in tissues or organs far away from the point of pathogen entry into the plant. When the symptoms of infection is not expressed externally, it is termed latent infection. Some fungal pathogens infecting unripe fruits do not induce any visible symptom, as they remain dormant. When the fruits begin to ripen, the pathogen proliferates, as the conditions become favorable resulting in the formation of characteristic symptoms. Such infection is known as quiescent which reflects the transient inactive stage of the pathogen.

It is essential to recognize infection of plants by microbial pathogens as rapidly as possible, preferably before the appearance of symptoms to eliminate the infected plants or planting materials to avoid disease incidence and to restrict further spread of the disease(s). In the case of several diseases, especially those infecting perennial woody plants, the first step to be followed for effective management of crop diseases, is the detection of microbial pathogens and diagnosis of the diseases caused by them, facilitating the elimination of infected plants and clonal materials. Detection of microbial pathogens refers to the process of establishing the consistent presence of a particular target organism(s) within the plant or in its environments, irrespective of the development of visible symptoms in the plant suspected to be infected by the pathogen(s) in question. Diagnosis, on the other hand, relates to the identification of the nature and cause of the disease problem under investigation.

Detection of microbial pathogens in crop plants and other host plant species and also in the environment such as soil, water and air may be required in order to (i) determine the presence and quantity of the pathogen(s) in a crop to initiate preventive or curative measures; (ii) assess the effectiveness of cultural, physical, chemical or biological methods of containing them; (iii) certify seeds and planting materials for quarantine and certification programs; (iv) quantify the pathogen population in the location concerned and for relating to consequent yield loss; (v) assess variations in pathogen infection in germplasm collections to select sources of resistance to disease(s) in question; (vi) identify rapidly new pathogens or strains of existing pathogens to restrict their further spread; (vii) study the taxonomic and evolutionary relationships of plant pathogens; (viii) resolve the components of complex diseases induced by two or more pathogens; and (ix) study the intricacies of interactions between plants and pathogens to have an insight into the phenomenon of pathogenesis and gene functions.

Microbial plant pathogens and also other microorganisms may be present in different natural habitats (substrates) such as plants (including phyllosphere and/rhizosphere), seeds (including spermosphere), soil, water and air. Pathogens may be able to infect a wide range of plant species or restricted to one or a few host species. Further, they may be either obligate parasites like pathogens causing powdery mildews, downy mildews or rust diseases requiring the presence of living plants for their development during their entire life cycle. On the other hand, most of the

fungus-like and fungal pathogens can be cultured on cell-free artificial culture media required for their growth and reproduction. They can lead a saprophytic life for short or long periods in the absence of their natural host plants. It is well known that a significant number of fungal pathogens can survive in the soil and infect the plants, when seeds are sown or young seedlings are planted in the pathogen-infested soil. Following infection of plants through roots, disease symptoms appear after a short or long incubation period during which no visible symptom is produced. Likewise, aerial plant parts such as leaves, stems, inflorescence and seeds may be infected by the pathogen propagules carried by wind. Fungal pathogens are disseminated by wind-borne fungal spores or bacterial cells. Rain water splashes, river water and irrigation water carrying fungal spores/mycelium form another important mode of disease spread. A comprehensive knowledge is essential on the nature of plant hosts and the manner in which the healthy plants get infected, in order to check the infiltration of host plant environment by different fungal pathogens. Maintenance of plant health to the desired levels is possible, if the presence of the microbial pathogen(s) in the crop plants, other plant species that can serve as sources of inoculum and other pathogen habitats, is detected, differentiated and quantified. Numerous methods based on biological, biochemical, immunological and molecular characteristics of the fungal plant pathogens have been developed to detect them in different plant sources with varying degrees of accuracy. The usefulness and the limitations of different detection techniques applicable to fungal pathogens are discussed in this chapter.

2.1 Detection of Fungal Pathogens in Plant Organs

2.1.1 Biological Methods

Among the microbial plant pathogens, fungal pathogens have well organized thallus (body) and mostly multicellular with distinctive spore forms produced during asexual and sexual reproduction stages in their life cycles. Traditional methods, applied commonly earlier, involve the isolation of the fungal pathogens in suitable standard agar media and studying the cultural characteristics such as colony morphology, color and production of asexual structures like sporangia, conidia, chlamydospores, sclerotia etc. Light microscopes may be used to examine the presence of sporangia, conidia, pycnidia or acervuli. The characteristics of the spore bearing structures such as oospores, ascocarps and basidia are considered for the taxonomy and classification of the fungal pathogens. Many fungal pathogens may be identified up to generic level and in some cases up to species level, depending on the experience of the observer. Such a simple procedure for examining the fungal pathogens may not be possible in the case of fungi that cannot be isolated in artificial media. In situ examination of the infected tissues may be performed by prefixing temporary or semipermanent mounts. A temporary mount of fungal pathogen

can be easily prepared by using a strip of transparent cellophane tape. A strip of transparent sticky tape (10 cm long) is held in between the thumb and the forefinger. The sticky side of the tape is firmly pressed onto the surface of a sporulating colony cultivated on an appropriate medium in the petridish. After gently removing the cellophane tape, the sticky surface carrying fungal spores and hyphae is carefully placed over drops of lactophenol cotton blue or aniline blue stain kept at the center of a clean glass slide. The tape is gently pressed and the extended ends of the tape may be held over the ends of the slide, if necessary. Using the light microscope, the characteristics of the spores and sporulating structures may be studied by this simple and rapid procedure.

The presence of fungal hyphae and sporulating structures in the infected tissues may become more discernible by using different stains. Intercellular mycelium and haustoria of *Ustilago scitaminea* causing sugarcane smut disease may be observed by staining with trypan blue. This method could be used for rapid detection of smut infection under field conditions (Sinha et al. 1982; Padmanabhan et al. 1995). A simple, effective technique to enhance visualization of the presence of fungal pathogen belonging to Deuteromycota, Ascomycota and Basidiomycota was developed by Hood and Shew (1996). Permanent slides are prepared using the microtomes to cut thin section of plant materials fixed in appropriate fixatives and embedded in paraffin wax. Details of the procedures are available in the earlier publications (Johansen 1940; Narayanasamy 2001). Field-collected plant tissues infected by pathogens such as *Peronospora tabacina*, *Blumeria graminis*, *Sclerotinia sclerotiorum* were autoclaved for 15 min at 121°C in 50 ml KOH followed by rinsing in deionized water. The tissues were mounted in staining solution containing 0.05% aniline blue dye in 0.067 M K_2HPO_4 at pH 9.0. The stained tissues were examined under the microscope fitted with UV light source. A high degree of resolution and contrast between the pathogen structures and host tissues provide a dependable basis for early recognition of fungal infection. Bright field and fluorescence microscopic methods were employed to detect the infection of wheat plants by *Pyrenophora tritici-repentis* causing tan spot disease. Wheat leaves were spot-inoculated with the pathogen. Cleared leaf pieces were stained with 0.06% aniline blue, 0.035% cotton blue or 0.2% Calcofluor White M2R New, as well as with 0.001% aniline blue fluorochrome or 1.0% acid fuchsin followed by 0.5% fast green. Sections embedded in L.R. White resin were stained with 1.0% acid fuchsin followed by 0.05 toluidine blue O or with 0.2% Calcofluor White M2R New. Initial stages of infection by *P. tiritici-repentis* were recognized by bright field microscopy. Stain retention of the infected epidermal cell wall was used to detect early infection. Aniline blue fluorescence was more sensitive and clearly revealed the presence of the pathogen in infected epidermal cells rapidly (Dushnicky et al. 1998).

Obligate fungal pathogens causing downy mildews, powdery mildews and rusts have to be examined only in the infected plant tissues. The presence of *Peronospora tabacina* causing blue mold disease of tobacco was detected in leaves and stems of tobacco plants. The samples were treated with 20 ml of methyl alcohol (80%) kept in test tubes and then dipped in boiling water until

complete evaporation of ethyl alcohol to remove the chlorophyll in the plant tissues. After washing the bleached plant tissues thrice in distilled water, they were immersed in NaClO (4%) for 30 min followed by rinsing thrice with distilled water. The tissues were transferred to NaClO solution and kept at 60°C for 15 min, followed by washing thrice in distilled water. The tissues were stained with lactophenol and examined under light microscope and/phase contrast microscope. Alternatively, samples (stem or leaves) were cut into small pieces (10 mm thick) fixed in formalin-propionic-propanol, followed by dehydration for 1 week in an isopropyl series. Thin sections stained with Conant's quadruple stain were examined under light microscope to observe the fungal structures at different stages of development (Caiazzo et al. 2006).

2.1.1.1 Isolation of Fungal Pathogens

Fungal pathogens are able to infect various plant parts such as roots, stems, leaves, flowers and fruits, inducing characteristic visible symptoms like spots, blights, anthracnose, wilts, rots etc. After washing the tissues thoroughly in sterile water, the causal fungi are isolated from plant tissues exhibiting clear symptoms. The infected tissues along with adjacent small unaffected tissue are cut into small pieces (2–5 mm squares) and by using flame-sterilized forceps, they are transferred to sterile petridishes containing 0.1% mercuric chloride solution used for surface sterilization of plant tissues for a period of 30–60 s. Alternatively, Clorox (10%), sodium hypochlorite (1%) or hydrogen peroxide (50%) may be used for surface sterilization of plant tissues. The sterilized pieces are aseptically transferred to petridishes containing standard medium like potato dextrose agar (PDA) supplemented with streptomycin sulfate, at the rate of three to five pieces of tissues per petriplate and incubated at room temperatures (25–27°C) that may favor the pathogen development. A portion of mycelium developing on the nutrient medium is transferred to the agar slants for purification and storage for further examination.

In the infected stems, roots or fruits, the fungal pathogens may be present in the deep-seated tissues. In such cases, the infected tissues have to be washed with sterile water thoroughly, followed by swabbing with cotton wool dipped in ethanol (80%) and exposure to an alcohol flame (from spirit lamp) for a few seconds. Using flame-sterilized scalpel, the outer layers of tissues are removed rapidly and small pieces from the central core of tissues in the advancing margin of infection are cut. They are then sterilized by dipping in alcohol (90%) and sterilized again by exposure to alcohol flame for a few seconds. The petridishes containing appropriate nutrient medium, after transferring the sterilized infected tissues, are incubated at required temperature and for optimum period. Actively growing mycelium from the medium is transferred to agar slants for further studies as mentioned above. Slow-growing and difficult-to isolate fungal pathogens may require specific or selective media for their development. Antibiotics may be incorporated in the media to prevent bacterial contaminants (Table 2.1).

Table 2.1 General/selective media and temperature requirements that favor the development of fungal plant pathogens

Pathogen	Nutrient medium	Incubation temperature (°C)	References
Alternaria yaliinficiens	Potato carrot agar (PCA)	20	Roberts 2005
Botrytis cinerea	Potato dextrose agar (PDA)	20–22	Mirzaei et al. 2008
Botryodiplodia theobromae	PDA	28	Fu et al. 2007
Botryosphaeria dothidea	PDA broth (Difco)	25	Ma et al. 2003
Cladosporium fulvum	PDA	24	Yan et al. 2008
Colletotrichum acutatum	PDA	20	González et al. 2006; Yoshida et al. 2007
Fusarium oxysporum cucumerinum	PDA	22	Lievens et al. 2007
F. oxysporum f.sp. *niveum*	PDA/lima bean agar	25	Zhang et al. 2005
F. oxysporum f.sp. *cucurbitae*	Fusarium selective medium (FSM)	25–37	Mehl and Epstein 2007, 2008
Monilinia fructicola	PDA broth (Difco)	25	Ma et al. 2003
Mycosphaerella melonis	Lima bean agar/PDA	25	Zhang et al. 2005
Phaeomoniella chlamydospora	Malt extract agar	25; exposure to UV light for sporulation	Martin and Cobos 2007
Phytophthora spp.	Difco corn meal agar	25	Polashock et al. 2005
Phytophthora cactorum, *P. infestans* and *P. phaseoli*	PDA	17–24	Causin et al. 2005
P. capsici	V8 agar	24	Larkin et al. 1995
P. bisheria	Selective medium	25	Abad et al. 2008

It is essential to purify the cultures of fungal pathogens stored in agar slants. Two procedures are commonly followed: (i) single hyphal tip method and (ii) single spore isolation method. A small segment of fungal growth in the agar medium is transferred to the center of petridishes containing nutrient medium, using a flame-sterilized inoculation needle and incubated at room temperature or optimum temperature for a few days. As the mycelium grows in the medium, the advancing edge of the mycelium will have hyphal tips well separated from each other and they are marked by a glass marking pencil by observing under the microscope. The marked hyphal tips along with bits of agar are carefully transferred individually to separate agar slants in tubes using sterile inoculation needle. The hyphal tips in tubes will develop into a pure colony of the fungal species under investigation. The fungus can be subcultured at regular intervals to maintain its vigor for various studies.

The fungal cultures may be purified alternatively by single spore isolation method. A spore suspension is prepared by transferring the fungal growth in the agar slant to sterile water kept in a sterilized test tube followed by vigorous shaking

for a few minutes in order to disperse the spores from the spore-bearing structures. A serial dilution of spores is prepared by transferring serially 1 ml aliquots to a series of tubes containing 9 ml of sterile water. Aliquots (1 ml) of spore suspension at optimal dilutions mixed with melted nutrient agar (at about 45°C) are transferred to sterile petridishes and the mixture is spread by tilting the dishes gently in different directions for uniformly covering the entire surface of the bottom plate. The petridishes are then incubated at temperatures that favor spore germination. The dishes are examined under the microscope at regular intervals and the locations of germinating spores are marked using a glass marking pencil. The marked germinating spores along with a small amount of medium are individually transferred to agar slants for development of colonies from the germinating spores. The fungal cultures contaminated with bacteria and mites may be purified using a simple technique that is based on the observation that the growth of bacteria and movement of mites are confined to the upper surface of the agar medium. The contaminated culture is placed upside down on a clean piece of paper. Then a thin layer of agar with the inverted culture is gently removed and transferred to plates containing sterile medium. Colonies developing on this medium are free of contaminants present in the original culture (Ko et al. 2001).

Morphological characteristics of fungal pathogens are studied at both asexual and sexual stages for the identification of these pathogens. In the case of some fungal pathogens, specific conditions have to be provided for sporulation (production of spores/conidia) and the morphological criteria are determined for appropriate placement in different species, genera, family etc (Hawksworth et al. 1995). In the cultures of some fungal pathogens, sporulating structures are formed only when they are exposed to certain treatments. Exposure of fungal cultures to wave lengths of near ultlraviolet (NUV) region of light is a very effective method of inducing sporualtion in *Alternaria solani* and *Septoria lycopersici*. The cultures of fungus-like *Phaeomoniella chlamydospora* associated with grapevine decline disease also required exposure to UV light for the production of sporulating structures. The sporulation characteristics formed one of the important criteria for identification of the pathogen up to genus or species level (Armengol et al. 2001; Martin and Cobos 2007).

In *Phytophthora capsici*, zoospore production in V8 agar cultures was favored by incubation at 24°C for 72 h in continuous light, whereas release of zoospores occurred when the cultures were placed at 5°C for 1 h and incubated at 24°C for 30–60 min. On the other hand, oospores were produced when opposite mating types of *Phytophthora capsici* were placed 5–6 cm apart in petridish and incubated at 24°C in the dark for 2 months (Larkin et al. 1995). Formation of sporangia in *Phytophthora bischeria* infecting strawberry was induced by placing small mycelial plugs from the edge of actively growing colonies in the nutrient agar into soil solutions (10%) and incubating under continuous fluorescent light at 22–25°C for 4–7 days. The isolates of *P. bischeri* were identified based on published keys for identification of *Phytophthora* species (Erwin and Ribeiro 1996; Abad et al. 2008).

Sporulation in *Fusarium oxysporum* f.sp. *niveum* was induced by incubating plate cultures in PDA at 25°C for 10 days in darkness (Zhang et al. 2005).

Alternation of light and darkness was required for some fungal pathogens for sporulation in cultures. *Botrytis* spp. with a wide host range was grown on PDA. Incubation of the isolates at 20–22°C for 7 days under light induced production of conidia. In contrast, the cultures produced scleriotia on incubation at 8 ± 1°C in darkness. The characteristics of both conidia and sclerotia are considered for the identification of different species of *Botrytis* (Mirzaei et al. 2008).

Some fungal pathogens grow slowly on nutrient media commonly used for isolation of fungi present in infected tissues. The saprophytic fungi or bacteria present along with fungal pathogens may overgrow, making it difficult to separate the target fungi from the saprophytes. Under such conditions, selective or specific media that differentially favor the development of target pathogen(s) have to be employed. A semiselective medium was used for isolating *Fusarium oxysporum* from infected gladiolus (Roebroeck et al. 1990). When Czapek solution agar containing 20% saccharose was used, differences in cultural and micromorphological characteristics were observed in *F. moniliforme*, *F. proliferatum* and *F. subglutinans. F. moniliforme* was differentiated from the other two *Fusarium* spp. on the basis of variations in colony color and texture. Further, bringing down the pH of the medium to 4.4 from 7.7, intensified the differences without altering the micromorphological characterics (Clear and Patrick 1992).

Isolation of *Gaeumannomyces graminis* var. *tritici* (Ggt) was facilitated by the use of a semi-selective and diagnostic medium (R-PDA) containing dilute PDA amended with 100 µg/ml of rifampicin and 10 µg/ml of tolclofosmethyl. In addition, the presence of Ggt could be detected by its ability to alter the color of rifampicin in R-PDA from orange to purple in about 24 h. This semi-selective medium was found to be more effective in isolating Ggt, compared to SM-GGT3 medium used earlier. *Rhizoctonia* spp. commonly present in the soil was inhibited by the combination of rifampicin and tolclofosmethyl, differentially favoring the growth of Ggt (Duffy and Weller 1994) (Appendix 1).

For the isolation of *Lasidiplodia theobromae* from the woody tissues of *Pinus elliotti*, a selective medium consisting of malt extract (33.6 g/l), tannic acid (300 µg/ml) benodanil (50 µg/ml), tridemorph (0.5 µg/ml) and water (1,000 ml) was successfully employed and found to be more effective than the simple malt extract agar medium in suppressing other fungi present along with *L. theobromae* (Cilliers et al. 1994). Petriplates containing a semi-selective medium were placed both inside and outside commercial greenhouses to detect and quantify the ascospores of *Sclerotinia sclerotiorum*, causative agent of collar rot disease affecting tobacco seedlings. The amount of ascospores trapped in the selective medium formed the basis for developing a forecasting system for this disease (Guitierrez and Shew 1998). Addition of tannic acid (300 ppm) as marker, to water agar was useful for isolation, identification and quantification of *Rhizoctonia solani*. The color of the agar plates or Czapek's liquid medium containing tannic acid turned light yellow to dark brown, as the biomass of the pathogen increased. Absorption values at 363 nm showed positive relationship with mycelial growth (Hsieh et al. 1996). Another kind of selective medium

(FSM) was found to be effective for isolation of *F. solani* f.sp. *cucurbitae* race 2 from cucurbits (Mehl and Epstein 2007).

The CW medium (a semi-selective medium) preferentially encouraged the growth of the pathogen *Alternaria brassicola* infecting cruciferous plants. Since the development of saprophytic fungi was suppressed by this medium, it was successfully employed for the detection of this pathogen in seeds (Wu and Chen 1999) (Appendix 1). Likewise, a semi-selective medium containing carrot leaf extract, glucose and minerals was developed for the isolation of *Alternaria dauci* and *A. radicina* from the seeds of carrot. Carrot leaf extracts stimulated profuse sporulation by both pathogens (Strandberg 2002). A semi-selective medium for isolation of *Colletotrichum gloeosporioides*, causing anthracnose disease of yam, was developed to prevent other fungi overgrowing the target pathogen. This medium has PDA as the basal medium amended with antimicrobial agents and the pH has to be adjusted to 5.0 (Appendix 1). The effectiveness of the medium was evident, due to its selective inhibition of contaminants, facilitating the development of distinct salmon-pink colonies of *C. gloeosporioides* (Ekefan et al. 2000). *Botrytis cinerea* is a polyphagous fungal pathogen infecting a wide range of economically important crops worldwide. A Botrytis selective medium (BSM) was found to be effective for isolation and enumeration of *B. cinerea* from plant materials and soil. BSM favored the development of significantly higher numbers of *B. cinerea* colony forming units (CFUs) in individual petriplates due to restricted radial growth of colonies. *B. cinerea* colonies on BSM were surrounded by a dark brown halo clearly visible against the pink color of the medium (Fig. 2.1). Botrytis spore trap

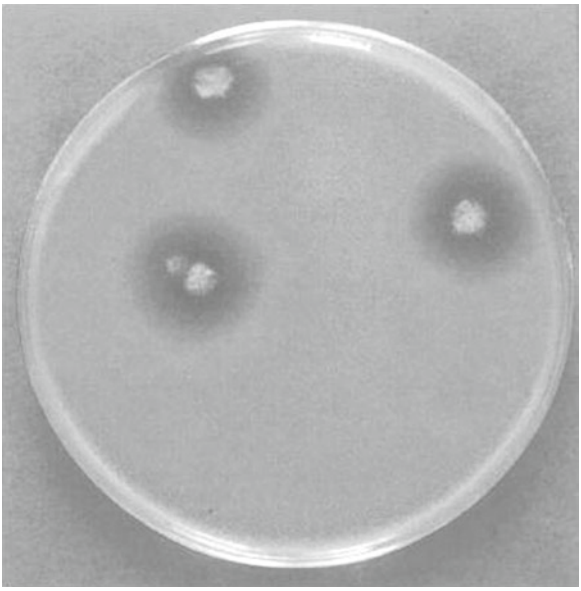

Fig. 2.1 Colonies of *Botrytis cinerea* (*Bc*) with dark brown halo on *Botrytis* selective medium seeded with *Bc* in soil suspension (Courtesy of Edwards and Seddon, 2001; Society for Applied Microbiology/Wiley-Blackwell, Oxford, UK)

medium (BSTM) containing fenarimol in place of Rose Bengal in BSM, was employed for trapping the conidia of *B. cinerea* in the air and for accurate enumeration of spores (Edwards and Seddon 2001).

2.1.1.2 Identification of Fungal Pathogens

Diagnosis of diseases caused by fungus-like and fungal pathogens induce certain characteristic symptoms based on which the causative agents may be inferred to some extent. The morphological characteristics of the fungal pathogens such as type, shape and color of sexual or asexual spore forms have been primarily used for their taxonomy and classification. Considerable expertise is required for such classification and establishing phylogenetic relationship among the related fungi. However, some cultural characteristics may also be useful belonging to the same genus as in the case of *Colletotrichum* spp. pathogenic to rubber. *Colletotrichum acutatum* has a slower growth rate at temperatures ranging from 15°C to 32.5°C and a higher level of tolerance to fungicides benomyl, carbendazim and thiophanate methyl compared with that of *C. gloeosporioides* (*Glomerella cingulata*) (Jayasinghe and Fernando 1998).

Types of Symptoms Induced by Fungal Pathogens

Both local (confined to infected plant organs/tissues) or systemic symptoms (present on or in organs away from point of infection) may be produced following infection by fungi or fungus-like pathogens. Infected leaves, stem, or flowers may exhibit localized symptoms. Spots, blights, shot-holes, anthracnose, rusts and powdery mildews are the symptoms observed commonly on leaves, whereas root rot, collar rot, stem rot, stem canker, club root and galls/tumors are the symptoms associated with stem infections. Fruit rot, capsule rot, grain discoloration and head rot symptoms are evident, when reproductive structures are infected. The symptoms are generally restricted to the tissues or organs in which infection is initiated. On the other hand, systemic nature of damping-off, wilt, smut and downy mildew diseases may be due to the ability of the pathogens to spread to different organs from the initial site of infection. The symptom expression may be recognized after a short or long incubation period that represents the time needed from infection to development of visible symptoms. In some cases, symptoms may be indistinct even after a long incubation period, as in the case of diseases affecting perennial crops. In such cases, as in pear leaf spot disease caused by *Alternaria kikuchiana*, use of indicator plant (progeny PS-95 of cross between Niitaka and Waseaka) has been reported to be useful for detecting the infection rapidly (Woong et al. 1996). Some of the types of symptoms induced by fungal pathogens are presented in Figs. 2.2–2.5.

Fungal pathogens induce symptoms, the severity of which may vary depending on the levels of resistance/susceptibility of the host species/cultivars, environmental factors and virulence (aggressiveness) of the pathogen. Drawings/color charts

Fig. 2.2 Symptoms of pearl millet downy mildew (right) and green ear (left) disease (Courtesy of International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India)

Fig. 2.3 Symptoms of banana Sigatoka disease (Courtesy of Dr. P. Narayanasamy)

representing different disease severity for foliar diseases are used for visual estimation of disease severity. A digital image analysis method was developed to measure the severity of several foliar fungal diseases. Images captured with a flatbed scanner or digital camera can be analyzed with a freely available software package, Scion Image to measure changes in leaf color caused by fungal sporulation or tissue

Fig. 2.4 Symptoms of rice sheath blight disease (Courtesy of International Rice Research Institute, Manila, Philippines)

damage. High correlations were recorded between the percent diseased leaf area estimated by Scion Image analysis and percent diseased leaf area determined by visual examination in the case of anthracnose pathogen *Colletotrichum destructivum* inoculated on *Nicotiana benthamiana*.

This method was adapted to quantify percent dieased leaf area ranged from 0% to 90% for anthracnose of lily-of-the valley, apple scab, phlox powdery mildew and golden rod rust diseases. Digital Image analysis using Scion Image can be adapted to detect early and quantify rapidly a wide variety of foliar fungal diseases (Wijekoon et al. 2008).

Morphological Characteristics of Fungal Pathogens

Among the microbial plant pathogens, fungi and fungus-like pathogens show distinct variations in the morphological characteristics that can be used for

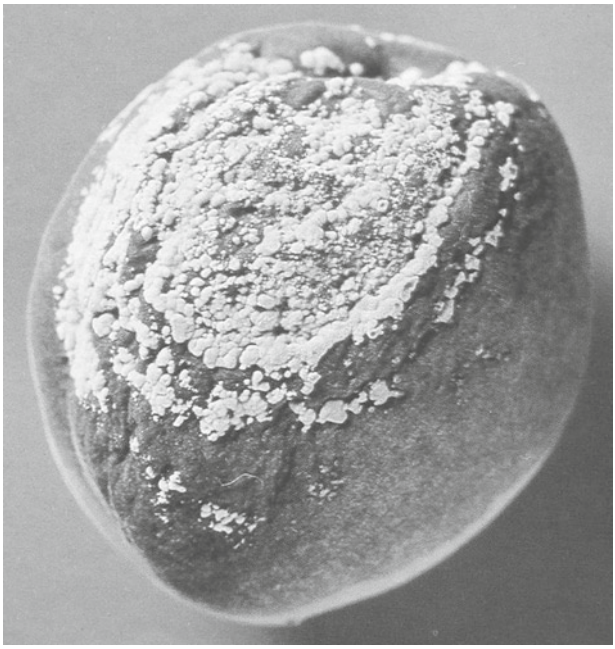

Fig. 2.5 Symptoms of peach brown rot disease (Courtesy of Dr. P. Narayanasamy)

identification of the fungus under investigation up to genus level and sometimes up to species level with some certainty. They are considered as microscopic plants without chlorophyll and conductive tissues. Of the 100,000 species, about 10,000 fungal species have been identified as plant pathogens, whereas about 50 species have been found to be pathogenic to humans and animals (Agrios 2005). Filamentous hyphae (tubular structures with or without cross walls [septa]) weave into thinner or broader segments of the mycelium. The fungal cells have well-defined cell walls enclosing one or two nuclei (binucleate) cell. The fungus-like Oomycetes characterized by the absence of cross walls, have coenocytic mycelium forming one continuous tubular, branched or unbranched multinucleate cell or multinucleate hyphae, when septa are formed below the sporulating structures (sporangia). Mycelial growth through elongation of hyphal tips leads to increase in biomass. Based primarily on the characteristics of spores and sporulating structures formed during asexual and sexual stages, the fungus-like and fungal pathogens are assigned to taxonomic positions indicated in Tables 2.2 and 2.3.

The fungal pathogens for which the sexual stage has not been discovered yet, are placed in the class Deuteromycetes (Fungi imperfecti). As and when the characteristics of sexual spores and the sporulating structures are produced, they will be transferred to and grouped with the fungi in appropriate class, genus or species already in existence. If the characteristics are distinct from other species/genus, a new species or generic name is proposed by the researcher concerned. *Sphaeropsis sapinea* (anamorph-*Diplodia pinea*), infecting conifers is a destructive pathogen in

Table 2.2 Classification of fungus-like pathogens infecting plants (Webster and Weber 2007)

Kingdom	Phylum	Class/Order	Family/Genus
Protozoa	Plamodiophoromycota	Plamodiphoromycetes/ Plasmodiophorales	Plasmodiophoraceae/ *Plasmodiophora*
Straminipila	Oomycota	Oomycetes/Pythiales	Pythiaceae/*Pythium*
		Peronosporales	Peronosporaceae/ *Peronospora*
			Peronosclerospora
			Plasmopara
			Sclerospora
			Albuginaceae
			Albugo

Table 2.3 Classification of fungal pathogens infecting plants Kingdom: Fungi (Agrios 2005)

Phylum	Class	Order	Genus
Chytridiomycota	Chytidiomycetes	Chytridiales	*Olpidium*
Zygomycota	Zygomycetes	Mucorales/	*Rhizopus*
			Choanephora
Ascomycota	Archascomycetes	Taphrinales	*Taphrina*
		Erysiphales	*Blumeria*
			Erysiphe
			Leveillula
			Sphaerotheca
			Uncinula
	Pyrenomycetes	Hypocreales	*Claviceps*
			Gibberella
		Microascales	*Certocystis*
		Phyllachorales	*Glomerella*
			Phyllachora
		Ophiostomatales	*Ophiostoma*
		Diaporthales	*Diapothe*
			Gaeumannomyces
			Magnaporthe
		Xylariales	*Rosellinia*
			Eutypa
	Loculoascomycetes	Dothideales	*Mycosphaerella*
			Elsinoe
		Pleosporales	*Cochliobolus*
			Pyrenophora
			Venturia
	Discomycetes	Helotiales	*Monilinia*
			Sclerotinia
			Diplocarpon
Basidiomycota	Basidiomycetes	Ustilaginales	*Ustilago*
			Tilletia
			Sphacelotheca

(continued)

Table 2.3 (continued)

Phylum	Class	Order	Genus
		Uredinales	*Cronartium*
			Hemileia
			Melampsora
			Puccinia
			Uromyces
		Exobasidiales	*Exobasidium*
		Ceratobasidiales	*Thanatephorus*
		Agaricales	*Armillaria*
		Aphyllophorales	*Ganoderma*

many countries. The isolates exhibited differences in conidial morphology and also in cultural characteristics. Thirty isolates of *S. sapinea* were examined using scanning electron microscope and these isolates were grouped into two types. Type A isolates had smooth surfaces, whereas the type B isolates exhibited pits distributed over the conidial surface. The cultural characteristics of types A and B correlated well with the morphological differences in the mature conidia of these two types. But differences in the conidial morphology of A and B types noted by observations under scanning electron microscope, could not be recognized by light microscopic observations. The presence of pits appeared to be a stable characteristic of all mature spores of B type isolates produced in vitro or in field-collected samples (Wang et al. 1985).

Identification of Vegetative Compatibility Groups

Variations may be seen in the biological characteristics of the isolates of a fungal species that cannot be differentiated by morphological characteristics. But they differ in their pathogenic potential (aggressiveness) or their ability to form 'heterokaryons' by fusion between genetically different strains existing within a morphologic species. Strains which are vegetatively compatible with one another are considered as members of the same vegetative compatibility group (VCG). On the other hand, forms or strains of a morphologic species may be identified, based on the host range or infection types induced on a set of differentials of host species or cultivars. Races or biotypes may be differentiated in fungal pathogens such as *Puccinia graminis* and *Pyricularia oryzae.*

Sexual and vegetative compatibility tests have been applied for routine identification of morphologically similar isolates or strains or clonal sub-populations or individuals within a species. Genetic systems governing vegetative compatibility may be of two types. The first type includes strains that are identical at a particular loci and are capable of forming a stable heterokaryon, while those that differ at any of these loci are incapable of forming a vegetatively stable heterokaryon. This type

of interaction is termed as allelic compatibility. In the case of nonallelic interactions, alleles at one locus interact with alleles at a second locus to block the formation of a stable heterokaryon. A vegetative compatibility group encloses strains of a morphologic species that can form stable vegetative heterokaryon, implying the identity of alleles by every *vic* locus (loci that govern vegetative compatibility). Vegetative compatibility involves fusion (anastomosis) of hyphae rather than the fusion of protoplasts or spheroplasts (Leslie 1993). Vegetative compatibility groups (VCGs) have been recognized in the fungal pathogens such as *Verticillium dahliae*, *Fusarium oxysporum*, *Armillaria* spp. and *Cryphonectria parasitica.*

The existence of vegetative compatibility system in *Verticillium dahliae* was first demonstrated by Puhalla (1979). Strains included in one VCG are capable of forming heterokaryons with one another, but not with strains in other VCGs. Two common types of mutants that have been used to study heterokaryons are UV-induced microsclerotial color mutants and spontaneous nitrate-nonutilizing (*nit*) mutants. Using color mutatnts, 19 strains of *V. dahliae* were classified into four VCGs. Later, 86 strains of *V. dahliae* isolated from several host plant species and geographical locations were classified into 16 VCGs. The strains earlier identified as defoliating and nondefoliating pathotypes infecting cotton consistently separated into different VCGs (Puhalla and Hummel 1983). Joaquim and Rowe (1990), using *nit* mutants characterized only four VCGs among 22 strains that were classified into 15 groups earlier by Puhalla and Hummel (1983).

As a nitrogen source, most fungi are able to utilize nitrate by reducing it to ammonium via nitrate reductase and nitrite reductase. Some fungi are unable to utilize nitrate and apparently cannot synthesize nitrate reductase (Garraway and Evans 1984). Chlorate, a nitrate analogue, has been frequently used for studying nitrate assimilation in fungi. The reduction of chlorate to chlorite by nitrate reductase may possibly result in chlorate toxicity in the fungi. In general, chlorate-sensitive strains can reduce nitrate to nitrite, whereas chlorate-resistant strains cannot do so. The *nit* mutants unable to utilize nitrate but able to use nitrite, ammonium, hypoxanthine and uric acid, were designated *Nit1* mutants. *Nit* mutants incapable of using nitrate and hypoxanthine, but capable of utilizing the remaining three nitrogen sources were named as *NitM*. *Nit1* mutants were considered to have arisen from a mutation at the structural locus of the gene for nitrate reductase, whereas the NitM phenotype originated from a mutation at one of several loci controlling the synthesis of a molybdenum-cofactor necessary for the activity of nitrate reductase and purine dehydrogenase (Correll et al. 1987). Utilizing the *nit* mutants of fungal pathogens, the strains or isolates have been assigned to different VCGs.

The wild type strains (187) of *V. dahliae* from potato plants and soil from 22 potato fields in Ohio, USA, were tested. Strains were assigned to VCGs based on pairings of complementary *nit* mutants induced on a chlorate-amended medium. Two strains were assigned to VCG1, 53 strains to VCG2 and 128 strains to VCG4. The remaining four strains did not yield *nit* mutants and hence they were not assigned to any VCG (Joaquim and Rowe 1990) (Appendix 2). In a subsequent study, two phenotypic classes of *nit* mutatnts were identified among the 126 *nit* mutants characterized. Eighty two *nit* mutants (65%) were able to utilize all nitrogen

sources except nitrate. These mutants were designated *nit1* mutants, whereas the mutants belonging to the second phenotypic class, were unable to utilize nitrate and hypoxanthine, but retained the ability to use other nitrogen sources tested. These mutants were named *NitM* (Joaquin and Rowe 1991).

In an another investigation, complementary auxotrophic *nit* mutants were used to determine vegetative compatibility with 27 strains of *V. dahliae* isolated from several hosts growing in Africa, Asia, Europe and the United States. About 500 *nit* mutants were generated from these strains and three VCGs 1, 2 and 4 were identified among them. When virulence of each strain was assessed on cultivars of *Gossypium hirsutum*, *G. barbadense* and *G. aroboreum*, the strains belonging to VCG 1 were of both the cotton-defoliating pathotype and race 3 (on cotton) and on tomato. The results indicated the existence of a relationship between the VCGs and the taxonomic position of host plants (Daayf et al. 1995). *V. dahliae* isolates (77) present in 87 fields intended for potato production were subjected to vegetative compatibility analysis. Isolates of *V. dahliae* present in 93% of fields belonged to VCG 4A group, while 23% of fields had isolates assigned to VCG 4B group and only one contained isolates related to VCG 2B group. Preplant assessment of nature of *V. dahliae* populations may be useful for disease management decisions (Omer et al. 2008).

In *Fusarium oxysporum*, pathogenicity to host plant species has been demonstrated to be useful for identification of specialized forms that can infect only certain plant species. However, virulence of isolates may be influenced by variables like temperature, host age and method of inoculation. Alternatively, use of vegetative compatibility as a means of subdividing *F. oxysporum* has been suggested. Puhalla (1985) could recover *nit* mutants from 21 strains of *F. oxysporum* at high frequencies without employing any mutagen. The same parent yielded *nit* mutants that were able to complement one another by forming a heterokaryon on a minimal agar medium that contained sodium nitrate as the source of nitrogen. The complementary *nit* mutants recovered from each strain were arbitrarily designated *nitA* and *nitB*. The development of dense aerial growth, where mycelia of the two thin *nit* mutant colonies anastomosed, indicated the heterokaryon formation between *nitA* and *nitB* mutants derived from the same parental strain. Further, a correlation between VCG and *forma specialis* was observed, as members of one VCG belonged to the same *forma specialis* (Puhalla 1985).

Using *nit* mutants, vegetative compatibility tests have been applied to identify *F. oxysporum* f.sp. *apii* race 2 from a population of *F. oxysporum* colonizing roots of celery (Correll et al. 1986a). *Nit* mutants have been useful to differentiate strain in the ubiquitous nonpathogenic potrtion of *F. oxysporum* population (Correll et al. 1986b). In a further study, a large number of *nit* mutants (over 1,300) was recovered from seven strains of *F. oxysporum* cultured on MMC and PDC media (Appendix 3). These mutants were grouped into three phenotypic classes by their growth on supplemented minimal agar medium. These classes appeared to reflect mutations at a nitrate reductase structural locus (*nit1*), a nitrate assimilation pathway-specific regulator locus (*nit3*) and loci that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase

activity (*NitM*). *Nit* mutants in each phenotypic class were generated from all the seven strains of *F. oxysporum*. Physiological complementation between six *nit* mutants with different mutations was indicated by the development of dense aerial growth where mycelia of the *nit* mutant colonies came into contact and anastomosed to form a heterokaryon. All *nit1* and *nit3* mutants from an individual strain were found to readily complement the *NitM* mutants derived from the same strain. The use of *nit* mutants may aid in comparing strains of *F. oxysporum* for vegetative compatibility worldwide. When combined with pathogenicity tests, vegetative compatibility tests may prove to be valuable to study genetic diversity of natural populations of the fungal pathogens (Correll et al. 1987).

Forty seven isolates of *Fusarium oxysporum* f.sp. *lactucae* infecting lettuce were isolated from infected plants and seeds in Italy, Japan, Taiwan and the United States. They were evaluated for their pathogenicity and vegetative compatibility. *Nit* mutants obtained from chlorate substrates were evaluated to determine the efficacy of different media in the production of *nit* mutants useful for VCG analysis. Based on the complementation pattern, all Italian isolates pathogenic on lettuce were assigned to VCG 0300. In addition, the American isolates type 2, Taiwanese isolates and one Japan race 1 also belonged to VCG 0300. But Taiwanese type 1 isolates were assigned to VCG 0301. VCG comparison established the similarity of isolates earlier identified as *F. oxysporum* f.sp. *lactucae* and *F. oxysporum* f.sp. *lactucum.* Hence, the adoption of the name *F. oxysporum* f.sp. *lactucae* was suggested to all isolates in VCG 030- (Pasquali et al. 2005).

The genus *Armillaria* causing root rot diseases of conifers, contains about forty morphologically distinct species or intersterility groups. Sexual and vegetative compatibility tests have been employed for routine identification of species and differentiation of unknown isolates of intersterile groups. *Armillaria* spp. have a heterothallic bifactorial sexual incompatibility system. The sexual compatibility or mating compatibility may be determined by pairing the unknown isolate with haploid tester strains (single spore isolates) that represent each species to which the unknown isolate may belong. The positive result in the production of genetically stable diploid (not dikaryotic) mycelium associated with changes in culture morphology. Nine *Armillaria* spp. and North American biological species (NABS) have been identified by mating compatibility studies. Four species *A. ostoyae*, *A. mellea*, *A. gallica* and *A. cepistepes* were present in North America and Europe. Interfertility testing allows clear separation from morphologically indistinguishable related strains or species. Production of pure cultures of the isolates and tester strains requires longer time and it is cumbersome compared with biochemical and molecular methods (Schulze and Bahnweg 1998).

Binucleate *Rhizoctonia* spp. cause several diseases such as damping-off, root rot, stem rot, leaf blight and fruit decay in a range of agricultural crop plants. They do not form a homogenous species. The binucleate *Rhizoctonia* spp. have been divided into 19 anastomosis groups (AGs) which are named as AG-A to AG-S (Ogoshi et al. 1983; Sneh et al. 1991). Three subgroups in AG-B, AG-Ba, AG-Bb and AG-Bo were differentiated based on frequency of hyphal anastomosis and cultural characteristics (Sneh et al. 1991). Later, two groups AG-D(I) and AG-D(II)

were identified based on cultural morphology and pathogenicity (Tanaka et al. 1994). Binucleate-like *Rhizoctonia* spp. were isolated from root rot and stem rot disease affected cut-flower roses (*Rosa* spp.). The isolates (670) were grouped into two colony morphology types, light brown to brown colony and whitish colony types which belonged to AG-G and AG-CUT respectively. AG-CUT group isolates did not anastomose with any tester strains of binucleate *Rhizoctonia*. Furthermore, none of the isolates of AG-G and AG-CUT did not anastomose with the tester strains of previously reported AG-MIN group collected from miniature roses, leaving the identity of this fungal pathogen in doubt (Hyakumachi et al. 2005), suggesting the need for employing molecular techniques for precise identification of the causative fungus.

Most VCGs have been shown to be stable through time and space including laboratory manipulations indicating the usefulness of VCG markers for population studies. Use of VCGs as a diagnostic tool has been explored based on the assumption that strains of a fungal pathogen belonging to the same pathogenic group (*forma specialis*, race or pathotype) are in one or only a few VCGs. The pathogen may be identified by the placement in a particular VCG, because pathogenicity tests using standard differential host species or cultivars are more laborious and time consuming. Identification based on VCGs has been reported to be advantageous in the case of *Fusarium oxysporum* f.sp. *apii* (Correll et al. 1986b), *F. oxysporum* f.sp. *conglutinans* (Bosland and Williams 1987) and *F. oxysporum* f.sp. *melon*s (Jacobson and Gordon 1990). The results of vegetative compatibility tests may be more reliable, if the strains contained within a particular VCG can be proved to be related to another by employing another corroborative test. Furthermore, the need to generate mutants of all field isolates to be examined is an important requirement reducing the possibility of large scale application of vegetative compatibility approach as a diagnostic technique.

Fusarium oxysporum f.sp. *radicis-cucumerinum* (FORC) was identified as a different *forma specialis* and it was distinguished from *F. oxysporum* f.sp. *cucumerinum* (FOC) based on symptomatology, cultivar susceptibility and epidemiology (Vakalounakis 1996). Of the 106 isolates of *F. oxysporum* collected from infected cucumber plants, 68 were identified by pathogenicity as FORC, 32 as FOC and six isolates were found to be non-pathogenic to cucumber. Isolates of FORC were vegetatively incompatible with FOC and *Fusarium* isolates. Among 68 isolates of FORC, 60 isolates belonged to VCG 0260, while five isolates were placed in VCG 0261. Bridging isolates (3) were vegetatively compatible with both VCGs. Confirmatory results were obtained by performing RAPD fingerprinting indicating that pathogenicity and vegetative compatibility tests could be as effective in distinguishing FORC as molecular techniques (Vakalounakis and Fragkiadakis 1999).

The existence of VCGs in *Botrytis cinerea*, the pathogen causing gray mold diseases of several fruit and vegetable crops was reported by Delcan and Melgarejo (2002) who described both *nit1* and *nitM* mutants that originated from the same isolates. But these mutants were unstable. A method, facilitating the recovery of both *nit1* and *nitM* pairs of *B. cinerea*, was developed by Beever and Parkes (2003). A large number of VCGs (59) among 82 field strains of *B. cinerea* in New Zealand

was recognized (Beever and Weeds 2004). Selenate-resistant mutants of *B. cinerea* were identified first by Weeds et al. (1998). The *sel* mutants were recovered spontaneously in the sector method from 21 *B. cinerea* strains grown in the presence of sodium selenate. Eighty one percent of *sel* mutants were also sulphate non-utilizing (*sul*) mutants. Mycelial incompatibility (barrage) is widespread in the populations of *B. cinerea* (Beever and Parkes 2003). The *sul* mutants are useful in defining VCGs in *B. cinerea*. One hundred and four *sul* mutants were divided into two complementary groups: resistant (66 mutants) and sensitive to chromate. Based on compatibility reactions, chromate-resistant and chromate-sensitive *sul* mutants of 12 strains were found to be compatible only with themselves and were each classified as belonging to different VCGs. No correlations could be deduced between VCGs and strain, host or geographic origin or colony morphology or pathogenicity. However, pathogenicity was dependent on the morphological characteristics of strains of *B. cinerea* (Korolev et al. 2008).

2.1.2 Pathogenicity and Host Range

Any microorganism(s) considered to be associated with plants showing visible symptoms has to be proved to be pathogenic. The ability of the microorganism to infect a plant species is known as pathogenicity which differentiates the pathogen from other microorganisms. Intensity of disease induced by the pathogen is referred to as virulence (aggressiveness). The isolates may vary in their virulence on a particular host species and the virulence is assessed by inoculating a set of plant species or cultivars that differentially respond to different isolates. Based on the disease intensity (infection types), the isolates may be classified into different subspecies, strains, races, biotypes or form species (*formae speciales*). The results of detection, identification and differentiation of microbial plant pathogens by various methods are expected to be validated by pathogenicity tests. The important limitation of many molecular methods is their inability to distinguish living and dead spore forms or pathogen structures from which protein or nucleic acid components are extracted for testing. The pathogen species may be able to infect a large number of plant species (wide host range), whereas another pathogen may have a restricted host range (capable of infecting only a few plant species). Determination of the host range of an unknown pathogen may be helpful to have a clue on the identity of the pathogen concerned.

Bait tests are conducted to detect the presence of the pathogen in the substrate (soil) by planting seeds or seedlings of the highly susceptible cultivar or host species on the soil to be examined. Duncan (1980) developed a root tip bait test for the detection of *Phytophthora fragariae* in strawberries. Root tips (25–50 mm) are cut from strawberry runners and mixed with soil-less planting mix in the ratio of one part: three parts of mix in pots. Highly susceptible *Fragaria semperflorens* var. *alpina* (Baron Solemacher) plants are planted in pots. After a period of 3–6 weeks, the bait plants collapse and their roots reveal the presence of oospores of the pathogen and

red coloration of the stele. This test is quite sensitive, but time-consuming. A similar test was applied for detecting *Phytophthora fragariae* var. *rubi* in infected raspberry plants (Duncan 1990). A modified method of Duncan et al. (1993) was employed for the detection of *P. fragariae* var. *rubi* in certified raspberry stocks. After removing soil adhering to the roots of test plants by shaking, the unwashed roots were cut into pieces (1–3 cm). A layer of about 2–3 cm of sterile peat/sand mixture (3:1) was placed in pots (15 cm diameter) and then the root pieces were kept as a top layer. A bait plant (highly susceptible cv. Glen Moy) was planted directly into the cut root pieces in each pot, followed by filling with sterile compost as required. The bait test was carried out in a glasshouse at 16°C and with an 18 h day length cycle. The bait test confirmed the infection of four raspberry stocks as did the nested polymerase chain reaction (PCR) assay. The results suggested that the bait test was more sensitive, requiring just only one third of the amount of infected roots necessary to give a reliable positive result by PCR. However, the bait test requires long time, expert knowledge for identification of oospores of *P. fragariae* var. *rubi* at the end of bait test (Schlenzig et al. 2005).

A pepper leaf disk assay was developed for detecting *Phytophthora capsici*. Five pepper leaf disks (0.5 cm diameter) were floated on the saturation water prepared from the pathogen-infested soil. After 24 h, the leaf disks were removed, surface-sterilized in 0.5% sodium hypochlorite solution for one min, rinsed in sterile water and plated on a medium that favored development of the pathogen. After 72 h, the colonies of *P. capsici* developing in the medium were identified and the percentage of leaf disks colonized by the pathogen was determined (Larkin et al. 1995). Baiting assays were carried out for the detection of fungus-like *Pythium ultimum* var. *sporangiferum*, *Phytophthora cactorum* and *Phytophthora cryptogea* using leaf disks (8 mm diameter) cut from the leaves of *Rhododendron ponticum* and seeds of *Cannabis sativa*. The baits were cleaned by wiping with 75% ethanol. The baits (10) were placed directly on each sample (water suspension) and incubated overnight (16 h) on the laboratory bench. For in situ bait tests, baits were placed in nylon mesh bags for easy retrieval and were left in place for 36 h. After incubation for required period, baits were taken out, blotted on sterile tissue paper and plated on PDA kept in petriplates supplemented with antibiotics rifamycin (30 mg/l) and pimaricin (100 mg/l). The baits with visible colonies growing from them were counted and expressed as number of baits colonized out of total (10). Baiting assays, dipstick immunoassay, zoospore trapping immunoassay (ZTI) and membrane filtration-dilution plating (MFDP) were compared for their efficacy. The ZTI was the most sensitive test for water samples, but MFDP provided more consistent results. For in situ testing, baiting assay and dipstick immunoassay were most effective and the latter test provided valuable, quantitative data on pathogen propagule numbers, useful for epidemiological studies (Pettitt et al. 2002).

Rhizoctonia solani (anastomosis group (AG) 8) and *R. oryzae* are important pathogens causing root and bare patch diseases in wheat and barley. Considerable difficulty is associated for the isolation of *R. solani* AG 8 from the root systems and quantifying pathogen population in soil. A quantitative assay of active hyphae using wooden toothpicks as baits was developed. The toothpicks are

inserted into the test soil samples and after 2 days they are placed in a selective medium.Then the number colonies developing after 24 h are counted under the dissecting microscope. *R. solani* and *R. oryzae* could be differentiated based on hyphal morphology. This simple and inexpensive technique can be used for detection of the pathgoen and diagnosis of the disease in commercial production areas (Paulitz and Schroeder 2005).

2.1.3 Biochemical Methods

2.1.3.1 Detection of GUS Activity

Constitutive expression of ß-glucuronidase (GUS) in the fungal pathogen transformed with GUS reporter gene has been exploited to establish the cause of certain diseases. Strains of *Cladosporium fulvum* infecting tomatoes and *Leptosphaeria maculans* infecting brassica crops expressing ß-glucuronidase activity were produced. The activity of this enzyme was used to detect histochemically the presence of the hyphae of these pathogens in infected host plant tissues. Further, the GUS activity of *C. fulvum* could be used as a measure of fungal biomass in the cotyledons of infected tomato seedlings (Oliver et al. 1993). In another investigation, GUS activity in leaves infected with an isolate of *C. fulvum* race 4 transformed with *uidA* gene was assessed by fluorimetric assay with methylumbelliferone as a substrate. GUS activity was useful for the detection and quantification of biomass of *C. fulvum* in tomato leaves and the efficacy of this method was compared with plate-trapped antigen (PTA)-ELISA format. Both GUS assay and PTA-ELISA could detect the pathogen at very low levels (<1 mg/g) in infected leaves immediately after inoculation. No GUS activity or pathogen-specific antigen (OX-CH1) could be detected in uninoculated plants. The course of infection by *C. fulvum*, as determined by these two techniques was similar up to 14 days after inoculation (Karpovich-Tate et al. 1998). For the detection of the endophytic *Fusarium moniliforme*, visual markers that may be recognized by histochemical staining were employed. Three strains of *F. moniliforme* transformed with a plasmid pHPG, containing the *gusA* reporter gene encoding GUS and the *hph* gene for hygromycin resistance as a selectable marker, were produced. The presence of the transformed strains in wheat plants and grains was detected by the GUS activity which was due to the fungus, but not due to the host plant. As this pathogen is capable of producing mycotoxins, the early detection of the pathogen is of great importance to human and animal health (Yates et al. 1999).

The cause of the malformation disease of mango has not been established unequivocally, since the involvement of *Fusarium subglutinans*, eriophyid mite (*Aceria mangifera*) and physiological factors has been suggested. GUS transformants of *F. subglutinans* expressing GUS reporter gene were used to inoculate mango floral and vegetative buds. GUS activity was monitored microscopically in inoculated/infected and noninoculated plant tissues. The GUS-stained mycelium

present in the mango tissue was observed after infiltration of a mixture containing X-Gluc (50 µg/ml), 0.1 M $NaPO_4$ (pH 7.0), 10 mM EDTA and 0.5 mM each of K ferri- and ferrocyanide in 0.05% Triton-X (v/v) into the tissues for 5 min. After clearing the tissues of chlorophyll by washing twice over a period of 48 h in chloral hydrate (120 g/100), the tissues were examined under a stereomicroscope to detect the presence of GUS transformants. GUS activity of the transformants was quantified in the extracts of the mycelium using 4-methyl umbelliferyl-ß-D-glucuronide (MUG) fluorimetric assay. The GUS transformed isolates were pathogenic to mango, causing typical vegetative and floral malformation symptoms in mango. The enzyme was detectable histochemically by using the substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide. As the GUS enzyme is absent in the wild pathogen isolate and mango, the GUS activity detected is attributed to the presence of transformed pathogen isolate providing definite evidence for its involvement in the development of mango malformation disease (Freeman et al. 1999).

Verticillium dahliae with a wide host range and *V. longisporum* were considered to be associated with Verticillium wilt on oilseed rape (*Brassica napus* subsp. *oleifera*). Controversy concerning the taxonomy of *V. longisporum* as a separate host-specific species of *Verticillium* was in existence (Fahleson et al. 2003). *V. dahliae* has been indicated as the causal agent of Verticillium wilt in *Brassica* crops (Söchting and Verreet 2004) and in horse radish (Babadoost et al. 2004). In order to study the infection patterns of *V. dahliae* and *V. longisporum* on the roots of *B. napus*, these two fungi were labeled with green fluorescent protein (GFP) from the jelly fish (*Aequorea victoria*) and their colonization of roots was visualized by confocal laser scanning microscopy (CLSM). GFP was stably expressed following *Agrobacterium tumefaciens* (At)-mediated transformation. This investigation revealed that *V. longisporum* upon penetration, readily spread into the vascular system. In contrast, the systemic growth of *V. dahliae* was strongly inhibited and the hyphae were loosely attached to the root surface only. The results confirm that *V. dahliae* was non-pathogenic on *B. napus* and the wilt disease was caused by *V. longisporum*, clearing the doubt on the aetiology of the Verticillium wilt disease of *B. napus* (Eynck et al. 2007).

2.1.3.2 Isozyme Analysis

Protein profiles of a fungal pathogen may be obtained by separating the proteins extracted from the target pathogen by employing electrophoresis techniques. These techniques are useful to separate and sometimes purify macromolecules such as proteins or nucleic acids that differ in size, charge or conformation. When charged molecules are placed in an electric field, they tend to migrate toward positive or negative pole according to their charge. Proteins can have either net positive or negative charge. On the other hand, nucleic acids have a consistent negative charge imparted by their phosphate backbone and hence migrate toward anode. Agarose or polyacrylamide gels are commonly used. Agarose is a polysaccharide extracted from sea weed used at 0.5–2.0% concentration. Agarose gels

are very easy to prepare and have a large range of separation, but relatively low resolution. Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer is determined by the concentration of the acrylamide (3.5–20%) used. As acrylamide is a potent neurotoxin, required care must be taken while handling. Polyacrylamide gels have comparatively small range of separation, but have very high resolving power.

Various electrophoretic techniques such as starch gel electrophoresis, polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) have been applied to separate isozymes. The starch gel electrophoresis is the simplest and relatively inexpensive technique, requiring fewer toxic chemicals than other methods. It is possible to use this method to screen many different enzyme activities simultaneously using appropriate stains. In this method, starch suspension is boiled and poured into a rectangular mold to form the gel (several centimeter thick). After cooling, a vertical slice is made through the gel close to one of the longer edges. Filter paper wicks dipped into the enzyme sample are placed side by side along one side of the slit. The two sides of the gel are squeezed back together facilitating the establishment of contact with the tray buffer, using cloth wicks. Depending on the buffer used, current is passed for 2.5–4 h. During electrophoresis, cooling is important to prevent enzyme degradation resulting in irregular migration pattern. After completion of electrophoresis, the gel is sliced both vertically and horizontally. About 4–45 stainable slices may be prepared from a single gel, depending on the number of samples and gel thickness. The gel slices are placed in specific activity stains kept in different trays (Bonde et al. 1993).

Isozymes may be defined as multiple molecular forms of a single enzyme and these forms have similar enzymatic properties, but slightly different amino acid sequences. The genetic locus may be monomorphic (expresses in a single allele). When the genetic locus is polymorphic, the isozymes formed by the expression of different alleles are called as allozymes. The usefulness of isozyme electrophoresis for the detection and identification of fungal pathogens has been demonstrated. Isozyme analysis is a powerful biochemical technique that can be used to detect, differentiate and identify morphologically similar or closely related species, varieties and *formae speciales*. Furthermore, this technique can be used to analyze genetic variability, trace pathogen spread, follow the segregation of genetic loci and identify ploidy level of the fungi and other pathogens. The ability of isozyme analysis to differentiate species and subspecies helps in identification of fungal pathogens and "fingerprinting" commercially important strains. The precise and rapid identification of an unknown pathogen may aid in early application of disease management strategies. Protein profiles of a fungal pathogen may be obtained by extracting proteins from a few milligrams of fungal cells, followed by running an electrophoresis of the denatured proteins in a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the separated proteins are stained and the patterns are compared visually or by computer-based analysis for differences between species of the fungal genus. Fingerprints obtained by this procedure based on the presence of multiple molecular forms (isozyme) of certain enzymes. These forms have similar, but not identical enzymatic properties.

Table 2.4 Application of isozyme analysis for identification of fungal pathogens (Adapted from Micales and Bonde 1995)

Fungal pathogens	Polymorphism (%)	References
Erysiphe graminis f.sp. *hordei*	0	Newton et al. 1985
E. graminis f.sp. *secalis*	20	Koch and Kohler 1990
E. graminis f.sp. *tritici*	20	Koch and Kohler 1990
Fusarium oxysporum	24	Bosland and Williams 1987
Peronoscleropsora sorghi	23	Bonde et al. 1984
Phytophthora cinnamomi	23	Old et al. 1984
P. infestans	54	Tooley et al. 1985
Puccinia graminis f.sp. *tritici*	0, 61	Burdon et al. 1983, 1986
P. recondita f.sp. *tritici*	9	Burdon et al. 1986
P. striiformis f.sp. *tritici*	0	Newton et al. 1985
Pyricularia oryzae	55	Leung and Williams 1986
Rhizoctonia solani	38	Liu et al. 1990
Rhynchosporium secalis	38	Newton 1991
Stagonospora nodorum	11	Newton 1991
Tilletia indica	44, 52	Bonde et al. 1985, 1989
Uromyces appendiculatus	67	Lu et al. 1987
Ustilago zeae	40	Newton 1991

Isozyme patterns are recorded according to their relative mobility and each band is considered as an allele of a specific locus. The bands are labeled alphabetically from the slowest to the fastest. The identification of an organism is based on the number of genes in common with those of known organism. The amount of interspecific variation determined by isozyme analysis is slight, because of only slight differences in enzyme structure, thus making accurate identification of a species or subspecies possible (Table 2.4) (Bonde et al. 1993). This technique has been found to be relatively cheap or at least cheaper and faster than immunoassays or restriction fragment length polymorphism (RFLP) procedure. Further, numerous genetic loci may be compared for discrimination at species level and less frequently between species (Schulze and Bahnweg 1998).

Maize crops are infected by downy mildews caused by three species of *Peronosclerospora*, the identity of which is difficult based on morphological characteristics, since extensive morphological variation exists within species. Isozymes from ten fungal cultures representing three *Pernosclerospora* spp. were compared electrophoretically on starch gels for species identification. None of the enzymes (11) tested was influenced by the host species on which the pathogen species was grown, the time of the year of conidial collection or the growth conditions (greenhouse or growth chamber). Isozyme analysis was found to be useful for distinguishing *P. sorghi*, *P. sacchari* and *P. sacchari-philippinensis* complex. Further, analysis of isozyme variations permits a quantitative interpretation of differences on the basis of genetics of pathogen species. However, it has to be borne in mind that isozyme analysis is not a substitute for, but an adjunct to, morphological characteristics which form the basis of systematics of fungal plant pathogens (Bonde et al. 1984). In addition to

detection of fungal pathogens, isozyme analysis has been demonstrated to be useful for recognizing development of resistance of tobacco to *Peronospora tabacina* by affecting the pathogen development (Pan et al. 1991).

Based on isozyme patterns, it was possible to distinguish the teliospores produced by *Tilletia indica* and *T. barclayana* present in stored grains in storage facilities and transportation vehicles (Bonde et al. 1989). Using the isoenzymes of pectin esterase and polygalacturonase, European *Armillaria* spp. were identified. The pectinase isozyme patterns of *A. mellea* were shown to be the most divergent, whereas *A. ostoyae* and *A. borealis* were the most closely related (Wahlström et al. 1991). Isozyme variation within among three species of *Phytophthora* was studied, using 162 isolates from a wide range of geographical locations and host plants. *P. cambivora*, *P. cinnamomi* and *P. cactorum* were compared using 18 isozyme loci by starch gel electrophoresis. These pathogens were clearly separated based on isozyme analysis and each species could be further subdivided into electrophoretic types (ETs). *P. cambivora* separated into eight ETs, while eight and two ETs were recognized in *P. cinnamomi* and *P. cactorum* respectively. By using cellulose acetate electrophoresis (CAE), three enzymes, phosphoglucose isomerase, malate dehydrogenase and lactose dehydrogenase were shown to be diagnostic, allowing clear identification of these three *Phytophthora* spp. (Oudemans and Coffey 1991a). Interspecific relationships that cannot be predicted on the basis of morphological characteristics, could be established among the twelve papillate species of *Phytophthora* based on isozyme analysis. *P. meadii* and *P. botryosa* clustered together indicating close genetic relatedness, while *P. kasturae* and *P. heveae* formed a single cluster (Oudemans and Coffey 1991b). The requirement of large quantities of the pathogen structures, as compared to immunoassays or nucleic acid-based techniques is considered as an important disadvantage of employing isozyme analysis. Extracts of mycelial proteins of *Phytophthora* sp. infecting *Chukrasia tabularis* were subjected to PAGE analysis. The mycelial proteins of this pathogen produced protein patterns identical to that of *P. nicotianae* infecting tobacco. The sporangial characteristics of *Phytophthora* sp. were similar to *P. nicotianae*, as confirmed by Common Wealth Mycological Institute (CMI), England, establishing the identity of the pathogen infecting *C. tabularis* as *P. nicotianae.* The results suggest that gel electrophoresis may be useful as an adjunct to the taxonomic characters for identifying species of *Phytophthora* (Aggarwal et al. 2001).

The differences in the pathogenic potential (virulence) may be used as the basis for identification of strains of fungal pathogens using isozyme analysis. Majority of the isolates (68) of *Leptosphaeria mauclans* contained a single isozyme of glucose phosphate isomerase (GPI) which migrated faster in starch gel (70 mm) than the isozyme of GPI present in the rest of the isolates (24) that could move only 65 mm in 11 h. Thus the isolates of *L. maculans* clustered into ET1 or ET2 reflecting fast or slow movement of GPI. Highly virulent strains had fast moving GPI (ET1), whereas the GPI in weakly virulent strains moved slowly (ET2) (Sippell and Hall 1995). A reliable procedure to identify *L. maculans* based on GPI electrophoresis on starch gels was developed. The extracts of lesions in infected oilseed rape (*Brassica napus*) were directly employed. Four different ET patterns ET1, ET2,

ET3 and ET4 of allozymes were recognized and these patterns correlated with virulence of the isolates of *L. maculans*. Group A isolates with high virulence showed ET1 pattern, whereas group B isolates (weakly virulent) exhibited ET2 pattern. On the other hand, the isolates in ET3 pattern induced a few typical and atypical lesions. The isolates of *Pseudocercosporella capsellae* were detected in the leaf lesions induced by this pathogen. They belonged to ET4 pattern, the allozyme being the fastest, allowing the differentiation of *P. capsellae* and *L. maculans* infecting rape plants (Braun et al. 1997).

Colletotrichum gloeosporioides causes anthracnose disease in a large number of crop plants, in addition to its infection on many weed species. The isozymes of nicotinamide adenine dinucleotide dehydrogenase (NAOH) and diaphorase (DIA) yielded the maximum number of electrophoretic phenotypes that clustered on the basis of host origin. The isolates from different plant species were grouped into three major groups (I, II and III) and four subgroups (IA, IB, IIIA and IIIB) (Kaufmann and Weidemann 1996). Resolution of allozyme genotypes of *Phytophthora infestans*, the historically important pathogen that ruined potato crops in Ireland, resulting in the migration of people in millions to other countries, was achieved by applying cellulose acetate electrophoresis (CAE) at two loci- glucose-6-phosphate isomerase (GPI) and peptidase (Pep). CAE system is more rapid, requiring only 15–20 min as against 16–18 h needed for starch gels. Further, it is possible to predict mating types and metalaxyl sensitivities of the isolates of *P. infestans* collected from the fields, using CAE system (Goodwin et al. 1995). The isolates of *P. infestans* (726) existed in Canada were grouped into eight genotypes, based on the allozyme banding patterns at two loci GPI and Pep with markers for mating types, metalaxyl sensitivities and cultural characteristics. Similarities between five genotypes present in Canada and United States were revealed by CAE analysis (Peters et al. 1999). Eighty five isolates of *P. infestans* collected from tomato and potato fields in North Carolina were classified into four allozyme genotypes at GPI and Pep loci (Fraser et al. 1999).

When 27 isolates of *Fusarium oxysporum* were analysed, polymorphism in 5 enzymes was detected by electrophoresis technique. Twenty six electrophoretic groups were identified (Paavanen-Huhtala et al. 1999). Using CAE technique, isozyme polymorphisms among various isolates of closely related *F. cerealis*, *F. culmorum*, *F. graminearum* and *F. pseudograminearum* present in different countries across the world were examined. The electrophoretic types (ETs) of adenylate kinase (AK), NADP-dependent glutamate dehydrogenase (NADPGDH), peptidase B (PEPB), peptidase D (PEPD) and phosphoglucomutase (PGM) were compared. PEPD alone was useful as marker for identification and differentiation of the four taxa investigated revealing its potential to be used as a rapid and simple CAE-based diagnostic tool. Uniform isozyme patterns were noted for different *Fusarium* species irrespective of the geographical origin of the isolates or the host/substrates from which they were isolated. Based on the similarity values, *F. graminearum* was considered to be more closely related to *F. cerealis* and *F. culmorum* than to *F. pseudograminearum.* This is in contradiction to the morphological similarity of *F. graminearum* and *F. pseudograminearum*. Morphological similarity of these

fungi does not seem to reflect their genetic relatedness (Láday and Szécsi 2001, 2002).

Cell wall protein analysis has been shown to be useful for identification of some fungal pathogens. Six different species viz., *Pythium graminicola*, *P. iwayamai*, *P. okanowganense*, *P. paddicum*, *P. vanterpoolii* and *P. volutum* cause the snow rot disease of winter cereal. The cell wall proteins of these pathogens (MW 25–40 kDa) form the major component among cell wall proteins of each species. The electrophoretic patterns of the glycoproteins, detected with Coomassie brilliant blue, lectin and antibody, exhibited sufficient interspecific polymorphism and intraspecific stability to allow identification and classification of the six *Pythium* spp. the causative agents of snow rot disease (Takenaka and Kawasaki 1994).

Changes in the enzyme activities have been reported to be a reliable basis for detection of certain fungal pathogens. The activities of peroxidase (PO), polyphenoloxidase (PPO), phenylalanine ammonia lyase (PAL), ß-1,3-glucanase (Glc), superoxide dismutase (SOD) and amylase (Amy) in healthy and *Verticillium*-infected eggplants were determined. Enhancement of the activities of Glc and Amy to significant levels in infected eggplants was recorded (Kawaradani et al. 1994). In a further study, increases up to 50 times in the activities of Glc in infected plants over healthy controls were noted, when the PNPG4 degradation method was followed. This method using *p*-nitrophenyl-ßD-laminarintetraside as a substrate was found to be easier and more precise. Hence it was suggested as a suitable method for diagnosing the disease in eggplants (aubergines) (Kawaradani et al. 1998).

Latent infections by fungal pathogens may be detected by treating the infected tissues with chemicals. Winter wheat leaves showing no visible symptoms are detached, washed, surface-sterilized and treated with paraquat (0.03–0.32% active ingredient (a.i.)). The treated leaves are placed in nutrient agar medium kept in petridishes and incubated. As the chlorophyll is degraded by the chemical, the presence of fungal pathogens in the treated leaves can be viewed easily under the light microscope. This procedure was successful in detecting latent infection by *Botrytis cinerea* in grapes (Gindrat and Pezet 1994).

Soil-borne pathogens initiate infection in the roots and/collar region below the soil level, making the recognition of infection in the early stages very difficult. Aerial plant parts exhibit visible symptoms, only when the pathogen has already well spread within the infected plants with a remote possibility of saving such infected plants. The efficacy of visual and infrared assessment of root colonization of apple trees by *Phymatotrichopsis omnivora* was assessed by Watson et al. (2000). The differences between the infrared readings of canopy temperature and air temperature were significant ($p < 0.01$) and this criterion was used as a basis for predicting infection of asymptomatic infected apple trees. Extensive tap root decay and infection of lateral roots were noted on visual observations. This study indicated the potential of employing infrared technique for early detection of fungal diseases affecting tree crops for assisting early disease management decisions.

2.1.4 Immunoassays

There is an imperative need to identify and eliminate all primary sources of inoculum from which plant pathogens may be introduced into new locations or fields, by early detection of pathogens. This step is essential to prevent introduction and to restrict subsequent spread of microbial plant pathogens. The conventional methods involving isolation of pathogens in appropriate media and identification by studying morphological characteristics are time-consuming and require knowledge of taxonomy. In addition, these methods cannot distinguish closely related species and strains of the same species reliably, necessitating the development of faster and more discriminative methods. Detection methods may be divided into two groups. The specific methods may be employed to detect particular species or group of pathogens after preliminary diagnosis, indicating the presence of target pathogen. Another group includes nonspecific methods which may be useful for detecting unknown pathogen(s) or when the presence of many pathogens is to be detected, as in the case of quarantine and certification programs (Chu et al. 1989). However, the terms specific or nonspecific to indicate the extent of reliability of the procedure to be applied for the detection of pathogens appears to be more appropriate. Immunoassays and nucleic acid-based techniques can be considered as more specific and reliable than most of the biochemical methods which are comparatively nonspecific and less reliable, though these methods are simpler (Narayanasamy 2001).

Immunology has developed as a multipurpose technology with innumerable applications in biological sciences in general and in medicine and agriculture in particular. Immunological techniques have been demonstrated to be highly specific, sensitive, simple, rapid and cost-effective and can be automated for large scale applications. Immunodiagnosis may be used for confirming visible symptoms and for the rapid detection of pathogens that cannot be easily identified by other routine methods. Further they permit early detection and precise identification of important pathogens, enabling application of fungicides that are specific only to certain pathogens or groups of pathogens at appropriate time (Narayanasamy 2001, 2005). The principles of immunological reactions, their applications, advantages and limitations of various immunoassays for the detection and identification of fungal pathogens are discussed below.

2.1.4.1 Principles of Immunological Reactions

Introduction of an antigen into an animal system results in a characteristic immune response. The antigens may be fairly large molecules or particles containing protein or polysaccharides. Antigens may be defined as compounds (macromolecules, cellular components or cells) that are capable of inducing production of specific antibodies (immunoglobulins) capable of reacting with the same antigen. This property of the antigen is called as immunogenicity. Another property of the

antigen known as antigenicity is its ability to react specifically with the antibody that was induced by the antigen concerned, in the animal system. The antigen should be both immunogenic and antigenic. Serum that is separated, after blood cells are removed following clotting, contains the antibodies and this is designated antiserum. Some small molecules with specific structures do not have the capacity to stimulate the production of antibodies. However, they are able to react with antibodies produced by other antigens containing the small molecule as part of their structure. Such small molecules are termed as haptens. Antisera containing antibodies specific to haptens can be produced by injecting into the animal system, haptens coupled with carrier molecule of required size. The lymphoid cells in the animal body contain receptors that can recognize the antigen, leading to proliferation of plasma cells that secrete antibodies specific to the antigen. This type of response is known as humoral immunity. Another response called as cell-mediated immunity involves the proliferation of immune lymphocytes possessing antigen specific receptors without any concomitant liberation of circulating antibodies (Sissons and Oldstone 1980).

Antigenicity of the antigen is due to the existence of specific parts of the antigen known as epitopes or antigenic determinants. An epitope has a three-dimensional structure that is complementary to the binding site present in the antibody molecule. The epitopes may be of a continuous determinant type containing a contiguous sequence of amino acid residues exposed at the surface of a native protein and distinctive conformational features. The second type of epitope, a discontinuous determinant possesses residues that are not contiguous in the primary structure of the native protein, but the distant residue may become contiguous through the folding of the polypeptide chain or by juxtaposing two separate peptide chains (Atassi and Lee 1978). The third type known as cryptotypes (present in viral capsid proteins) may become antigenically active only after breakage, depolymerization or denaturation of the antigen (Jerne 1960). Serological properties may be affected by changes in the amino acid sequence of proteins. If the substitution of a particular amino acid leads to variation of antigenic reaction, the amino acid in question is considered to contribute to the structure of the epitope (Van Regenmortel 1982).

Antibodies induced by different antigens belong to a group of proteins designated immunoglobulins (IgG). They have the ability to specifically bind to the antigen that induced their production in animals. They are produced by lymphoid (B) cells or ß-lymphocytes and they make their appearance in the serum of immunized animals. The B-cells may be present in the spleen, lymph nodes, Peyer's patches of the digestive tract and peripheral (circulating) blood. Each B-cell produces one type of antibody specific for one epitope (antigenic site) present on the antigen.

The basic structure of all IgGs is quite similar. The IgG molecule present in the rabbits contains one pair of identical light (L) chain (MW 25,000) and heavy (H) chain (MW 50,000). These two chains are linked together by noncovalent forces and disulfide bonds. A variable and constant region has been identified in each light and heavy chain. The amino acid sequences of a variable region (V_L) is located in the N-terminal half of the different antibodies. This V_L region shows extensive

variations. On the other hand, the amino acid sequences of the constant region (C-terminal half) may reveal variation only to a lesser extent. The sequence variability of the V_L region is not uniformly distributed along the V_L region, but confined to three regions totalling about 25 residues called as hypervariable regions. The nature of the antibody combining sites coming in contact with epitopes present on the antigens is determined by the hypervariable residues. The N-terminal quarter of the H chains is also variable (V_H). The variable regions (V_H and V_L) are located in the two arms of the Y-shaped IgG molecule. The L chains are placed externally in the arms and have one variable and a constant (CH1) regions in the arms and two parts (CH2 and CH3) toward the C-terminal end. The CH1 and CH2 domains are separated by a short region known as the hinge region which is sensitive to proteolytic enzymes (Fig. 2.6).

There are different classes of antibodies defined, based on the constant region located in the C-terminus of the antibody molecule. These classes of antibodies are named as IgG, IgM, IgA, IgD and IgE and the subclasses such as IgG1, IgG2a, IgG2b, IgG3, IgG4, IgM1, IgM2, IgA1 and IgA2 have also been distinguished, using commercially available isotyping reagents. IgG and IgM are produced generally in response to antigenic stimulation. The serum containing the antibodies (immunoglobulins) induced in response to the antigen, is known as the antiserum that specifically reacts with the antigen concerned. The sensitivity of the immunoassays can be markedly increased by using purified immunoglobulins in place of whole antiserum. During the antigen–antibody interaction, the noncovalent intermolecular forces that hold together the antibody-combining sites (paratopes) and antigenic determinants (epitopes) are similar to those involved in the stabilization and specific configuration of proteins. These forces become operative as the paratopes and epitopes are brought together. The closer the contacts established between the reactants, stronger will be the antigen–antibody bond (Van Regenmortel 1982).

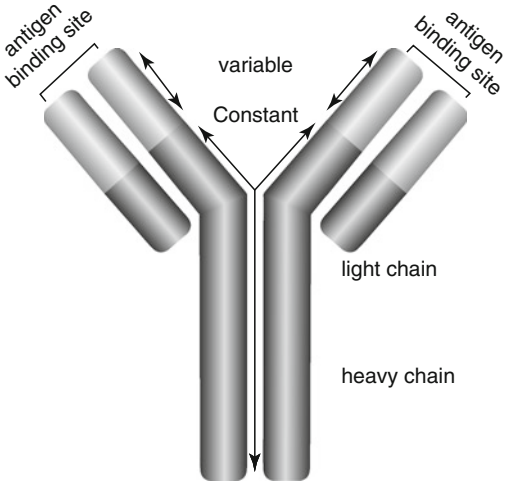

Fig. 2.6 General structure of antibody (Department of Immunobiology, University of Arizona, Tucson, USA)

2.1.4.2 Preparation of Antisera

The basic requirement for performing various immunoassays for detection, identification, quantification and differentiation of microbial plant pathogens is the availability of suitable antisera. Depending on the size and structure of the antigens, immunogenicity of the antigen concerned varies considerably. Antisera containing polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) have been prepared to suit the required levels of sensitivity and specificity of serological reactions.

Polyclonal Antisera

Among the microbial plant pathogens for which PAbs have been generated, viruses have the simplest structure, whereas the bacteria, being mostly single-celled, have complex structural elements. The complex nature of the antigen, further increases in the case of fungal pathogens which produce various spore forms and sporulating structures at different stages of their life cycle. Hence, the antiserum produced using the spores or mycelium in the early stages, may not be useful for detection of the pathogen in the later stages of its life cycle. Different animals such as rabbits, mice, fowl and horses have been used for producing antisera. However, rabbits are the widely used test animals for producing the antisera against plant pathogens. The protocols for preparing the PAbs for various fungal pathogens differ considerably and some of them are presented in Appendix at the end of this chapter.

The antigen solution may be emulsified with Freund's complete or incomplete adjuvant. The complete adjuvant contains killed and dried cells of *Mycobacterium butyricum*, in addition to a mixture of mannide monooleate (1.5 parts) and paraffin oil (8.5 parts). The incomplete adjuvant does not contain the bacterial cells. The adjuvant stabilizes the antigen and prevents rapid translocation of the immunogen. Furthermore, the adjuvant induces an inflammatory reaction which favorably influences the formation of antibodies. The number of injections, quantity of immunogen needed for each injection, interval between injections and interval between the last injection and bleeding the animal vary considerably with immunogens. The immunized rabbits are allowed a suitable period of rest. The marginal veins in the ear are cut with a sterile razor and the blood is collected in sterile tubes. After coagulation of red blood cells and centrifugation, the clear supernatant antiserum is stored in small vials at 5°C. Preservatives such as glycerol, phenol or sodium azide may be added to preserve the serological activity of the antiserum.

Monoclonal Antisera

The hybridoma technology introduced by Köhler and Milstein (1975) is hailed as a revolutionary advancement in the process of antibody production that avoids many problems associated with the use of PAbs. The principal advantage of hybridoma

technology is the possibility of continuous supply of monoclonal antibodies (MAbs) secreted by hybridomas obtained by fusion of ß-lymphocytes (antibody producing cells) and myeloma cells (capable of multiplying indefinitely). Each hybridoma clone can produce identical antibodies that are specific for a single epitope present in the immunogen.

BALB/c mice of 6 weeks age are given two to four intraperitoneal injections with the antigen solution emulsified with an equal quantity of complete Freund's adjuvant (for initial immunization) or incomplete Freund's adjuvant for subsequent injections at suitable intervals. After a rest for required period, two intravenous booster injections of the antigen are given. The spleens are excised and the spleen cells (ß-lymphocytes) are used for fusion. Myeloma cell lines deficient in their ability to synthesize hypoxanthine (or adenosine) phosphoribosyl-transferase (HPRT or APRT) or thymidine kinase (TK) are used as cell fusion partners for ß-lymphocytes. The myeloma cells are provided by the American Type Cell Culture Collection (ATCC). Specific medium (Dulbecco's modified Eagle's medium (DMEM)) has been used commonly. DMEM contains glutamine-pyruvate antibiotic ß-mercaptoethanol (GPAM) plus 10% fetal calf serum (FCS). The mouse peritoneal macrophages from peritoneal cavities and thymocytes from thymus of mice are suspended in DMEM-GPAM-HAT 10% FCS medium and used as feeder cells for hybridoma clones.

Cell fusion is performed by following procedures using polyethylene glycol (PEG). The cell suspension containing spleen cells and myeloma cells in appropriate proportions are placed in the 96-well Falcon plates and incubated for required period in an incubator. The supernatant solution in each well is tested for the presence of antibodies using the enzyme-linked immunosorbent assay (ELISA). The desired hybridomas are cloned by limiting dilution procedure. Large quantities of MAbs may be obtained from mouse ascites which is found in the fluid accumulating in the peritoneal cavity (abdomen) injected with hybridoma cells in DMEM. For further details Narayanasamy (2005) may be referred.

Phage-Displayed Recombinant Antibodies

Bacteriophages, viruses infecting bacteria, replicate in and are released from the infected bacterial cells following the lysis of bacterial cell wall. M13 phage infecting *Escherichia coli* has single-stranded (ss)-DNA genome and flexuous (flexible) filamentous shape. Components of M13 such as phage DNA, gene 8 coat proteins, gene 3 attachment proteins and other proteins that may be fused with the phage components, are continuously produced in the bacterial cells without undergoing lysis. By genetically linking the DNA from antibody-producing ß-lymphocytes or hybridomas with phage gene 3 DNA, the recombinant antibodies can be produced. *E. coli* cells are infected by M13 phage carrying the gene 3 DNA-antibody fusion. Proteins encoded by antibody DNA are coexpressed along with gene 3 DNA. Any antibody DNA linked to phage DNA and antibody proteins fused to phage proteins

will be assembled and secreted just like phage proteins. Antibodies displayed on phage can be produced more rapidly and at lower cost compared to MAbs. These recombinant antibodies are as specific as MAbs in the identifying microbial plant pathogens.

2.1.4.3 Immunodetection Techniques for Fungal Pathogens

Fungus-like and fungal pathogens are known to infect numerous plant species including economically important crops. Comparatively immunodiagnostic techniques are more precise, sensitive, specific and rapid than biological methods of detection and identification of fungal pathogens. Although several immunoassays have employed PAbs, a high degree of specificity has not been obtained without sacrificing sensitivity. Many types of immunogen preparations containing whole cells (Kraft and Boge 1994), crude mycelial or spore extracts (Harrison et al. 1990), extracellular culture filtrates (Brill et al. 1994), secreted toxins (Ward et al. 1990), crude or partially purified soluble proteins (Velichetti et al. 1993) and cell wall extracts have been reported to offer varying degrees of specificity for the detection of target fungi. Further, no consistent advantage has been observed in using any particular life-stage of the target fungus (producing asexual or sexual spores) for the preparation of polyclonal antisera. The specificity of a polyclonal antiserum may be increased to a certain extent by absorbing it with heterologous antigens to remove cross-reacting antibodies or dilution of antiserum to suitable level. The presence of immunodominant high molecular weight polysaccharides may possibly adversely affect specificity of PAbs. In certain cases, use of various purified fungal components such as ribosomes (Takenaka 1992), mycelial proteins after electrophoresis (Lind 1990) and lectins (Kellens and Peumans 1991) has been attempted to overcome the problem of specificity.

Among the various immunoassays available, the type of assay to be employed has to be determined based on (i) the fungal tissue type (spores, mycelial extract etc.), (ii) type of plant tissue or plant parts to be tested (seeds, roots, leaves, flowers etc.), (iii) laboratory equipments and expertise available, (iv) number of samples to be tested and duration for which testing has to be extended, (v) the level of specificity and sensitivity required, and (vi) nature of antibodies available (PAbs or MAbs). Antisera against fungal pathogens may be generally produced using rabbits or mice for the generation of PAbs or MAbs respectively. However, chicken egg yolk was used to generate antibodies against *Colletotrichum falcatum* and *Fusarium subglutinans* infecting sugarcane. PAbs raised in egg yolk were shown to be equally effective as PAbs produced in rabbits (IgG) by performing indirect double antibody sandwich (DAS)-ELISA. Further, IgY preparation and purification procedures are less laborious requiring less time compared to IgG. The strains of the above mentioned sugarcane pathogens could be identified, quantified and differentiated by employing IgY (Vöhringer and Sander 2001). Phage-displayed antigens specific against surface epitopes of *Phytophthora infestans*, destructive potato pathogen were raised by Gough et al. (1999).

When the antigen and antibody are brought together, formation of complexes that can be inferred or visualized macroscopically, occurs. Production of complexes forms the basis of immunodiagnosis. The immunodiagnostic techniques fall into two categories: (i) direct methods and (ii) labeled methods. Precipitation, agglutination, diffusion, neutralization of infectivity and complement fixation tests are included in the category of direct methods. Labeled methods include enzyme immunoassays, immunofluorescence tests and radioimmunoassay. Special techniques for localization of pathogen antigens in the host plant tissues and for studies on the distribution of pathogens in plant hosts rely on labeling the antigens of the pathogen origin and visualization using light microscope or electron microscope. The direct methods were employed in the earlier years. As these methods require large volumes of antiserum and more time to obtain results, it becomes necessary to make suitable modifications to improve the sensitivity and reliability of immunoassays. Tempel (1959) developed the gel diffusion test for detection and differentiation of *formae speciales* of *Fusarium oxysporum.* Later more sensitive techniques were developed utilizing monoclonal antibodies that are specific at species or subspecies levels. Use of techniques involving labeled antibodies has become predominant because of higher levels of specificity and sensitivity in addition to the possibility of obtaining results rapidly.

Radioimmunosorbent assay (RISA), one of the labeled immunodiagnostic procedure, was employed for the detection of *Botrytis cinerea* antigens in homogenized samples from grapes. I^{125}-labeled γ-globulin was used as the marker to infer the reaction between the antigen and antibody. The mycelial suspensions of the pathogen were incubated with the labeled antibody and the radiation was counted by liquid scintillation.

The assay was specific for *B. cinerea*, although positive reaction was noted in the reactions with *B. allii*, *Sclerotinia* sp. and *Monilinia* sp. (Savage and Sall 1981). As the half-life of radioactive labels is short and existence of health hazards involved in handling radioactive materials, RISA test has only limited application in pathogen detection process.

Standard enzyme-linked immunosorbent assay (ELISA) and its variants have been used to detect and quantify the pathogenic fungi and also their metabolic products like mycotoxins. Species-specific and subspecies-specific MAbs have been produced for *Phytophthora cinnamomi* (Hardham et al. 1986) and inclusion of glutaraldehyde in the fixative improved the specificity of the reaction (Hardham et al. 1991). Commercial kits have been produced for on-site detection of fungal pathogens. The turf diseases due to *Pythium* sp., *Rhizoctonia solani* and *Sclerotinia homeocarpa* could be diagnosed by employing visible immunodiagnostic assay kits for plant "side testing" (Rittenburg et al. 1988). Using a species-specific MAb, *Pythium ultimum*, causing pre-emergence damping-off disease of strawberry was detected in roots of infected plants. Monoclonal antibodies raised against *P. ultimum* P201 were used in indirect ELISA format. *P. ultimum* isolates among 246 isolates of *Pythium* sp. from sugar beet roots and soil were readily identified on the basis of reactivity to antibody E5 in ELISA. All 188 isolates identified as *P. ultimum* at the asexual stage based on morphological characteristics reacted positively with E5 in

ELISA (Yuen et al. 1993). Banks and Cox (1992) precoated the walls of microplate wells with poly-L-lysine and glutaraldehyde to immobilize fungal hyphae onto walls of the wells. The hyphae were attached to the walls by overnight drying. The attached hyphae were uniformly coated and remained reactive. The plates could be stored at −20°C. This procedure was applied for the detection of *Penicillium aurantiogriseum* var *melanoconidium* (Banks et al. 1992) (Appendix 4).

Enzyme-linked Immunosorbent Assay (ELISA)

Development of the enzyme-linked immunosorbent assay (ELISA) is considered as an important milestone in the advancement of serological diagnosis of diseases caused by microbial plant pathogens, especially by viruses (Clark and Adams 1977). ELISA technique has been extensively employed for the detection, identification and quantification of pathogen propagules in plant tissues and other substrates. Because of its sensitivity, economical use of antiserum, availability of quantifiable data and amenability for automation for large scale application, ELISA has become a popular and preferred technique. There are different modifications and adaptations of standard ELISA to suit the requirements of the experiments and researcher's preference. The standard format is known as double antibody sandwich (DAS)-ELSIA. Other widely applied adaptations are direct antigen coating (DAC)-ELISA, Protein A (extracted from the cells of *Streptococcus aureus*) coating (PAC)-ELISA and indirect ELISA format using pathogen antibody in addition to labeled antiglobulin conjugate. The DAS-ELISA format is highly strain-specific and demands the use of different antibody conjugate for each pathogen (or strain) to be detected. In contrast, the indirect ELISA method can be performed using a common conjugate for different pathogen species and the antirabbit globulin conjugate or antigoat globulin conjugate available commercially can be used. This is a distinct advantage of using indirect ELISA over DAS-ELISA method. In the case of PAC-ELISA format, using the optimal concentration of protein A is an important requirement, since higher concentrations may result in nonspecific reactions and lower concentrations may give false negative results. Among the labeled methods of pathogen detection, ELISA technique has been employed extensively for the detection of several fungus-like and fungal pathogens. The oomycete pathogen *Phytophthora cinnamomi* infecting azalea was detected using commercial ELISA kits under greenhouse conditions. The multiwell kit detected the pathogen in root samples containing as little as 1.0% infected root tissue. The rapid assay kit was easy to use and provided results in short time compared to culture plate method (Benson 1991).

The antiserum produced with ß-D-galactosidase-labeled antirabbit IgG was used as the secondary antibody and cell fragments of strain *Fusarium oxysporum* f.sp. *cucumerinum* attached to balls (Amino Dylark) was employed as the solid-phase antigen in the ELISA test which was found to be highly specific and sensitive in detecting the pathogen (Kitagawa et al. 1989). The PAbs reacting with purified exopolygalacturonase (exoPG) produced by *F. oxysporum* f.sp. *radicis-lycopersici*

(FORL) were employed to detect the pathogen in wilt disease-affected tomato plants (Plantiño-Álvarez et al. 1999). An ELISA procedure was developed to detect and quantify antibodies to *Ophiostoma* (*Certocystis*) *ulmi* in polyclonal antisera and for its use as a screening assay to detect MAbs in hybridoma supernatants. The assay was effective in detecting pathogen antigen in saline extracts of diseased plant tissues (Dewey and Brasier 1988). In a later investigation, Dewey et al. (1989b) isolated two cell lines (hybridomas) that could produce MAbs capable of differentiating mycelial antigens of the virulent isolates of *O. ulmi* from those of non-aggressive subgroup isolates. Most of the MAbs (11) appeared to have the potential diagnostic value, the absorbance values being two- to tenfold higher with extracts from diseased than from healthy elm trees.

The anastomosis groups of *Rhizoctonia solani* have been identified based on the ability or inability to anastomose with the known isolates (standards). Using PAbs in immunodiffusion tests, attempts have been made to distinguish anastomosis groups of *R. solani* (Abe et al. 1969; Adams and Butler 1979). In a later study, PAbs raised against total secreted proteins cross-reacted in immunoblotting assays. Hence, MAbs to the secreted proteins were generated and they reacted with fewer proteins and exhibited greater degree of specificity for AG-8 isolates and proteins of lower MW in isolates from other anastomosis groups. Another MAb was still more specific reacting with 38-, 40-, and 50-kDa proteins from AG 8 isolates and cross-reacted only with few isolates of other anastomosis groups (Matthew and Brooker 1991). The presence of *R. solani* in poinsettia could be detected by ELISA (Benson 1992). The mycelial proteins of *Verticillium dahliae* were purified. The PAbs generated against the fungal proteins reacted positively with 11 of 12 *V. dahliae* isolates from potato, cotton and soil. However, this antiserum did not react with tomato isolate of *V. dahliae.* The PAbs used in double antibody sandwich (DAS)-ELISA, detected *V. dahliae* and *V. albo-atrum* in infected roots and stems of potato (Sundaram et al. 1991).

The efficacy of combination of baiting and double MAb-ELISA for detection of *R. solani* in soils was tested. This technique was rapid providing results in 3 days from the receipt of soil samples containing *R. solani.* This format involves recovery of *R. solani* isolates from colonized baits for the determination of their anastomosis group affiliation and pathogenicity. The isolates pathogenic to lettuce were identified as AG4 group (Thornton et al. 1999). A genus-specific MAb (NG-CF10) generated against *Nectria galligena* was successfully employed to detect *N. lugdunensis* (anamorph – *Heliscus lugdunensis*) infecting alder trees (*Alnus glutinosa*) in a plate-trapped antigen (PTA)-ELISA procedure. The pathogen biomass in infected leaves and roots was assessed by the immunoassay. Root tissues had lower pathogen biomass compared with leaf tissues (Bermingham et al. 2001).

Pythium violae, a soil-borne oomycete, was detected in field-grown carrots using PAbs (Lyons and White 1992). An MAb specific to *P. ultimum* was highly reactive to 21 of its isolates, but not to any of the 16 species of *Pythium* tested by ELISA. The test was effective in detecting *P. ultimum* in roots of sugar beet seedlings with more than two infections/10 cm of root (Yuen et al. 1993). The PAbs generated against crude cell wall fractions of *P. aquatile* or *P. coloratum* associated

with root rot of tomato were employed in ELISA test. Specific reactions with closely related isolates were discernible (Rafin et al. 1994). The PAb prepared against the cell walls of *P. ultimum* as the capture antibody and an MAb specific for recognition were used in indirect DAS-ELISA test for the detection and quantification of the pathogen. Strong positive reactions were noted when culture filtrates of seven isolates of *P. ultimum* were tested. The presence of the pathogen in the roots of sugar beet, beans and cabbage seedlings grown in infested soils was detected, even with one infection/100 cm of root tissues (Yuen et al. 1998). The monoclonal antibodies raised against surface antigens of *Pythium sulcatum* were highly specific to the isolates of *P. sulcatum*. These MAbs recognized glycoproteins in the cell walls and they could be employed to detect this pathogen in naturally infected carrot tissues and soil by applying indirect competitive ELISA (Kageyama et al. 2002).

Monoclonal antibodies were generated against components on the surface of glutaraldehyde-fixed zoospores and cysts of *Phytophthora cinnamom*i. These MAbs were used as isolate-specific-, species-specific- and genus-specific markers (Hardham et al. 1986). A dip-stick immunoassay based detection (Azodye) of MAb-labeled cysts attached to a nylon membrane was effective in detecting *P. cinnamom*i in a wide range of soil samples collected from beneath a diverse range of host plant species (Cahill and Hardham 1994). The antiserum raised against *P. cinnamomi* was employed to detect *Phytophthora* spp. in commercial nurseries equipped with water recirculation systems. DAS-ELISA format was effective in detecting the pathogen. However, *Rhododendron* leaf test trapped the widest range of *Phytophthora* spp. and more efficient than DAS-ELISA test (Themann et al. 2002). Commercial immunoassay kits have been developed for the detection of other *Phytophthora* spp. also. The efficacy of the Albert *Phytophthora* "flow thorough" immunoassay and multiwell ELISA kits (Agri-Screen) for detection of *P. capsici* and *P. cactorum* was assessed. The former test could be carried out easily and also rapidly (taking 10 min) for detection of *P. capsici* in pepper and cucurbit crops. On the other hand, the latter technique was effective in detecting the pathogen only in pepper tissues, but had higher absorbance values for healthy samples. The results of ELISA tests were corroborated by the plating method, using a semi-selective medium (Miller et al. 1994).

Soluble protein extracts of chlamydospores and mycelium of the soil-borne pathogen *Thielaviopsis basicola* causing cotton black root rot disease were used as immunogen to raise polyclonal ascites antibodies. The purified IgG fraction was biotin-labeled and used in DAS-ELISA test. Both brown and gray cultural types could be detected with negligible cross-reactivity with other soil-borne fungi encountered in cotton field soils. The detection limit of DAS-ELISA was between one and 20 ng of *T. basicola* protein, the earliest time of detection being 2 days after inoculation of roots. The results of immunofluorescence assay were also similar to that of ELISA test (Holtz et al. 1994).

Macrophomina phaseolina is a soil- and seed-borne pathogen capable of infecting numerous host plant species. A double-antibody sandwich (DAS)-ELISA method was developed for the specific detection and quantification of

M. phaseolina in plant tissues. PAbs were generated against the immunogens in mycelium and culture filtrate of the pathogen. PAbs raised against mycelium were more sensitive than the PAbs raised against the culture filtrate. The detection limit was 15–30 ng protein/ml. In cowpea plants showing symptoms of infection, *M. phaseolina* was quantified in leaves, epicotyl, hypocotyls and roots by DAS-ELISA at 1 month after seed or soil inoculation. Furthermore, DAS-ELISA format could be employed to detect *M. phaseolina* in asymptomatic plants, indicating cowpea plants may carry infection without expressing the symptoms for sometime (Afouda et al. 2009).

Propagule densities of fungus-like pathogens such as *Phytophthora citrophthora* have been detected and quantified in plant roots and soil samples using commercial ELISA kits (Timmer et al. 1993). In addition, *P. fragariae* var. *rubi* was detected in the root tissues of raspberry using a commercial multiwell assay kit at 4 days after inoculation (DAI), with a detection limit of about 0.25% of simulated infection level (percentage of infected tissue/healthy tissue, w/w) (Olsson and Heiberg 1997). In a later investigation, PAbs and MAbs were raised against specific proteins of this pathogen in strawberry roots (Pekárová et al. 2001). The diagnostic values of ELISA tests for the detection of *Phytophthora* at genus level and *P. ramorum* at species level were assessed along with polymerase chain reaction (PCR). TaqMan PCR and ELISA had higher sensitivities for genus level detection than species-specific detection, making them useful for prescreening of pathogen isolates (Kox et al. 2007).

In order to facilitate implementation of disease management strategies effectively, early and reliable detection of the target pathogen is important. Indirect ELISA format was employed for the detection of *P. infestans* causing the potato late blight disease even before the first appearance of visible symptoms. *P. infestans* was detected in potato shoots of 5–9 weeks old plants at about 39 days before disease outbreak under field conditions. However, no correlation between ELISA results and symptom development on single plants in the field could be established, possibly because of infection by zoospores transported in soil water following heavy rainfall (Schlenzig et al. 1999). Using mouse MAbs (phyt/G147P) raised against *P. infestans*, the comparative efficacies of plate-trapped antigen (PTA)-ELISA and subtractive inhibition ELISA were assessed. These two formats were specific to *P. infestans* showing no or only limited cross-reactivity against air-borne spores of fungi belonging to Ascomycetes, Deuteromycetes and Basidiomycetes. The MAbs phyt/G1470 was incorporated in a subtractive inhibition surface palsmon resonance (SPR) immunosensor for detection of sporangia of *P. infestans.* The SPR assay involves the preincubation of MAb and sporangia, centrifugation step to remove sporangia-bound MAb and quantification of remaining MAb by SPR. The assay had a detection limit of 2.2×10^6 sporangia/ml, requiring 75 min for obtaining the results. This procedure appears to be superior to other immunoassays available for detection of *P. infestans* (Skottrup et al. 2007b).

Colletotrichum falcatum, causative agent of sugarcane red rot disease could be detected by employing the antisera raised against the unfractionated fungal protein and also against a 101 kDa polypeptide present in all pathotypes in ELISA tests.

The pathogen was detected in different tissues such as root eyes, buds, leaf scar and pith region of the stalk (Viswanathan et al. 1998). *Ganoderma lucidum* considered to be the causative agent of a coconut disease (differently named as Thanjavur wilt, bole rot, Anabe, Ganoderma disease etc.) was detected in roots of infected trees by employing PAbs raised against the basidiocarp mycelial proteins in indirect ELISA format. The results of the immunoassay were confirmed by PCR technique (Karthikeyan et al. 2006).

Detection of *Pestalotiopsis theae* causing gray blight disease of tea by using PAbs in indirect ELISA was possible as early as 12 h after inoculation. This technique was found to be effective in assessing very low levels of infection enabling early initiation of suitable disease management practices (Chakraborty et al. 1996). *Sclerotinia sclerotiorum* infects a wide range of crop plants and different plant parts of the same plant species are involved in the attack. By using DAS-ELISA technique, infection of young petals of rapeseed was recognized (Jamaux and Spire 1994). In a further study, mycelium and ascospores of *S. sclerotiorum* were employed as immunogens to raise anti-mycelium serum (Smy) and anti-ascospore serum (Ssp). Smy serum was found to be more sensitive than Ssp serum in detecting the pathogen in mycelial extract. However, both antisera exhibited similar sensitivity, when exposed to ascospore antigen. These antisera showed cross-reactivity with *Botrytis cinerea*, indicating lower level of specificity of the antisera (Jamaux and Spire 1999). A commercial kit was used for detection of *S. sclerotiorum* on canola petals as part of a disease prediction model by Bom and Boland (2000).

Winter cereals are seriously affected by the eye spot disease caused by *Pseudo cercosporella herpotrichoides*. Early detection of *P. herpotrichoides* and its differentiation from other stem-base pathogens is essential to initiate effective fungicide application schedule. The DAS-ELISA test was developed using a highly specific MAb (PH-10) as the capture antibody and genus-specific rabbit PAb as the detector antibody. The presence of *P. herpotrichoides* in artificially inoculated and naturally infected plant samples was detected by this assay. The assay tested positively against all isolates of *P. herpotrichoides*, including W-type and R-type isolates. The presymptomatic plants also tested positive to the assay. Removal of high MW proteins and glycoproteins from the mycelioid extract significantly enhanced the specificity and eliminated cross-reaction with other stem-base fungi such as *Rhizoctonia cerealis* and *Microdochium nivale* and *Fusarium* spp. (Priestley and Dewey 1993).

Following interaction with the pathogen metabolites, the host protein profile may show distinct variation compared with healthy plants. The depletion of a specific host protein fraction in response to infection by *Pseudocercosporella herpotrichoides* has been used as an indicator of disease severity. In the stem bases of healthy wheat plants, a specific protein (Pc) is abundantly produced, whereas this protein fraction in the infected plants could not be detected, because of its possible depletion. Coff et al. (1998) developed an ELISA format for quantitative estimation of the Pc protein. The degree of host plant tissue degradation by *P. herpotrichoides* was negatively correlated with Pc protein contents. The ELISA assessments of Pc protein contents could be used as the estimates of disease severity. During the 'Septoria Watch' diagnostic survey to assess the incidence of *Septoria* diseases of

wheat crop caused by *Septoria tritici* and *S. nodorum*, ELISA tests were found to be effective and the results formed the basis for the timing of fungicide application against these diseases (Kendall et al. 1998). The MAbs were generated against the surface eiptopes present on the conidia of *Stagonospora nodorum* inducing leaf and glume blotch disease in cereals. By employing two MAbs in plate-trapped antigen (PTA)-ELISA format, the compositional differences in the stage-specific secretion and development of extracellular matrices (ECMs) secreted by *S. nodorum* could be assessed (Zelinger et al. 2004).

Monoclonal antibodies specific for the ubiquitous pathogen *Botrytis cinerea* were employed to detect the pathogen in strawberries. ELISA technique was effective in recognizing mycelial fragments, saline extracts of mycelia and germinating conidia. The results were corroborated by those of immunofluorescence test (Bossi and Dewey 1992) (Appendix 5). Specific MAbs were employed to detect and distinguish highly virulent and less-virulent strains of *Leptosphaeria maculans*, the causative agent of canola black leg disease (Stace-Smith et al. 1993). The PAbs specific to *Phomopsis longicola* infecting soybeans were produced using the culture filtrate (CF) and mycelial extract (ME) and their specificity was tested in DAS-ELISA and indirect ELISA formats (Dewey et al. 1989a). The DAS-ELISA was more specific and 100-fold more sensitive than indirect ELISA in detecting the pathogen in the *Diaporthe-Phomopsis* complex and the variability in the specificity was less, when DAS-ELISA was applied (Brill et al. 1994).

Different compounds of pathogen/host origin are produced during the interaction between fungal pathogens and their host plants. These compounds may be detected by immunoassays making them useful for the detection and identification of the target fungal pathogen. An MAb (MAb 57D3) could specifically bind with a 16-kDa protein that was produced by rice blast pathogen *Pyricularia oryzae*. This protein was present only in some of the isolates of *P. oryzae*. By employing this MAb, *P. oryzae* isolates representing the predominant races occurring in the United States were detected. MAb 57D3 reacted with intercellular and cell wall antigens of this pathogen as well as with extracts of blast lesions on rice leaves (Nannapaneni et al. 2000). *Botrytis cinerea*, causing the grapevine gray mold disease, induces the activity of the enzyme invertase. In addition, another invertase (BIT) was also produced in infected berries. The anti-BIT, IgY antibodies generated in chicken were found to be very specific to BIT, indicating the possible use for these antibodies to detect BIT in berries, thereby the infection by *B. cinerea* (Ruiz and Ruffner 2002). In the case of fungal pathogens like *Alternaria alternata*, melanins derived from 1, 8-dihydroxy naphthalene (DHN) play an important role in pathogenicity and their survival. Phage-displayed antibody (scFv) was demonstrated to bind specifically to 1,8 DHN located in the septa and outer (primary) walls of wild-type *A. alternata* conidia. Use of M1 antibody to detect melanized fungal pathogens in plant tissues may be a distinct possibility in future (Carzaniga et al. 2002).

A genus-specific MAb OX-CH1 was raised against surface washings of *Cladosporium herbarum.* This MAb recognized an epitope present in the species of *Cladosporium* including *C. fulvum* causing tomato leaf mold disease. The PTA-ELISA test, using this MAb was effective in detecting and quantifying the

biomass in infected tomato leaves. Detection limit of the PTA-ELISA test was about 1 mg fresh weight of *C. fulvum*/g of fresh weight of leaf tissue. The procedure developed was found to be robust and simple to use (Karpovich-Tate et al. 1998). *Colletotrichum acutatum* causing the anthracnose disease is responsible for significant losses in strawberry and hence it is an important quarantine pathogen to be intercepted. Both PAbs and MAbs were raised for detecting the pathogen in different plant parts such as roots, crowns, petioles and fruits. Four techniques PTA-ELISA, dot-blot, immunoprint and immunfluorescence microscopy were applied to test the specificity and sensitivity of antibodies produced. *C. acutatum* was detected by PTA-ELISA format in roots and crowns of all cultivars tested at 7 days after inoculation, when no visible symptom of infection appeared. However, the pathogen could be detected in the petioles of only one cultivar (Elsanta) (Krátká et al. 2002).

Mycosphaerella fijiensis, causative agent of black Sigatoka disease, the most dangerous devastating disease of banana, has to be detected at presymptomatic stage for effective disease management. A specific PAb generated in rabbit against *M. fijiensis* antigen was reactive to the fungal secreted proteins and it was able to discriminate naturally infected tissues from healthy plant tissues, in addition to other concomitant fungi present on banana leaves. An MAb was reactive to a high MW antigen from *M. fijiensis* mycelial single ascospore in vitro culture. This MAb did not react with mycelial antigens from *M. musicola*, *M. musae* and *M. minima*. A triple antibody system (TAS)-ELISA procedure was developed, using the MAb as a capturing reagent and the PAb as a second antibody was developed. TAS-ELISA technique was able to detect and quantify mycelial protein antigens in a range of from 10 to 40 μg/ml. This TAS-ELISA format has the potential for use in epidemiological studies for development of forewarning systems (Otero et al. 2007) (Appendix 6).

Specific detection of the resting spores of *Plasmodiophora brassicae*, causing club root disease of crucifers, present in the plant tissues was achieved by applying ELISA test. The resting spores of *P. brassicae* were separated by homogenization of plant tissues followed by centrifugation and purified by using sucrose density gradient column. The antiserum was prepared by immunizing rabbits with the antigen prepared as mentioned above and its efficacy was evaluated for detecting *P. brassicae* in infected root tissues. ELISA format detected the resting spores up to 1×10^2 to 1×10^3 spores/ml in homogenates of club root-infected roots (Orihara and Yamamoto 1998) (Appendix 7). *Polymyxa graminis*, a soil-borne pathogen infecting roots of monocots and dicots, functions also as the vector of 12 different plant viruses. Direct antigen coating (DAC)-ELISA method was developed for detecting *P. graminis* in spiked root samples at one sporosorus/mg of dried sorghum root tissues. Majority of isolates of *P. graminis* from Europe, North America and India reacted strongly with the PAb raised against the fungal pathogen. DAC-ELISA format was successfully employed for the detection of various stages in the life cycle of *P. graminis* and detection of the pathogen in naturally infected and artificially inoculated plants (Delfosse et al. 2000).

Preparation of antiserum against obligate fungal plant pathogens pose certain special problems, because of their inability to grow in artificial media. They have to be maintained on live hosts in glasshouses or growth chambers under controlled conditions to

obtain specific isolates or strains of pathogens. Polyclonal and monoclonal antisera were produced against the conidia of four genetically distinct isolates of *Uncinula necator*, causative agent of grapevine powdery mildew disease. The MAbs reacted specifically to the antigens present on the conidia, as well as in the pathogen mycelium. Three antigens with MW 21-, 29-, and >250-kDa present on the conidia were recognizable. The PAbs detected homologous *U. necator* conidial antigens in a plate-trapped antigen (PTA)-ELISA format, with a linear range of detection extending from 1,000 to 9,000 conidia/ml at a 1:5,000 dilution of antiserum. MAbs were generated against the 21-kDa conidial antigen to avoid cross-reactivity with other fungal pathogens associated grapevine. In addition to detection and identification of this powdery mildew pathogen, grape cultivars, on the basis of disease intensity, could be grouped using the isolate-specific MAbs (Markovic et al. 2002). MAbs raised using partially purified extracts of the sunflower downy mildew pathogen *Plasmopara halstedii* recognized three fungal antigens (68-, 140- and 192-kDa). The presence of *P. halstedii* in sunflower seeds was detected by the specific MAbs in ELISA tests (Bouterige et al. 2000).

Puccinia striiformis, causing yellow rust disease accounts for significant losses in wheat crops. A library of novel MAbs was developed against urediniospores of this obligate pathogen. Two specific MAbs were employed in a competitive ELISA (Pst MAb 4) and a subtractive inhibition ELISA (Pst MAb 8). The subtractive inhibition ELISA exhibited higher level of sensitivity, with a detection limit of 1.5×10^5 spores/ml. However, subtractive inhibition ELISA format showed positive cross-reaction with spores of other *Puccinia* spp., suggesting the presence of common epitopes among different *Puccinia* spp. (Skottrup et al. 2007a).

Dipstick Immunoassay

In order to make the application of immunodiagnostic methods under field conditions possible, simple procedures that produce visible reactions were developed. The 96-well microtiterplates used in ELISA formats are replaced by dipstick formats. This dipstick immunoassay is based on the phenomena of chemotaxis and electrotaxis to attract the zoospores of fungus-like pathogens (oomycetes) to a membrane on which they encyst and they are subsequently detected by immunoassay. Chemicals such as amino acids, alcohols, phenols and isovaleraldehyde, pectin and phytohormone abscissic acid influence the chemotactic properties of the zoospores. Positively charged nylon membranes strongly attract the zoospores. Detection limit of the dipstick assay may be as few as 40 zoospores/ml and the results may be available within 45 min. By viewing under the low power lens of the light microscope, immunolabeled cysts attached to the membrane can be recognized, after silver enhancement of the gold-labeled secondary probe. *Phytophthora cinnamomi* was rapidly detected by employing the MAbs (Cahill and Hardham 1994). Dipsticks prepared from cellulose nitrate membrane were used for the detection of *Pythium ultimum* var. *sporangiferum* and *Phytophthora cactorum*. The antibody-based dipstick assay provided results comparable to the conventional plant tissue baits in

sensitivity for detection of both *Pythium* spp. and *Phytophthora* spp. and shows potential for in situ testing in restricted sampling sites. Dipstick immunoassays can provide epidemiologically valuable, quantitative data on pathogen propagule numbers (Pettitt et al. 2002).

Dot Immunobinding Assay

Dot immunobinding assay (DIBA) is quite similar to ELISA in principle. Nitrocellulose or nylon membrane that is used for immobilizing the antigen (pathogen protein), replaces the polystyrene plates used in ELISA tests. But the free protein-binding sites present in the membranes have to be blocked by employing bovine serum albumin (BSA) or nonfat dry milk powder or gelatin. The unconjugated pathogen-specific antibody is allowed to react with the immobilized antigen in the membrane. The trapped antibody is then probed with appropriate enzyme and in suitable substrate which allows visual detection of a colored product. DIBA method has been widely applied for detection of plant viruses because of several advantages over ELISA. DIBA procedure was applied for the detection of the resting spores of *Plamodiophora brassicae*, causing club root disease of crucifers. Samples (2 μl) were spotted onto a nitrocellulose membrane sheet (Trans-Blot, BIO-RAD, USA) and air-dried. The non-binding sites were blocked overnight at 4°C in a buffer solution containing 2% polyvinyl pyrrolidone (PVP) and 2% BSA followed by treatment with anti-resting spore IgG. The alkaline phosphatase-conjugated goat anti-rabbit IgG and the substrate nitro-blue tetrazolium and 5-bromo-1-chloro-3-indolyl phosphate-*p*-toluidine prediluted with *N*,*N*-dimethylformamide were applied to detect the positive reaction. The detection efficiency of DIBA was similar to that of ELISA. Dilution end point of detection of spores in root homogenates was 1×10^2 to 1×10^3 spores/ml (Orihara and Yamamoto 1998).

Tissue Blot Immunoassay

Tissue blot immunoassay (TBIA) is useful to detect the antigen of pathogen origin, transferred from freshly cut plant tissue surface to nitrocellulose membranes. A tissue imprint of the cut tissue surface is made on the membrane by gently pressing the plant tissue. The unoccupied protein binding sites present on the membrane are blocked as in DIBA test. The blots are then probed with specific antibodies raised against the pathogen (antigen). A direct tissue blot immunoassay (DTBIA) procedure was developed to detect *Fusarium* spp. in the transverse sections from stems or crown of tomato and cucumber plants by employing a combination of the MAb (AP19-2) and FITC-conjugated antimouse IgM-sheep IgG. Positive reactions were discernible within 4 h indicating the usefulness of DTBIA for rapid diagnosis of *Fusarium* spp. (Arie et al. 1995). Fescue toxicosis in grazing animals was found to be highly correlated with the presence of an endophytic fungus *Acremonium coenophialum* in tall fescue (*Festuca arundiancea*). In order to assess the infestation of

Table 2.5 Comparative efficacy of tissue-print immunoblot (TPIB) and protein A-sandwich (PAS)-ELISA tests in detecting *Acremonium coenophialum* in tall fescue plant tissues (Gwinn et al. 1991)

	Percentage of endophyte infestation	
Pasture No.	TPIB	PAS-ELISA
1	84[a]	84[a]
2	72	90
3	28	32
4	88	92
5	72	72

[a]Differences between methods were not significant (P = 0.05)

tall fescue by *A. coenophialum*, a tissue print-immunoblot (TPIB) technique was developed. The results of TPIB and protein A-sandwich ELISA tests were similar, indicating the level of accuracy of TPIB method. TPIB was comparable to other detection techniques (Table 2.5). TPIB assay has the potential for the routine detection of the endophyte in tall fescue tissues (Gwinn et al. 1991).

The role of endopolygalacturonase (endoPG) in the pathogenicity of *Fusarium oxysporum* has been demonstrated. A PAb APG1 was produced using the purified preparation of the enzyme elaborated by *F. oxysporum* f.sp. *lycopersici* race 2. The presence of the endoPG in the stem tissue of inoculated tomato plants was detected by applying the TBIA procedure (Arie et al. 1995) (Appendix 8). Roots including hypocotyls from healthy and club root-infected turnip plants were cut transversely starting 1 cm downward from the basal part of the stems. The cut surfaces of root discs from healthy and infected plants were stamped onto nitrocellulose membrane sheets for processing as in the case of DIBA technique described above. The membranes blotted with infected tissue disks produced strong positive reactions throughout the tissue blotting marks, indicating the presence of resting spores of *Plasmodiophora brassicae* in all tissues (Orihara and Yamamoto 1998). The possibility of distinguishing the live from the dead fungal spores using DIBA test is a distinct advantage over ELISA technique.

Western Blot Analysis

Proteins of pathogen origin, after electrophoresis, are transferred from the gel matrix onto nitrocellulose membranes and subsequently probed by specific antibodies raised against the pathogen (antigen). Water soluble proteins from *Phytophthora cinnamomi* mycelium were used as antigens to prepare a polyclonal antiserum (A 379). This antiserum reacted positively with mycelial proteins from *Phytophthora* spp. and *Pythium* spp. But a species-specific protein 55-kDa was immunodecorated only in *P. cinnamomi* samples providing an unambiguous identification of *P. cinnamom*i. Two diagnostic bands of 55- and 51-kDa were formed only in *P. cinnamomi.* This antiserum was found to be effective for specific detection and identification of *P. cinnamomi* emerging in distilled water from infected tissues of chestnut, blueberry and azalea (Ferraris et al. 2004).

Immunofluorescence Assay

Immunofluorescence (IF) assays have been performed using PAbs and MAbs. Specific MAbs raised against *P. cinnamomi* was used successfully for the detection of this pathogen (Gabor et al. 1993). Likewise, IF assay was applied for the detection of *Botrytis cinerea* in infected plant tissues. The MAbs were generated against whole conidia, their extracellular matrix (ECM) and a putative cutin esterase isolated from the conidia. Three selected MAbs recognized the conidia of 43 isolates of *B. cinerea* from different hosts brought from 6 countries. The percentage of conidia emitting fluorescence ranged from 55% to 100%. There was no fluorescence in reactions with healthy gerbera flowers as well as with spores of other common air borne fungi and bacteria (Salinas and Schots 1994). It was possible to generate MAbs against compounds present on the surface of zoospores. Three MAbs reacted with a polypeptide (>205 kDa) which was distributed on the entire zoospore surface including that of two flagella (Robold and Hardham 1998). In the case of *Mycosphaerella brassicola*, causing ringspot disease in cruciferous vegetables, a PAb specific for ascospores produced by the pathogen was found to be effective in detecting *M. brassicola* under field conditions (Kennedy et al. 1999).

Fusarium oxysporum, causing wilt diseases of several economically important crops, exists in the form of many *formae speciales* that are pathogenic to only one or a few plant species. By employing IF assay, the MAb AP 19–2 and FITC-conjugated antimouse IgM-sheep IgG, the presence of *Fusarium* spp. in the infected stems, crown and roots of tomato and Japanese honewort plants was consistently detected. This assay was found to be an useful tool for the observation of behavior of *Fusarium* spp. in infected host plant tissues (Arie et al. 1995) (Appendix 9).

Immunofluorescence microscopy was used to localize and quantify the internal mycelial colonization of infected leaf tissues of cyclamen (*Cyclamen persicum*) by *Botrytis* spp. The pathogen mycelium was labeled specifically by indirect immunofluorescence using an MAb specific for *Botrytis* spp. and an antimouse fluorescein conjugate. Wheat germ agglutinin conjugated to the fluorochrome TRITC was used to label the fungal mycelium. Image analysis procedure, was applied to measure the relative surface area of the cryostat section covered by the fluorescing hyphae of *Botrytis* spp. A mathematical conversion was derived and used to calculate the relative mycelial volume of the pathogen (Kessel et al. 1999).

Resting spores of *Polymyxa graminis*, an obligate root parasite of sorghum were detected by applying fluorescein 5-isothiocyanate (FITC)-labeled antibodies in fluorescent antibody technique (FAT). The sporosori of *P. graminis* fluoresced with typical apple green color following staining with FITC-labeled specific antibodies. The majority of the specific staining was restricted to the outer layers of the resting spores and the inner part of the spores was orange-brown in color. FAT assay was applied for the detection of different isolates of *Polymyxa*, including those that gave weak reaction in ELISA. A clear distinction between those that reacted strongly and those that reacted weakly was inferred by the results of FAT assay. Isolates that reacted weakly in ELISA, showed specific staining in FAT assay (Delfosse et al. 2000) (Appendix 10).

Phakopsora pachyrhizi causes the rust disease of soybean. Polyclonal antisera were raised in rabbit against intact non-germinated (SBR1A) or germinated (SBR2) urediniospores of *P. pachyrhizi*. Both antisera specifically reacted with *Phakopsora* spp., but not with other common soybean pathogens or healthy soybean leaf tissues in ELISA tests. An indirect immunofluorescence spore assay (IFSA) was developed to detect the rust pathogen. SBR1A and SBR2 bound to *P. pachyrhizi* and *P. meibomiae* were detected with goat anti-rabbit Alex Flour 488-tagged antiserum, using a Leica DM IRB epifluorescent microscope. The presence of *P. pachyrhizi* urediniospores in the passive air was also detected by capturing them on standard glass slides fixed with double-sided tape or thin coating of petroleum jelly. Double-sided tape was found to be superior in retaining the urediniospores and in immunfluorescence test (Baysal-Gurel et al. 2008).

Immunosorbent Electron Microscopy

Serologically specific electron microscopy (SSEM) was developed by Derrick (1972, 1973) and it was later renamed as immunosorbent electron microscopy by Roberts and Harrison (1979). This technique was first applied for the detection of plant viruses and then modified for the detection of fungal and bacterial pathogens. This method involves the production of antibodies against the fungal pathogen/antigen and linkage to the antibodies to protein A-gold complexes to locate the antigen. The antibodies produced against the extracellular protease of *Nectria galligena* were employed to detect the enzyme produced by this pathogen in infected apple tissues (Rey 1984). Protein A-colloidal gold labeling and an antiserum specific to the surface antigens of *Botrytis cinerea* were effective in detecting this ubiquitous pathogen. Thin sections of leaves of *Vicia faba*, uninfected and infected with *B. cinerea* were treated with the antiserum prepared against the fimbriae of the smut fungus *Ustilago violacea*. Then a suspension containing protein A–gold complexes were applied to the sections. Further, sections of hyphae of *B. cinerea* were placed on electron microscope grids and treated with either anti-*Botrytis* or fimbrial antiserum and then by a protein A-colloidal suspension. Gold labeling of the fungal surface and cytoplasm was observed, when either antiserum was used. Thin sections of host cells were strongly labeled, at 12 and 16 h after inoculation, whether the fungus was present in the host cell or not. Gold labeling in the sections revealed that chloroplasts and host cytoplasm were strongly labeled, whereas vacuoles, mitochondria and walls had lesser amounts of labels. In addition, antigens of the type found on the surface of *B. cinerea* were present inside host cells some distance away from the nearest fungal hypha at 8 and 12 h after inoculation or 1 and 5 h after fungal penetration of the epidermal cells. It was suggested that infection of *B. cinerea* may stimulate a response by the host that includes synthesis of host proteins with antigenic regions similar to those of surface proteins (Svircev et al. 1986) (Appendix 11).

The MAbs were generated against the species-specific eipitopes on the surface of zoospores and cysts of *Phytophthora cinnamomi*. The MAbs possessed a valuable

spectrum of taxonomic specificities and they were tested against six isolates of *P. cinnamomi* and six species of *Pythium.* The MAbs indicated the presence of spatially restricted antigens on the surface of the zoospores and they have the potential for application in the investigations on the biology and taxonomy of *P. cinnamomi* (Hardham et al. 1986).

2.1.5 Nucleic Acid-Based Detection Techniques

The presence of nucleic acids (DNA and/RNA) is one of the important characteristics of all living organisms, including the structurally simple viruses and viroids that were earlier placed in the no man's land between living and nonliving entities. The characteristics of nucleic acids and other organelles of organisms have been studied for detection, identification and differentiation of the microbial plant pathogens.

2.1.5.1 Fluorescence Microscopy

Fluorescence microscopy has been useful in studying the presence of microbial pathogens in different tissues of the infected plants. Four different *Rhizoctonia* spp. infect turf grasses, causing the 'patch' diseases. The number of nuclei present in each cell of the *Rhizoctonia* spp. has been used as one of the primary characteristics for identification and differentiation of closely related species. The fluorescence microscopy, using the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) was used to determine the number of nuclei/hyphal cell. The hyphae were fixed by flooding the hyphae with formaldehyde (3%) for 2 min, then rinsed with sterile distilled water by flooding for 1 min. Hyphae were then flooded with DAPI in distilled water (1 ppm), followed by rinsing in distilled water to remove excess of DAPI. The stained hyphae revealed the nuclei clearly, when viewed under fluorescence microscope (Zeiss Epiillumination System), using ultraviolet excitation. Nuclei within isolates of *R. solani*, *R. cerealis*, *R. zeae* and *R. oryzae* were clearly differentiated. *Rhizoctonia* spp. can be tentatively identified within 24 h of receipt of the specimen. This procedure is simple and rapid and it can be used as a preliminary diagnostic tool (Martin 1987).

2.1.5.2 Hybridization-Based Nucleic Acid Techniques

During evolution, mutations in the nucleic acids might have occurred, resulting in fairly random changes in nucleotide sequences and certain changes accumulate giving rise to genetic variations. The characteristic genetic constitutions of individual organisms are due to many generations of mutations and recombinations.

It is generally accepted that closely related organisms share a greater nucleotide similarity than those that are distantly related. Techniques based on nucleic acid hybridization involve the identification of a highly specific nucleotide common to a given strain or isolate of the microbial plant pathogen species, but absent in other strains or isolates or species and this selected sequence of the organism is used to test for the presence of the target organism. Likewise, a highly conserved sequence present in all strains or species in a genus may be employed to probe for the presence of any member of that genus. The selection of a specific sequence as a probe may be based on one of several methods, but is distinctly derived from the sequential data and screening of related organisms to determine its specificity.

Detection of microbial plant pathogens by nucleic acid hybridization techniques is based on the formation of double-stranded (ds) nucleic acid molecules by specific hybridization between the single-stranded (ss) target nucleic acid sequence (denatured DNA or RNA) and complementary single-stranded nucleic acid probe. Sequences of either RNA or DNA have been used as probes. If the probe strand in the duplex is labeled with a detectable marker like ^{32}P, information of the duplex can be assayed after removal of unhybridized sequences. Hybridization reaction may be performed in solution (solution hybridization), in situ (in situ hybridization) and on solid filter supports (filter hybridization). The filter and in situ hybridization methods have been more frequently employed for detection of microbial pathogens. Probes of different types such as cloned and uncloned nucleic acid molecules, oligonucleotides, in vitro RNA transcripts, radioactive and nonradioactive probes have been employed in various investigations. Probes for plant viruses are mostly cDNA, since the genomes of a majority of plant viruses are RNA. Transcription vectors to produce RNA probes can be developed to yield RNA:RNA or RNA:DNA hybrids which are more stable than DNA:DNA hybrids.

Detection of fungal plant pathogens by employing nucleic acid (NA)-based techniques provides certain distinct advantages over immunodetection methods. The fungus-like and fungal pathogens are complex antigens, the nature of which may vary, depending on the stage in their life cycle. The antisera produced against one type of spores or mycelium formed at a particular stage may not actively react with spores or mycelium produced at all stages in the life cycle of the pathogen. However, the presence or absence of spore-bearing structures or the slow growing nature of some fungal pathogens will not affect their detection by NA-based techniques, since the nature of the genomic elements remains constant, irrespective of the stages of life cycle of the pathogen to be detected. It is possible to detect, identify, differentiate and quantify the fungal pathogens concerned, using appropriate DNA probes, even in the case of pathogens that are not amenable for detection by other methods. For example, fungal pathogens causing nonspecific, generalized rotting and death of plants and obligate fungal pathogens that cannot be cultured may be rapidly detected and differentiated by employing suitable probes. Adoption of PCR-based assays allows enhancement of sensitivity and specificity of detection and quantification of fungal pathogens in plant tissues and assessment of relatedness of pathogens.

Dot Blot Hybridization Assay

The presence of fungal pathogens has to be detected in different substrates, such as plants, soil, water and air. It is also necessary to monitor the buildup of pathogen populations that have a bearing on the incidence and spread of the disease in a geographical location. *Leptosphaeria korrae*, causative agent of spring dead spot disease of bermudagrass (*Cynodon dactylon*) was detected and identified based on the presence of a unique banding pattern obtained by digestion of total RNA with restriction enzyme *Eco*RI. Total DNA from 37 North American isolates and one Australian isolate of *L. korrae* was fractionated after digestion by agarose gel electrophoresis and stained with ethidium bromide. Strongly stained DNA bands corresponding to sizes of 1.1, 1.3 and 2.4 kb were recognized for all 38 isolates tested. The banding pattern of *L. korrae* could be readily differentiated from *Eco*RI digest of total DNA of 26 other fungal species included in the investigation (Tisserat et al. 1991) (Appendix 12). The genomic DNA of *Ophiosphaerella herpotricha* (also involved in spring dead spot disease of bermudagrass) was digested with *Xba* 1 restriction enzyme and the digest was fractionated in 0.7% agarose gels. A 1.5 kb clone (pOH 29) was selected from a genomic library for its specificity and strong hybridization to the total DNA of 29 isolates of *O. herpotricha.* The ability of this multicopy probe pOH29 to detect *O. herpotricha* in diseased plants was tested by probing slot-blots of total DNA from field- and greenhouse-grown bermudagrass roots colonized by the fungus. The probe pOH 29 was specific to *O. herpotricha* and it did not hybridize to other fungi commonly associated with roots and stolons of bermudagrass including *L. korrae* and *Gaeumannomyces graminis* var. *graminis.* The detection limit of the probe was found to be 200 ng (wet weight) of infected bermudagrass roots or 1 µg of lyophilized mycelium. Nonsporulating cultures of *O. herpotricha* were also identified using this probe (Sauer et al. 1993).

Rhizoctonia solani AG-8, causative agent of root rot and damping-off diseases affecting several crops, was detected by using a specific DNA probe pRAG12. The specificity and high copy number of AG-8 probe accounted for the reliable and sensitive detection of this pathogen (Whisson et al. 1995). *R. solani* AG-2-2 IV strain infects Zoysia grass causing the large patch disease. A plasmid DNA fragment named as PE-42 was shown to hybridize to DNA of all isolates of *R. soani* AG-2-2 IV, but not to the DNA of other pathogens infecting Zoysia grass, indicating the specificity of the hybridization assay with PE-42 plasmid DNA. The results of Southern hybridization using PE-42 plasmid DNA fragment as a probe, revealed the feasibility of employing this fragment as a marker to distinguish *R. solani* AG-2-2-IV from other intraspecific groups of *R. solani* and for diagnosis of large patch disease affecting Zoysia grass (Takamatsu et al. 1998).

A plasmid DNA (pG158) probe was designed for use in a slot-blot hybridization technique for the detection of *Gaeumannomyces graminis* var. *tritici* (Ggt) inducing the destructive take-all disease of wheat. This probe pG158 specifically and strongly hybridized to pathogenic isolates of Ggt and moderately to *G. graminis* var. *avenae* (Gga). Nevertheless, there was no hybridization of the probe to non-pathogenic isolates of Ggt and other soil fungi associated with wheat rhizosphere.

Differentiation of pathogenic isolates of Ggt from the morphologically similar nonpathogenic isolates has practical utility, because of the possibility of relating soil population of pathogenic Ggt isolates with incidence of take-all disease. It is possible to employ pG158 for the detection of Ggt, in wheat roots, as well as in the soil and also for intraspecific classification of *G. graminis* isolates (Harvey and Ophel-Keller 1996).

A specific probe was selected from a library of genomic DNA of *Phytophthora cinnamomi*, infecting avocado roots. Detection and quantification of *P. cinnamomi* was achieved by using the specific probe in dot-blot and slot-blot hybridization assays, with detection limit of 5 pg of pathogen DNA. The extent of colonization of avocado roots by *P. cinnamomi* was assessed by determining relative amounts of pathogen and host DNAs over a period of time (Judelson and Messenger-Routh 1996). For the identification of pathogens belonging to Oomycetes, a reverse dot-blot procedure was developed based on oligonucleotides labeled with digoxingenin (DIG) as probes. This procedure exhibited far fewer cross-hybridization than the one based on entire amplified internal transcribed (ITS) fragments. New or unknown oomycetes species may be detected and identified, by observing the positive hybridization reaction between the DNA labeled directly from the sample and the specific oligonucleotides immobilized on nylon membrane. Thus the different species of *Pythium*, *P. aphanidermatum*, *P. ultimum*, *P. acanthum* and *Phytophthora cinnamomi* were identified (Lévesque et al. 1998). DIG-labeled probes were employed in dot-blot hybridization method for the detection of *Sporisorium reiliana*, incitant of head smut disease and *Ustilago maydis*, causative agent of common smut disease of maize (Xu et al. 1999).

2.1.5.3 Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism (RFLP) analysis has been useful in the identification and taxonomy of fungal pathogens. The RFLP technique is based on the natural variations in the genomes of different groups or strains of organisms. Variations (polymorphisms) in fragment sizes may be generated by loss or gain or by other events that influence fragment sizes such as deletions or insertions in the DNA sequences. The DNA of the target organism is digested with restriction enzymes and the fragments are separated by electrophoresis in agarose or polyacrylamide gel to detect the differences in the size of the DNA fragments. The number and size of the fragments formed after digestion are determined by the distribution of restriction sites in the DNA. Hence, depending on the combination of each restriction enzyme and target DNA, a specific set of fragments that can be considered as the ‘fingerprint’ for a given strain is formed. The specific sites of fragments are usually identified by Southern blot analysis (Hamer et al. 1989; Leach and White 1991). They can also be directly observed by staining the gels with ethidium bromide and observing under ultraviolet light (Klich and Mullaney 1987). The DNA fragments are then transferred to nitrocellulose or nylon membrane and hybridized with an appropriate probe.

It has been demonstrated that multiple-copy DNA probes prepared from the chromosomal DNA have several advantages. The sensitivity of the assay can be enhanced by using highly repetitive DNA sequences because of the presence of signal in multiple copies and the reliability of the assay also increases due to the lack of influence of variation in one copy in the genome of the total signal available in a hybridization assay. In addition, repetitive DNA has a very high probability of being species-specific. The genomic DNA of *Phytophthora* spp. extracted from frozen mycelium was digested with a restriction enzyme and subjected to electrophoresis in 1% agarose gels. After staining with ethidium bromide, the discrete bands appeared over a faint smear. Thirty nine isolates belonging to 12 species of *Phytophthora* were examined. Isolates belonging to a single species exhibited the same digestion pattern, whereas different species had distinctively different patterns. Repetitive DNA profile analyses discriminated between morphologically similar *P. cryptogea* and *P. drechsleri*. Complete homogeneity was discernible among profiles of 12 isolates of *P. parasitica*, including 8 isolates from tobacco. As this method is simple, it may be useful for investigations on taxonomy and for rapid identification of fungal pathogens (Panabieres et al. 1989) (Appendix 13).

Cloned DNA probes prepared from chromosomal DNA of *Phytophthora parasitica* hybridized to *P. parasitica* only, but not to DNA of other *Phytophthora* spp. and *Pythium* spp. DNA from all isolates of *P. parasitica*, including *P. paraistica* var. *nicotianae*, hybridized strongly with probes, indicating their species-specific nature (Goodwin et al. 1989, 1990). *P. parasitica* var. *nicotianae*, incitant of tobacco black shank disease does not elaborate elicitin which is involved in the development of resistance to the disease. The avirulent isolates and nontobacco isolates of *P. parasitica* produce elicitin (TE^+). The TE^- isolates are generally highly virulent on tobacco. RFLP analysis of both mitochondrial and nuclear DNA could be applied for detection and differentiation of isolates of *P. parasitica*, infecting tobacco (TE^+) from other isolates (TE^-), incapable of infecting tobacco (Colas et al. 1998). Identification of isolates that cannot produce elicitin may be helpful in disease management programs.

Development of similar species-specific DNA probes has been achieved for the detection of *Gaeumannomyces graminis* (Henson et al. 1993) and *Phoma tracheiphila* (Rollo et al. 1987). Cloned mtDNA probes generated from mtDNA of *G. graminis* (Henson et al. 1993) and *Peronosclerospora sorghi* (Yao et al. 1991) were highly specific and did not hybridize with DNA of other fungi. In contrast, some probes prepared from the DNA of *Pythium* sp. hybridized to a subset of isolates sharing the same mitochondrial restriction map, whereas many probes hybridized to DNA of more than one *Pythium* sp. (Martin 1991). When the relationship of two host-adapted pathotypes of *Verticillium dahliae* was examined by RFLP analysis, it was observed that isolates obtained from and adapted to peppermint formed a subgroup (M) distinct from the non-host adapted specific group A of *V. dahliae*. Likewise, isolates of *V. dahliae* from cruciferous hosts constituted another group D. By using two specific probes, the isolates from cruciferous plants could be distinguished on the basis of the various polymorphisms (Okoli et al. 1994).

The probe specific for a dispersed repeated sequence (known as MGR) was employed for constructing genotype-specific *Eco*RI restriction fragment length profiles (MGR-DNA fingerprints) from field isolates of rice blast pathogen, *Magnaporthe griesea* in the United States. The MGR-DNA fingerprints could be used as the basis for distinguishing major pathotypes of *M. grisea*, identifying the pathotypes precisely and defining the organization of clonal lineages within and among pathotype groups (Levy et al. 1991). The genetic relationships among isolates of *Pyricularia grisea* from rice and other grass hosts were examined by RFLP analysis by using the repetitive probe MGR586. Rice blast isolates representing four distinct races differentiated by inoculation on Korean differential rice varieties showed multiple bands hybridizing to the probe MGR586. This study indicated that *P. grisea* population from nonrice hosts could be sources of inoculum for the rice crops (Han et al. 1995).

Binucleate-like *Rhizoctonia* spp. was isolated from cut-flower roses (*Rosa* spp.) showing root and stem rot disease. The *Rhizoctonia* spp. isolates (670) were grouped based on cultural characteristics and hyphal anastomoses reactions. RFLP analysis of a ribosomal (r) DNA internal transcribed spacer (ITS) region was performed to establish the identity of the isolate groups, anastomosis group G (AG-G) and AG-CUT. Among the eight restriction enzymes used, *Hae*III produced DNA banding patterns for AG-CUT that differed from those of tester strains (used for anastomosis). AG-G isolates from cut-flower roses had the same RFLP pattern as the tester strains. The results of RFLP analyses have been used as a criterion for designation of anastomosis groups of this *Rhizoctonia* spp. (Hyakumachi et al. 2005).

A total of 486 isolates of *Glomerella cingulata*, *Colletotrichum gloeosporioides* and *C. acutatum* were grouped based on morphological characteristics, vegetative compatibility and RFLP analysis. Using mtDNA, seven different mtDNA RFLP haplotypes were identified and differentiated within isolates of *G. cingulata*, two within isolates of *C. gloeosporioides* and two within isolates of *C. acutatum*. Each species was demarcated as distinct groups (Gonzáles et al. 2006). Development of anthracnose-like fruit rots in tomato was attributed to different species of *Colletotrichum*, *Alternaria*, *Fusarium*, *Phomopsis* and *Mucor. Colletotrichum* spp. was the most abundant pathogen (136 of 187 isolates) associated with the disease. The fungal isolates could be identified by using the ITS sequence analysis in combination with RFLP pattern analysis. The fungal isolates (187) were classified into six groups. The isolates with unique banding patterns were sequenced. Sequence analysis of amplified products indicated high levels of sequence identity with five different genera. The fungal isolates could be rapidly classified up to genus level by concomitant application of RFLP and sequence analyses (Gutierrez et al. 2006).

Restriction landmark genomic scanning (RLGS) technique was developed to detect DNA polymorphism, using restriction sites as landmarks. RLGS method identifies the landmark through direct end-labeling two-dimensional electrophoresis and autoradiography, giving a profile with many spots to allow the scanning of numerous DNA loci. Isolates of *Colletotrichum acutatum* and *C. gloeosporioides*

were subjected to RLGS by digesting total genomic DNA with three restriction enzymes *Not* 1, *EcoR* V and *Mbo*1. Specific profiles of about 400–1,600 spots for each isolate were obtained. A polymorphic spot appearing to reflect a genetic difference between the two species of *Colletotrichum* was recognized in the isolate profiles. No other common spots could be seen in any combination of isolates, indicating that other spots on the profiles might be considered as unique to each isolate. The results suggested that RLGS technique may be useful for identifying DNA markers required for taxonomic and genomic investigations (Tomioka and Sato 2001).

2.1.5.4 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a simple, ingenious technique capable of amplifying specific DNA sequences exponentially by in vitro DNA synthesis. Amplification is carried out rapidly with very high specificity and fidelity using oligonucleotide primers and a DNA polymerase in an automated reaction. The discovery by Nobel Laureate Arthur Kornberg, of a cellular enzyme designated DNA polymerase triggered the use of this enzyme in many investigations, resulting in the development of PCR assay (Mullis and Faloona 1987; Mullis 1990) which is being applied widely in all biological sciences, including plant pathology. The usefulness of PCR rests in its ability to amplify a specific DNA or cDNA transcript in vitro from trace amounts of complex templates. Specific DNA or cDNA sequences from as short as 50-bp to over 1,000-bp in length can be amplified to more than a million folds in a few hours, in an automated DNA thermocycler. There are three essential steps in PCR amplification: (i) melting of target DNA or cDNA, (ii) annealing of two oligonucleotide primers to the denatured DNA strands and (iii) primer extension by a thermostable DNA polymerase. Heat enaturation of the target ds-DNA is followed by hybridization of a pair of synthetic oligonucleotide primers to both strands of the target DNA, one to the 5′ end of sense strand and one to the 5′ end of the antisense strand by an annealing step. A thermostable *Taq*DNA polymerase from *Thermus aquaticus* (*Taq*) is used for the synthesis of new DNA strands. Newly synthesized DNA strands serve as targets for subsequent DNA synthesis, as the 3 steps are repeated up to 50 times. The DNA sequences between the primers are produced with high fidelity and efficiency of up to 85% per cycle (Weier and Gray 1988). This procedure is amenable for automation, if required, using heat-stable *Taq* DNA polymerase at temperatures between 60°C and 85°C.

The products of PCR can be used for three different purposes: (i) as a target for hybridization, (ii) for direct sequencing of the DNA to determine strain variations and (iii) as a specific probe. Rasmussen and Wulff (1991) appear to be the first to apply PCR approach for the detection of a bacterial pathogen in diseased plants. PCR assay may be preferred by researchers because of several advantages over conventional methods involving isolation and examination of cultural characteristics. The pathogen(s) need not be isolated in pure culture before detection in infected plant materials. It is enough, if the pathogen DNA is extracted. Using PCR

assay, it is possible to detect a single pathogen or many related pathogens, as in the case of immunoassays. However, serological methods comparatively are more expensive and time-consuming. Large number of different PCR primers can be artificially synthesized at costs comparable to the methods for preparing only a few monoclonal antibodies (Henson and French 1993). Gene Releaser, a commercial product has been found to be preferable for preparing the extracts suitable for PCR amplification without the use of organic solvents, alcohol precipitation or additional nucleic acid purification techniques. This protocol appears to be effective especially for the detection of viruses, viroids, and phytoplasmas infecting woody hosts. In addition, the samples can be prepared within 1–2 h, as against 1–3 days required for other extraction methods (Levy et al. 1994). High levels of sensitivity and specificity, in addition to simplicity, have made the PCR-based assays as the technique of choice for routine and large scale application in quarantine and certification programs.

Although PCR is a highly sensitive technology, the presence of inhibitors in the plant tissues and soil, greatly reduces its sensitivity. The inhibitors are believed to interfere with the polymerase activity for amplification of the target DNA. Another problem with the conduct of PCR arises from the possible DNA contamination leading to false negative results. Hence, it is essential to adopt stringent conditions during all operations and to have proper negative controls. Further, it would be desirable to allot separate dedicated areas for pre- and post-PCR handling. The DNA-based detection methods have yet another limitation. In addition to determining the presence or absence of the pathogen in the plants or in the environment, the pathogen detection system has the principal goal of ascertaining the viability of pathogen propagules. In the event of positive result, it is necessary to know whether the pathogen detected poses a threat to crop production, public health or food safety. The lack of discriminating viable from dead cells is a pitfall commonly recognized, while applying nucleic acid-based systems, including PCR and microarrays (Keer and Birceh 2003; Call 2005). Development of the method involving enrichment culturing (BIO-PCR) prior to PCR, addresses this problem to some extent (Schaad et al. 2003).

Designing suitable primers is a critical step in PCR assay. Generally, short sequences (100–1,000 bp) are more efficiently amplified and resolved by agarose electrophoresis. Specific primers are derived from sequences of either amplified or cloned DNA (cDNA) or RNA from target pathogen species or strains or isolates. Primer specificity for target sequences is affected by many factors which include primer length, annealing temperature, secondary structure of target and primer sequences. Ribosomal genes and the spacers between them provide targets of choice for molecular detection and phylogenetic investigations, since they are present in high copy numbers, contain conserved as well as variable sequences and can be amplified and sequenced with universal primers based on their conserved sequences (Bary et al. 1991; Bruns et al. 1991; Stackebrandt et al. 1992; Ward and Gray 1992). Sequence variation in the internal transcribed spacer (ITS) regions between the rRNA repeat unit is significant. Similarly, greater sequence differences have been noted in the nontranscribed spacer (NTS) regions between the rDNA repeat units

and also in the intergenic spacer (IGS) region. Sequence differences in the ITS region of *Verticillium dahliae* and *V. albo-atrum*, incitants of wilt diseases were used for designing primers that could amplify the DNA of each species (Nazar et al. 1991). Specific amplification of the DNA of either weakly or highly virulent isolates of *Leptosphaeria maculans* was achieved by employing primers designed based on the differences in ITS1 sequences of the pathogen DNA (Xue et al. 1992).

The sensitivity of the detection method has to be at high levels in order to detect fungal pathogens, especially those causing vascular wilts, as they have to be detected rapidly, when their populations in infected tissues or soil are very low. PCR-based assays have been demonstrated to be well suited in the case of several diseases incited by fungal pathogens (Narayanasamy 2001). Some of them detected by PCR, are *Phytophthora parasitica* in infected tomato roots and soil (Goodwin et al. 1990), *Gaeumannomyces graminis* var. *tritici* in wheat (Henson et al. 1993), *Verticillium* spp. in potato (Moukhamedov et al. 1994), *Phytophthora fragariae* var. *fragariae* in roots of strawberry (Hughes et al. 1998), and *Phytophthora ramorum* in oak (Martin et al. 2004) and quercus (Schena et al. 2006).

Rapid, small-scale methods that have sensitivity, speed and automation potential of PCR assay are required for timely analysis of economically important plant pathogens. The DNA from healthy and infected plant tissues and microbial pathogens has to be extracted and purified to remove all DNA polymerase inhibitors, such as polysaccharides, phenolic compounds or humic substances. If the target sequences are available as many copies, simple boiling of the sample for a few minutes may suffice for qualitative detection. Boiling the mycelium of *Phoma tracheiphila* obtained from lemon trees (Rollo et al. 1990) and boiling barley leaves infected by *Pyrenophora teres* (Henson and French 1993), yielded sufficient DNA for amplification in a PCR test. Many DNA extraction and purification procedures have been developed to suit the requirements of various experiments.

The genomic DNAs of *Verticillium albo-atrum* from alfalfa and *V. dahliae* from sunflower were extracted either by hexadecyltrimethylammonium bromide (CTAB) or SDS-phenol method. The rRNA genes are highly repetitive, making attractive targets for PCR-based assays (Hu et al. 1993). An efficient method of extracting DNA of *Fusarium oxysporum* f.sp. *ciceris* involving disruption of fungal tissues by grinding it with dry soil using its abrasive properties in the presence of skimmed milk powder, which prevented the loss of DNA by absorption to soil particles was developed. Further, the skimmed milk powder reduced the coextraction of PCR inhibitors along with pathogen DNA. Specific detection of *F. oxysporum* f.sp. *ciceris* was possible in spiked and natural soils (Garcia Pedrajas et al. 1999). A rapid extraction protocol for extraction of DNA from *F. oxysporum* f.sp. *canariensis* was applied. This procedure involves grinding the mycelium in liquid nitrogen followed by freezing and thawing to 22°C and precipitation with chloroform/octanol. Additional steps were followed in the case of some isolates for further purification of fungal DNA using isopropanol/ethanol (Plyler et al. 1999) (Appendix 14). Freezing of fungal cells in liquid nitrogen followed by grinding in a mortar with a pestle has been shown to be a reliable method of DNA extraction from fungal cells. The limitation of this method, however, is the inability to process

multiple isolates simultaneously and to prevent sample cross-contamination, if separate sets of pestle and mortar are not used for different samples.

A simple and effective method for extraction of fungal genomic DNA involving freeze-drying of mycelium was developed by Al-Samarrai and Schmid (2000). The initial steps involved suspension of freeze-dried mycelium in a buffer containing SDS, detachment of DNA from polysaccharides by mild shearing, NaCl precipitation of polysaccharides and protein, chloroform extraction and ethanol precipitation. This procedure was repeated again and it could be completed in an hour, yielding 8–32 μg of high MW DNA/30 mg of freeze-dried mycelium of six fungal species, such as *Fusarium graminearum* and *Aspergillus flavus*. The DNA was digestible with *Eco* RI, *Hind* III, *Sal* I and *Bam* HI. As the methods already available did not produce amplifiable fungal DNA from *Claviceps* spp. a novel method was developed for extraction of DNA from *C. africana*, causal agent of ergot disease of sorghum, involving the use of magnetic beads and high salt extraction buffer. Reliable PCR amplification of the ITS regions of rDNA of *C. africana* was successfully obtained. Magnetic microparticles specific for DNA molecules were used apparently to separate inhibitors and the DNA. Biomagnetic separation has the potential for high throughput and automation for handling large number of samples (Scott Jr et al. 2002) (Appendix 15).

A method for rapid extraction of fungal DNA from small quantities of tissues in batch-processing format was developed. The fungal mycelium was suspended in the buffer (AP1) and subjected to several rounds of freeze/thaw using crushed ice/ethanol bath and a boiling water bath. After boiling for 30 min, the fungal tissue was rapidly ground against the wall of the microfuge tube using a sterile pipette tip. The commercially available Qiagen DNeasy Plant Tissue Kit was used as per the manufacturer's instructions to purify the DNA for PCR/sequencing studies. This method is simple and rapid without the need for specialized equipments that are not conducive for batch-processing (Griffin et al. 2002) (Appendix 16).

Depending on the host plant species and the target fungal pathogen(s), different methods have been applied to obtain high quality DNA for use in PCR-based assays. Different species of *Phytophthora* have been detected using primers designed based on the repetitive sequences. *P. infestans*, causing the potato late blight disease of historical importance, was detected in potato leaves at 1 day after inoculation (Niepold and Schöber-Butin 1995). A region in the ITS specific to *P. infestans* was used to construct a PCR primer (PINF) which could detect the pathogen in the infected tomato and potato field samples (Trout et al. 1997). Later, based on sequences of ITS2 region of DNA of *P. infestans* and *P. erythroseptica* (causative agent of pink rot disease of potato tubers), primers were designed for use in PCR assay. The presence of both pathogens in potato tubers could be detected as early as 72 h after inoculation, well in advance of expression of any visible symptoms of infection (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 of pure DNA of *P. infestans* was detectable in leaves, stem and also in tubers of potato before visible symptoms could be seen (Hussain et al. 2005).

By employing the primers developed based on sequences of ITS region of ribosomal gene repeat (rDNA), *Phytophthora fragariae* was detected more efficiently when comparerd to ELISA test (Bonants et al. 2000). The primers P-FRAGINT and the universal primer ITS 4 were used for the detection of *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* in the roots of strawberry and raspberry respectively. The detection efficiency was maximum between 1 and 5 days after inoculation (Hughes et al. 1998). In a later investigation, primers designed on DNA sequences of various parts of the rDNA were employed for the detection of *P. fragariae* var. *rubi* in infected roots of raspberry and the detection efficiency was compared with that of bait test. PCR assay is a rapid, specific and very sensitive method and it does not require special knowledge of the pathogen. PCR results correlated very well with those of the bait test. However, PCR detected 10 positive samples from four different stocks, whereas the bait test gave positive result in two additional samples coming from the same four stocks. PCR provided the results within 3 days, while bait test needed 6 weeks. Comparison of the sensitivity tests suggested that bait test was more sensitive, needing only a third of the amount of infected roots required by PCR to provide positive results (Schlenzig et al. 2005).

A *Phytophthora* sp. associated with root rot of strawberries from greenhouse grown plants (USA), root rot of roses (Netherland) and root rot of raspberry (Australia) was identified base on the morphological characteristics and sequence analysis of ITS regions of rDNA. Two oligonucleotide universal primers ITS5 and ITS4 were used to amplify the ITS rDNA region (ITS1, 5.8S and ITS2). PCR amplification was performed and amplicons were purified with the Qiagen Kit as per the instructions of the manufacturer. Sequences of the amplified DNA fragments were compared. Morphological comparison with descriptions of *Phytophthora* spp. reported earlier and alignment of ITS sequences of *Phytophthora* sp. isolated in this investigation showed that this one was different from all other species of *Phytophthora* already reported. Hence, this isolate was proposed as *P. bisheria* sp. nov. (Abad et al. 2008).

A PCR assay, based on the amplification of 5.8S rDNA gene and ITS4 and ITS5 primers, was employed for the rapid detection and identification of economically important *Phytophthora* spp. belonging to six taxonomic groups. The pathogens detected include *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. erythroseptica*, *P. fragariae*, *P. infestans*, *P. megasperma*, *P. mirabilis* and *P. palmivora*. For the detection of *P. medicaginis*, a pair of oligonucleotide primers (PPED 04 and PPED 05) that amplified a specific fragment within the intergenic spacer (IGS) 2 region was highly effective in detecting this pathogen in stems and roots of lucerne, even at a dilution of 1: 1,000,000 of pathogen DNA (Liew et al. 1998). *P. nicotianae*, causing tobacco black shank disease was detected by employing two specific primers designed from internal transcribed spacer (ITS) regions ITS1 and ITS2. A PCR fragment of 737-bp was amplified by PCR from the DNA of all isolates of *P. nicotianae*, but not from other *Phytophthora* spp. tested, revealing the specificity of the PCR assay (Grote et al. 2000).

The sequences of mitochondrially encoded genes *cox I* and *II* provided for designing primers FM75 and FM77/83 that were able to amplify the target DNA

from all 142 isolates of 31 species of *Phytophthora*. The amplicons were digested with restriction enzymes for generating species-specific RFLP banding profiles. Digestion with *Alu* I alone resulted in specific detection and differentiation of most species of *Phytophthora* tested. However, single digests using four restriction enzymes were used to increase the accuracy level of identification of isolates of *P. capsici*, *P. infestans*, *P. megasperma* and *P. palmivora* causing important diseases. They could be identified by obtaining RFLP banding profiles from PCR amplicons (Martin and Tooley 2004). In another study, PCR primers capable of amplifying the DNA from 27 different *Phytophthora* spp. were employed. The amplicons, following digestion with restriction enzymes provided specific restriction patterns or fingerprints that were characteristic of each species. Thus 27 different species of *Phytophthora* could be detected and identified precisely (Drenth et al. 2006).

Phytophthora capsici infecting pepper and several weed plant species was detected by using the primer PCAP in combination with the universal primer ITS1. A 172-bp product was amplified from all infected plants revealing the presence of *P. capsici* (French-Monar et al. 2006). Primers based on nuclear rDNA sequences have been employed to detect the infection by *Phytophthora* in vegetable and fruit crops. *P. capsici* in pepper and zucchini plants, *P. infestans* in tomato (late blight) and *P. nicotianae* in tomatoes (buckeye rot) and *P. cactorum* in strawberry (buckeye rot) were efficiently detected by PCR assay. The frequency of infection in Clementine trees by *P. citrophthora* and *P. nicotianae* was found to be similar forming a major factor in the incidence of decline disease in this citrus type (Camele et al. 2005).

Phytophthora ramorum causes a devastating disease, sudden oak death in oak trees in many countries in Europe and also in the United States. Almost all European isolates are mating type A1, while those existing in California and Oregon belong to type A2. Presence of these two mating types in one location may result in sexual recombination paving way for generation of new source of diversity. Hence, a rapid, reliable and discriminating diagnostic technique became necessary to detect and distinguish the two populations. Based on the DNA nucleotide sequence difference in the mitochondrial cytochrome c oxidase subunit 1 (*cox 1*) gene, a single nucleotide polymorphism (SNP) method was developed to detect and differentiate isolates of *P. ramorum* from Europe and those originating in the United States. By employing the SNP protocol, 137 isolates of *P. ramorum* were accurately identified as European (83 isolates) and as US populations (54 isolates), demonstrating the practical utility of the technique in restricting the spread of *P. ramorum* populations from one geographical location to another (Kroon et al. 2004). In another study, a molecular system was developed based on the mitochondrial sequences of *cox I* and *cox II* genes for the detection of *P. ramorum* and also *P. nemorosa* and *P. pseudosyringae* associated less frequently with sudden oak death disease. Two primer pairs, one for amplification of host plant DNA and another pair for the amplification of *Phytophthora* spp. DNA sequences were employed. The presence of plant primers did not interfere with the amplification of pathogen DNA (Martin et al. 2004).

Phytophthora cinnamomi infects a wide range of plant species including eucalypt, causing root rot or die-back diseases. Primers LPC2 and RPC3 were

synthesized as per the manufacturer's (Pacific Oligos) recommendations. The primers were found to be species-specific in detecting *P. cinnamomi*. An internal standard DNA fragment amplified by the same PCR primers, but giving an amplicon of a different size, was added to reaction mixture to detect false negative reactions caused by inhibition of amplification. The use of PCR in the detection of *P. cinnamomi* effectively eliminates the limitations of the traditional baiting method. In addition, large numbers of samples can be processed in a short time, resulting in the possibility of efficient management of eucalypt die-back disease (O'Brien 2008).

Performance of PCR-based assays, AFLP analysis and **t**argeting **i**nduced **l**ocal **l**esions **i**n **g**enomes (TILLING) requires high quality DNA. TILLING is applied for large scale survey of fungal pathogens like *Phytophthora* spp. from natural environments. A strategy to recover high MW genomic DNA from large number of isolates (5,000–10,000) of *Phytophthora* spp. was developed. The DNA extracted was consistently of high MW with total yields varying based on the amount of starting material used. The DNA isolated was suitable for carrying out standard PCR, fluorescently labeled nested PCR, real-time PCR and reverse genetics labeled AFLP analysis. It is possible to process a large number of samples in relatively short period, using a fraction of space required for traditional methods, in addition to being less expensive (approximaterly less than 85% of the currently available commercial kits). Furthermore, handling hazardous solvents such as chloroform or phenol can be avoided, if TILLING method is adopted. Extraction of DNA from biological materials including plants, fungi and nematodes has been successfully accomplished by employing this protocol (Lamour and Finley 2006) (Appendix 17).

Collaborative studies were taken up for the validation of detection protocols targeting the regulated oomycete pathogens such as *Phytophthora ramorum*, *P. fragariae*, *P. rubi*, *Plasmopara halstedii* and the fungus *Monilia fructicola*. Seven protocols based on species-specific PCR tests were selected from published works and their performance was evaluated by participants (16) recruited by European Mycological Network (EMN) members. Accuracy, qualitative repeatability and reproducibility of the selected protocols were assessed. All selected protocols were found to be accurate and provided sensitive and specific results on DNA extracts containing various concentrations of target DNA/µl of host DNA. The results of the evaluation showed that the selected protocols fulfilled the requirement to be considered fit for regulatory purpose (Ioos and Iancu 2008).

Phytophthora sojae, causing root and stem rot in soybean crops has to be specifically and rapidly detected in plants and soils where it may survive for several years. The ITS regions of eight *P. sojae* isolates were amplified by using the universal primers DC6 and ITS4 in a PCR assay. By aligning sequences of PCR products, a region specific to *P. sojae* was identified and specific primers amplified a product of ~330 bp exclusively from the isolates of *P. sojae* among the 245 isolates representing 25 species of *Phytophthora*, revealing the specificity of the PCR assay. The pathogen could be detected from the diseased soybean tissues and residues. The detection limit of this PCR assay was 1 fg of purified DNA of *P. sojae* (Wang et al. 2006).

The usefulness of PCR-based assays for detection and differentiation of fungal pathogens causing wilt and root rot diseases, has been demonstrated. The primers Fov1 and Fov2 designed based on the difference in the ITS sequences between 18S, 5.8S and 28S rDNAs reliably amplified a 500-bp DNA fragment of all isolates of *Fusarium oxysporum* f.sp. *vasinfectum*, incitant of cotton wilt disease. This assay has the potential for use in disease diagnosis and also in disease monitoring and forecasting programs (Morrica et al. 1998). For the detection of *F. oxysporum* f.sp. *canariensis* (FOC) causing Canary Island date palm wilt disease, a PCR-based assay was developed. A partial genomic library of FOC isolate 95–913 was used to identify a DNA sequence diagnostic for a lineage containing all tested isolates of FOC. Two oligonucleotide primers amplified a 567-bp fragment of FOC in all isolates tested. Further, a rapid DNA extraction procedure, developed in this study, was useful for the correct identification of 98% of the tested FOC isolates (Plyler et al. 1999).

Watermelon Fusarium wilt and gummy stem blight diseases are due to *F. oxysporum* f.sp. *niveum* (FON) and *Mycosphaerella melonis* (MM) respectively. PCR assays for rapid and accurate detection and identification of these two pathogens in infected plant tissues as well as in the infested soils, were formulated. Two pairs of species-specific primers Fn-1/Fn-2 and Mn-1/Mn-2 based on the differences in the ITS sequences of *Fusarium* spp. and *Mycosphaerella* spp. were synthesized. The primers specific to FON amplified only a single PCR band of ~320 bp only from FON isolates (24), whereas MM-specific primers yielded a PCR amplicon of about 420-bp product only from MM isolates (22). No amplicon of DNA from any of 72 isolates from other Ascomycotina, Basidiomycotina, Deuteromycotina and Oomycota occurred, indicating the specificity of the primers, the sensitivity of detection being one fg of genomic DNA of both pathogens. The PCR assay developed in this study can be applied for disease diagnosis and pathogen monitoring (Zhang et al. 2005).

Panama wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (FOC), is one of the most serious diseases affecting banana production all over the world. Early and reliable detection of banana infected by FOC is an important component of integrated disease management system. FOC is known to exist as four races that can differentially infect various banana cultivars. A primer set Foc-1/Foc-2 derived from the sequence of a random primer OP-AO2 amplified-fragment produced by PCR amplification, a 242-bp (Foc_{242}) DNA fragment specifically from FOC race 4 present in tropical countries. The primer set Foc-1/Foc-2 was able to amplify the marker fragment Foc_{242} at a concentration as low as 10 pg of pathogen DNA, indicating the sensitivity of the assay. The primer set specific to FOC race 4 isolates could detect the pathogen in field-collected, naturally infected banana pseudostem tissues. In addition, the presence of marker fragment in symptomless banana leaves was amplified positively, although the bands were very faint in the gel after electrophoresis. In combination with Southern hybridization procedure, the sensitivity of PCR assay was increased by 100-fold. Further, the PCR assay using the race-specific primer set, was useful to differentiate FOC race 4 from other races of FOC (Lin et al. 2009).

Wilt diseases caused by *Verticillium* spp. occur in all countries around the world. The rRNA genes of *Verticillium albo-atrum* infecting alfalfa and *V. dahliae* infecting sunflower have been cloned and the DNA sequences of suitable regions have been determined. Complementary oligonucleotide primers were designed based on the sequence difference in the intervening ITS1 and ITS2 regions. These primers were used for detection and quantification of biomass of these pathogens in plant tissues. The assay was able to assess the extent of colonization of tissues by *V. albo-atrum* and *V. dahliae* comparatively. Substantial differences between two pathogens were revealed more rapidly and accurately by the PCR assay than by conventional cytological or maceration of tissues and plating procedures (Hu et al. 1993). Likewise, based on the sequence difference in the rRNA genes, *V. albo-atrum*, *V. dahliae* and *V. tricorpus*, suitable primers were designed. These primers were employed for reliable detection of these pathogens. A diagnostic set of primers are available for investigation and monitoring of the Verticillium-potato pathosytem (Moukhamedov et al. 1994).

The relative sensitivity and rapidity of detection of *V. albo-atrum* in potato stem tissues and soil by PCR assay employing specific primers and media-plating method was assessed. Whereas the PCR assay was faster and more efficient, requiring only 2 days for detection and identification, media-plating method was slow and required a period of more than 4 weeks (Mahuku et al. 1999). Two arbitrarily primed oligonucleotide primers (15–16-mer) were employed in the arbitrarily primed (AP)-PCR analysis to obtain genome polymorphic patterns from total DNA extracted from *Verticillium dahliae*. A 350-bp fragment (designated MGC), unique for recognition of *Verticillium* spp. proved specific for *V. dahliae* in the Southern blot. This fragment could be used as a diagnostic tool for *V. dahliae* (Cipriani et al. 2000). *V. albo-atrum* hop pathotypes were detected and identified rapidly by the PCR assay, using the primers PG-1 and PG-2 designed from 16 sequences. Polymorphic amplified fragment length polymorphism (AFLP) markers were converted into pathotype-specific sequence characterized amplified region (SCAR) markers (Radiseˇk et al. 2004).

Rhizoctonia solani is known to cause root rot disease in several economically important crops. Root infection by *R. solani* is not usually recognized till symptoms appear on the foliage and at this stage it is practically not possible to save the infected plants. Hence, early detection of the pathogen causing root rot disease is essential to initiate strategies to contain the disease spread. *R. solani* has many anastomosis groups (AGs) within the morphologic species. *R. solani* AG1 IA causing rice sheath rot disease could be detected and identified rapidly by using primers designed from unique sequences within the ITS regions of rDNA. The pathogen was rapidly detected and precisely identified in infected rice tissue as well as in paddy field soils by the PCR assay (Matsumoto and Matsuyama 1998). Similarly, *R. solani* AG2 was detected by the PCR protocol involving amplification of 5.8S rDNA and part of the ITS region. The designed primers in combination with the general fungal primers ITS IF and ITS 4B were used. Six primers specifically amplified the DNA of *R. solani* AG2, the subgroups AG2-1, AG2-2 and AG2-3 and the ecotype AG2-t. The DNAs from *R. solani* AG2 and AG4 in

infected radish plants were amplified as in the case of DNA from axenic cultures (Salazar et al. 2000).

A universally primed (UP)-PCR cross hybridization assay was developed for rapid detection, identification and placement of isolates of *Rhizoctonia solani* into the correct anastomosis group (AG). A single UP primer that generated multiple PCR fragments for each isolate was employed to amplify 21 AG tester isolates belonging to 11 AGs of *R. solani.* The amplified products were spotted onto a filter, immobilized and used for cross-hybridization against amplification products from different isolates. Isolates within AG subgroups cross-hybridized strongly, whereas between different AGs little or no cross-hybridization occurred. Sixteen isolates of *Rhizoctonia* from diseased sugar beets and potatoes were identified using the UP-PCR format. The results were corroborated by RFLP analysis of the ITS1-5.8S-ITS2 region of nuclear encoded ribosomal DNA (Lübeck and Poulsen 2001).

Gaeumannomyces graminis has three speicialized varieties *tritici*, *avenae* and *graminis* within the morphologic species. The rDNA fragment from *G. graminis* was amplified in PCR assay. This fragment, after labeling, was used as a probe which hybridized to *Eco* RI digests of target DNA. Consistent differences in the band pattern among three varieties of *G. graminis* were observed, indicating that such probes have considerable potential for use in the identification of these pathogens (Ward and Gray 1992). A DNA fragment (188-bp) was obtained from boiled mycelium of *G. graminis* for amplification by PCR as a probe for detection and identification of the pathogen from different grass hosts. Amplification of the specific DNA and presence of lobed hyphopodia in culture are the dependable characteristics essential for the identification of *G. graminis* (Elliot et al. 1993).

A single-tube PCR assay was developed to identify *G. graminis* varieties, *avenae* (Gga), *graminis* (Ggg) and *tritici* (Ggt), based on the nucleotide base sequences of avinacinase-like genes from isolates of these three varieties, Gga, Ggg and Ggt. Three 5′ primers specific for these varieties and a single 3′common primer allows amplification of variety-specific fragments of 617-, 870- and 1086-bp respectively. Each 5′ primer was specific in mixed populations of primers and templates. No PCR amplicons were detected from other related fungi. This single-tube PCR format could be used for rapid detection and differentiation of the three varieties of *G. graminis*, causing the take-all diseases in cereals (Rachdawong et al. 2002). In another investigation, the universal primers NS5 and NS6 that amplified the middle region of 18S rDNA of *Gaeumannomyces* spp. and varieties were used in a PCR-based assay. Primer pair GGT-RP/GGA-RP developed by sequence analysis of cloned NS5:GGT-RP amplified a single 410-bp fragment from the isolates of Ggt, a single 300-bp fragment from the isolates of Gga and no amplicon from the isolates of Ggg or other *Gaeumannomyces* spp. The primer pair NS5:GGA-RP amplified a single 400-bp fragment from the isolates of Ggt and Gga. Two sets of primer pairs (NS5:GGT-RP and NS5:GGA-RP) were used in PCR assays to detect and identify Ggt and Gga either colonizing wheat, oats or grass roots or in culture. DNAs from plants colonized by other pathogens were not amplified, revealing the specificity of the PCR format developed in this study (Fouly and Wilkinson 2000).

The clubroot disease of cruciferous plants, caused by *Plasmodiophora brassicae*, has a worldwide distribution. *P. brassicae* is an obligate endoparasite of the roots. Chee et al. (1998) developed a PCR-based technique useful for the detection of isolated resting spores of *P. brassicae*, but its effectiveness for *in planta* detection was not tested. Later, an *in planta* molecular marker for the detection of *P. brassicae* was developed, using an oligonucleotide primer set from the small subunit gene (18S-like) and ITS region of rDNA. This primer set was used specifically to detect *P. brassicae* in plant tissues and a 1,000-bp fragment was amplified by this primer. The high copy number of rDNA gene sequences from which the primer set was designed, provides for the detection of small quantities of the target pathogen DNA in the total DNA extracts of infected Chinese cabbage plants. The DNAs of test plant and other soil-borne fungi and bacteria were not amplified, indicating the high specificity of the PCR assay developed by Kim and Lee (2001).

In order to detect *Plasmodiphora brassicae* infecting canola (*Brassica napus*), a simple, one-step PCR format was developed using the primers TC1F/TC1R based on a *P. brassicae* partial 18S rRNA gene sequence from GenBank. This primer pair generated a 548-bp product in the optimized PCR. A second primer pair TC2F/TC2R amplified a fragment of 18S and ITS1 regions of the rDNA repeat and this primer produced a 519-bp product. Both primer pairs amplified the DNA of *P. brassicae* only, but not the DNA from unifected plants, noninfested soil or common soil fungi and bacteria tested. This assay detected *P. brassicae* in symptomless root tissues of plants at 3 days after inoculation with the pathogen, providing a reliable and early detection results reliably. Hence, this protocol may be useful for routine detection of *P. brassicae* in plant and soil samples (Cao et al. 2007).

The ITS regions have been shown to be useful targets, since they seem to be conserved within species, but may vary sufficiently among several plant pathogenic species to allow the construction of unique primer sequences. Highly specific primer was designed for the detection and identification of *Pythium violae* and *P. myriotylum* based on amplification of a fragment from the ITS region by PCR (Wang and White 1996; Wang et al. 2003). Twenty PCR primers were designed from the sequences of the rDNA ITS1 region from 34 *Pythium* spp. Five species-specific primers were selected, after testing the specificity of these forward primers paired with ITS2 or ITS4 and reverse universal primers. The specific amplifications, using these species-specific primers, allowed the identification and differentiation of nine *Pythium* species. *P. aphanidermatum* in infected carrot tissues and *P. dimorphum* in infested soil were detected rapidly and reliably (Wang et al. 2003a, b).

Universal primers are employed for the amplification of sequences of specific regions like ITS of rDNA by PCR. This approach was adopted for detection and differentiation of *Pythium* spp. that are difficult to identify based on the morphological characteristics. The restrtiction fragment probes from ITS1 exhibited a high degree of species specificity to *P. ultimum*, when tested by dot-blot hybridization against 24 different *Pythium* spp. No distinct difference could be seen among the 13 isolates of *P. ultimum* var. *ultimum* and var. *sporangiferum* from eight countries and two isolates of *Pythium* group G, later classified as *P. ultimum* (Lévesque et al. 1994). The tandem arrays of 5S genes unlinked to the ribosomal DNA repeat unit

are present in some *Pythium* spp. Probes specific for *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiferum* were found to be species- or variety-specific. The results indicate that 5S rRNA gene spacer sequences may be useful in defining species boundaries in the genus *Pythium*, as these sequences diverged rapidly after speciation (Klassen et al. 1996).

Rice leaf sheath is invaded by *Sclerotium hydrophilum*, in addition to *R. solani*, *Ceratobasidium oryzae-sativae* and *Waitea circinata* which induce overlapping symptoms in infected rice plants, making disease diagnosis very difficult. Hence, it is necessary to have a reliable and rapid diagnostic test for identification of the pathogen(s) involved in the disease(s) occurring in different locations. Twenty two representative isolates of the above mentioned pathogen species were used for DNA extraction. Universal primers ITS1 and ITS4 were employed to amplify the ITS regions of the respective pathogens. The primers PS-F/PS-R amplified a 540-bp DNA fragment for all isolates of *S. hydrophilum*, but not for any other fungal species tested. The presence of *S. hydrophilum* could be reliably detected as early as 3 days after inoculation. These primers did not amplify DNA of rice plant or any other fungal pathogens infecting rice leaf sheaths, indicating the specificity of the PCR assay and its ability to clearly differentiate *S. hydrophilum* from other fungal pathogens causing similar symptoms in rice leaf sheath (Hu et al. 2008).

Potato smut disease caused by *Thecaphora soalni* induces galls in the lower stems, stolons and tubers, as the principal symptom. The fungus produces sponge-like mycelial mass in PDA medium after about 40 days. DNA profiles were generated by PCR assay, using sequence-repeated primers CAG5, GTG5, TCC4, GACA4, TGTC4 and GACAC3. The DNA profiles revealed a close relationship between the teliospores and the sponge-like mycelial mat originated from the teliospores, thus indicating both structures are the same. The results of DNA fingerprinting and partial sequencing of the large subunit (LSU) rDNA region lend support to this conclusion (Andrade et al. 2004).

Ophiosphaerella korrae (= *Leptosphaeria korrae*) and *O. herpotricha* are ectotrophic root-rotting fungi, causing the spring dead spot disease of bermudagrass. The universal primers ITS4 and ITS5 amplified the ITS regions of the rDNA of both pathogens. The OHITS1 and OHITS2 primers amplified a 454-bp fragment from DNA of *O. herpotricha*, whereas OKITS1 and OKITS2 primers amplified a 454-bp fragment from the DNA of *O. korrae* isolates only. The primers (OHITS and OKITS) detected *O. herpotricha* or *O. kerrae* respectively in total DNA preparations from greenhouse-inoculated or naturally infected bermudagrass roots. These primers have the potential for detection of these pathogens rapidly in infected bermudagrass without the need for culturing the pathogens (Tisserat et al. 1994). *Leptosphaeria maculans*, causative agent of blackleg disease of oilseed rape is a component of the species complex. *L. maculans* could be detected by amplifying the ITS region directly from the intact conidia as a substrate (Balesedent et al. 1998).

Dead spot disease of creeping bentgrass and hybrid bermudagrass, caused by *Ophiosphaerella agrostis*, is difficult to diagnose, because of unclear symptoms induced by the pathogen in the initial stages. Hence, a PCR assay was developed

using oligonucleotide primers specific for *O. agrostis*, based on the ITS rDNA regions (ITS1 and ITS2) of three previously sequenced isolates of *O. agrostis*. The primers (22-bp) amplified a 445- or 446-bp region of 80 isolates of *O. agrostis* obtained from different states of USA, but not from the DNA of other common turfgrass pathogens, including other species of *Ophiosphaerella*. The primers successfully amplified the pathogen DNA from the field samples of creeping bentgrass and hybrid bermudagrass plants. Positive amplification occurred, if the pathogen DNA content was between 50 ng and 5 pg level. The entire process of detection including DNA isolation, amplification and amplicon visualization can be completed within 4 h, indicating the potential of this PCR format for rapid and accurate detection and identification of *O. agrostis* in plant hosts (Kaminski et al. 2005).

Phaemoniella chlamydospora is the most important endophyte associated with a decline (esca and esca-like syndromes) disease causing serious losses in grapevines in Europe and North American countries. A potential marker in the New Zealand isolate A21 was identified. Sequencing of the region flanking the 1,010 bp marker revealed a single nucleotide polymorphism in the 3′ border of the marker band. Primers were designed to amplify a 487-bp fragment encompassing this polymorphic site. Digestion of this product with the restriction enzyme BsrI produced three bands only in isolate A21 and two bands in all other isolates tested (Ridgway et al. 2005).

Eutypa dieback caused by *Eutypa lata* is an important grapevine disease in several countries. As this pathogen does not have diagnostic features and conidial production is erratic, development of tests for rapid detection and identification was considered essential. PCR-based tests using primers designed with ITS sequences and sequence characterized amplified regions (SCARs) were employed. These primers were used to detect DNA of *E. lata* in supernatant obtained by boiling shavings of diseased grapevine wood in water (Lecomte et al. 2000). Later, a PCR-RFLP based procedure was developed involving amplification of DNA using the universal ITS1 and ITS4 PCR primers and digestion of the PCR product with the restriction enzyme *Alu*I (Rolshausen et al. 2004).

In another investigation to determine the identity of fungi associated with esca, black dead arm or eutypa diseases, contributing to grapevine decline, several fungi were isolated. *Botryosphaeria*-like spp. *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. were found to be predominantly associated with the grapevine decline. DNAs extracted from the fungi were amplified by PCR using ITS4-ITS5. Based on amplification products, the components of the decline problem were resolved. *Botryosphaeria*-like species viz., *Diplodia seriata* (= *Botryosphaeria obtusa*), *Neofusicoccum parvum* (= *B. parva*), *B. dothidea* and *D. mutila* generally infected young vines, whereas matured vines harbored only *D. seriata* and *D. mutila* (Martin and Cobos 2007).

The isolates of *Dothistroma pin*i (*Mycosphaerella pini*) present in New Zealand appeared to be isogenic. The possibility of introduction of genetically diverse isolates of *D. pini* from other countries, necessitated the development of a DNA-based monitoring system. Two informative microsatellite loci were noted in the sequence data available. For each loci identified, specific primers were designed to flank the

repeated sequence subsequently used to generate DNA profiles for the *D. pini* strains. The profiles of five microsatellite loci were used to identify most isolates of *D. pini* tested (Ganley and Bradshaw 2000). Collar rot disease of red and jack pines caused by *Diplodia pinea* and *D. scrobiculata* has to be diagnosed in the early stages of infection for effectively protecting the pines. Primer pairs were designed utilizing the variation among mitochondrial small subunit ribosome gene (mt SSU rDNA) sequences of *Botryosphaeria* species and related anamorphic fungi. Two forward primers DpF and DsF, when used separately with nonspecific reverse primer BotR, amplified DNA of *D. pinea* and *D. scrobiculata* respectively. Absence of amplification of the DNA of *B. obtusa* by these primers revealed the specificity of the PCR assay with a detection limit of 50–100 pg and 1 pg in red and jack pine wood tissues respectively. The pathogens could be detected in seedlings and saplings exhibiting Diplodia collar rot disease symptoms (Smith and Stanosz 2006).

Septoria tritici (*Mycospherella tritici*) and *Stagonospora nodorum*, causative agents of leaf blotch and glume blotch diseases of wheat constitute the Septoria complex. The ITS regions of these pathogens were cloned and sequenced. Conserved sequences of the rDNA were used for designing specific primers that successfully amplified similar sized fragments from wheat leaves infected by both pathogens (Beck and Ligon 1995). In combination with ELISA, the PCR-based assay was adapted to a microplate format for quantification of pathogen population, reflecting disease pressure (Beck et al. 1996). A robust and quantitative PCR-based assay using the ß-tubulin gene as target for the detection and quantification of *S. tritici* infection levels in wheat leaves was developed. Specific primers were designed by aligning and comparing ß-tubulin sequences from other fungi. The final primer set was selected after testing against several fungi and also against *S. tritici*-infected and healthy wheat leaves from different localities. A single DNA fragment (496-bp) was amplified from DNA of *S. tritici*, but not from DNA of wheat plant or other fungi tested, the detection limit being ~2 pg of pathogen DNA. The amounts of PCR-amplified products were directly quantified in a microtiter plate wells with a fluorimeter using the dye PicoGreen which fluoresced specifically upon binding with ds-DNA. The PicoGreen assay required ~10 pg of *S. tritici* DNA/reaction, showing that this assay was less sensitive compared to conventional PCR assay. However, PicoGreen was shown to be quantitative, rapid and easy to perform in a microtiter plate format, allowing high-throughput screening. In addition, the PCR/PicoGreen assay was found to be useful to study the colonization, infection and subsequent disease development of *S. tritici* on wheat under in vitro, glasshouse and field conditions (Fraaije et al. 1999). In order to detect the pathogens *Rynchosporium secalis* and *Pyrenophora teres*, causing leaf blotch disease in barley leaves, two primer sets were designed based on the sequences of ITS regions of rDNA of *R. secalis* and from the amplified fragment length polymorphism (AFLP) marker in *P. teres.* The primer set RS1/RS3 detected *R. secalis* in infected leaves and also in the barley seeds, whereas RS8/RS9 effectively detected *P. teres* in barley leaves. The intensity of the corresponding DNA band after amplification with primer pair RS1/RS3 was higher than that amplified with the primer pair RS8/RS9 (Gubis et al. 2004).

Phomopsis azadirachtae causes the dieback disease of neem trees (*Azadirachta indica*) which provide many products with medicinal and pest-repellant properties. As the conventional methods depending on isolation and studying morphological characteristics of the pathogen are time-consuming and laborious, a PCR-based assay was developed using primers based on the sequences of 5.8S rDNA fragment of *Phomopsis* spp. The expected 141-bp PCR product was detected in all isolates of *P. azadirachtae* obtained from different states of India. As this pathogen is transmitted through neem seeds, early and reliable detection of pathogen can check the spread of the disease to new areas through infected seeds (Prasad et al. 2006).

Probes generated from the ITS region of rDNA of *Colletotrichum gloeosporioides* (*Glomerella cingulata*), incitant of anthracnose diseases of several fruit and vegetable crops, amplified by PCR were employed to study 39 different isolates. These isolates were classified into 12 groups linked to host plant species and geographical origin (Mills et al. 1992). Likewise, by using species-specific PCR primers for ITS regions, *Cylindrocarpon heteronema* (Brown et al. 1993) and *Monosporascus* spp. (Lovic et al. 1995) were detected. *Phialophora gregata* infects many legumes such as soybean, mungbean and adzuki bean in several countries. A unique banding pattern, after digestion of PCR-amplified ITS and the 5′ end of the large subunit rDNA with restriction enzymes, was recognized in all the 79 isolates of *P. gregata* obtained from diverse locations. The isolates from soybean in the Midwestern states of the United States and Brazil possessed identical ITS sequences, whereas the ITS sequence of the isolates from adzuki bean from Japan exhibited 98% homology with soybean isolates (Chen et al. 1996).

Watermelon is affected commonly by two fungal pathogens *Mycosphaerella melonis* (MM), causing gummy stem blight disease and *Fusarium oxysporum* f.sp. *niveum* (FON), inducing wilt disease resulting in heavy losses. Two species-specific PCR assays were developed for the detection of these two pathogens. Two pairs of specific primers Fn-1/Fn-2 and Mn-1/Mn-2 were designed based on the differences in the ITS sequences of FON and MM respectively. The primer pair Fn-1/Fn-2 amplified only single PCR band (~320-bp) from FON, while a product (~420-bp) was obtained for MM DNA, due to the amplification by Mn-1/Mn-2 primer pair. The detection limit of the PCR assays was one fg of genomic DNA for both FON and MM (Zhang et al. 2005).

Winter oilseed rape, when infected by *Pyrenopeziza brassicae* (anamorph – *Cylindrosporium concentricum*), remains symptomless for a long time until the first visible necrotic lesions appear. Only when infected plants are incubated for several days at high humidity, necrotic lesions can be seen. PCR amplification of *P. brassicae* DNA of isolate NH10 using primers Pb1/Pb2 produced a 753-bp amplicon. But ITS primers PbITSF and PbITSR amplified a 461-bp product. Detection limit of the primers Pb1 and Pb2 was 1 ng of *P. brassicae* DNA. On the other hand, ITS primers were more sensitive and could detect as little as 1 pg of pathogen DNA. Furthermore, the new primers PbITSF/PbITSR could detect the pathogen in symptomless plant tissues. This PCR-based test has the potential for large scale application for testing field-infected plants in which symptoms could be observed only after a long time (more than 2 months) (Karolewski et al. 2006).

Botrytis cinerea causes grey mold diseases in several economically important crops including grapes, pineapple, strawberry, vegetables and ornamental plants. Two sibling species named *transposa* and *vacuma* in the *B. cinerea* complex have been recognized, because of the presence of the transposable elements *Boty* and *Flipper* in *transposa*, but absent in *vacuma* (Giraud et al. 1999). Some of the strains of *B. cinerea* were not amplified by primers designed for the DNA marker (Rigotti et al. 2002). They did not amplify the DNA fragments of this group of strains under standard conditions. In a later investigation, new improved specific primers capable of amplifying the DNAs of all strains of *B. cinerea* were developed. Two new internal primers BC_{108}^{+} and BC_{563}^{-} were designed, in addition to $C_{729}^{+/-}$ primer used earlier. The new primers amplified a DNA fragment of 0.48 kb for the main group of 26 *B. cinerea* strains and a shorter fragment of 0.36 kb for the smaller group of 13 strains, due to the detection of 0.12 kb, which was not detected with primers $C_{729}^{+/-}$. All the strains were amplified to detect the presence or absence of *Boty* and *Flipper* transposable elements. Other related *B. allii* and *B. fabae* species were not amplified by these primers, indicating the specificity of the PCR assay for *B. cinerea* (Rigotti et al. 2006). Isolates (363) of different *Botrytis* spp. were examined by PCR, using necrosis and ethylene-inducing protein (NEP2) and C729 primers. The DNAs of *B. cinerea*, *B. fabae* and *B. pelargonii* were amplified using NEP2 producing a 835-bp band, but not in other *Botrytis* spp. tested. The primer C729 amplified a band of 700-bp band from *B. cinerea* and *B. pelargonii* and a 600-bp band in *B. fabae*. The primers were effective in detecting and differentiating the *Botrytis* spp. (Mirzaei et al. 2008).

Magnaporthe oryzae, causes the grey leaf spot disease of perennial ryegrass. A PCR-based detection system was developed, using a commercially available kit for extraction and amplification of plant DNA from leaf tissue. Primers were designed to amplify a 687-bp fragment of the Pot2 transposon that is present in multiple copies in the genome of the pathogen. The isolates of *M. grisea* and *M. oryzae* had Pot2 transposon. The expected 687-bp amplicon was detected for all isolates tested. The primers selected for the assay amplified amounts of purified DNA as low as 5 pg and detected specifically *M. oryzae* in single diseased leaf blades as well as in field samples of infected perennial ryegrass within a period of 4–8 h (Harmon et al. 2003) (Appendix 18). Fusarium crown rot (FCR) disease is caused primarily by *Fusarium culmorum* and *F. pseudograminearum*. A real-time quantitative PCR (QPCR) assay was developed for the detection of these fungal pathogens, using primers and probes specific for a segment of the trichodiene synthase (*tri5*) gene. The utility of the QPCR assay was tested under field conditions by inoculating spring and durum wheat cultivars to produce different levels of disease severity. Plants harvested 2 weeks after harvest were analyzed by QPCR assay. Disease severity scores (DSS) based on visual assessment and *Fusarium* DNA contents determined by QPCR were positively correlated with each other for all three cultivars in 2004, but only for durum cultivar in 2005 ($P < 0.005$). Grain yields for both spring wheat cultivars were negatively correlated with *Fusarium* DNA contents (Hogg et al. 2007).

Three related ascomycetous fungal pathogens *Mycosphaerella fijiensis*, *M. musicola* and *M. eumusae* are involved in the banana Sigatoka disease complex.

Diagnosis based on the symptomatology is unreliable and likely to hamper disease management strategies. Hence, a rapid and robust species-specific molecular diagnostic procedure for detection and quantification of the pathogen species was considered essential. Conventional species-specific PCR primers were developed based on the actin gene that detected DNA as little as 100, 1 and 10 pg/µl from *M. fijiensis*, *M. musicola* and *M. eumusae* respecitively. Further, TaqMan real-time quantitative PCR assays were developed based on the ß-tubulin gene and detected quantities of DNA as low as 1pg/µl for each *Mycosphaerella* spp. from pure cultures and DNA at 1.6 pg/µl/mg of dry leaf tissue for *M. fijiensis* that was validated using naturally infected banana leaves (Arzanlou et al. 2007). The presence of *Mycosphaerella fijiensi*s, causing banan black Sigatoka disease was detected, using PCR assay to amplify, the simple sequence repeat 203 $(GTT)_7$ specific to *M. fijiensis*. The primers Mf-SSR-203 (forward and reverse) were employed for amplification of SSR sequences. A band consistent with the expected 227-bp product was revealed after agar gel elelctrophoresis (Peraza-Echeverría et al. 2008).

Some fungal pathogens elaborate host-specific (selective) toxins that have crucial role in the development of primary symptoms characteristic of the disease induced by the pathogen concerned. *Alternaria alternata*, apple pathotype (= *A. mali*) induces Alternaria blotch symptoms due to the production of the host-specific toxin, AM-toxin (AMT). Considerable difficulty has been experienced in the identification of some *Alternaria* species, because of high level of variability among the isolates of different *Alternaria* spp. A gene with a crucial role in the biosynthesis of AMT was identified, cloned and characterized. The *AMT* gene was detected only in the isolates of *A. alternata* apple pathotype. A PCR-based method using primers designed based on the sequences of AMT gene was developed. This protocol detected specifically AM-toxin producing isolates of *A. alternata* apple pathotype (Johnson et al. 2000).

Smut disease symptoms are expressed generally at the time of heading, although infection may take place much earlier. Smut whips in place of inflorescence are produced in sugarcane infected by *Ustilago scitaminea*. Detection of the presence of the pathogen in infected plant tissues by PCR assay was positively correlated with eventual production of smut whips later (Schenk 1998). Specific primers were employed for the detection of *Sporisorium reiliana*, causing head smut and *Ustilago maydis*, causing common smut disease in maize. Primer pairs SR1 and SR 3 specific for *S. reiliana* and UM11 specific for *U. maydis* (= *U. zeae*) amplified the respective pathogen sequences. *S. reiliana* could be detected in the extracts of pith, node and stalk, but not in leaves of infected plants, the detection limit being 1–6 pg of fungal DNA, irrespective of the presence of maize DNA (Xu et al. 1999). Likewise, *U. hordei* could be detected using the primer pair designed based on the sequences of ITS region of rDNA. The pathogen DNA was amplified from leaf tissues of inoculated susceptible and resistant plants at different stages of plant development. The presence of *U. hordei* in the first three to four leaves was detected, but not in leaves produced later, in the case of resistant barley plants. The possibility of detecting *U. hordei* prior to heading would be very useful in assessing resistance to barley smut disease (Willits and Sherwood 1999).

The direct amplification of DNA from single ungerminated teliospores of *Tilletia indica* and *T. tritici* was effective for the detection and identification of these smut fungi infecting wheat. There is no need for DNA extraction step to perform this PCR assay (McDonald et al. 1999). The primer pair Tcar 2A/Tcar 2B, developed in a later study, detected *T. caries* (= *T. tritici*) in the shoots and also in leaves of infected wheat plants with a detection limit of 16 pg DNA/100 mg of plant fresh weight. The DNA in the extracts of teliospores was positively amplified by the primer pair. However, the spore extract did not test positive by ELISA test (Eibel et al. 2005a). Wheat dwarf bunt caused by *Tilletia controversa* is an important international quarantine disease. As the teliospore morphology and genomic structure of *T. controversa* are very similar to that of *T. caries*, the need for differentiation these two closely related pathgens was realized. A random amplified polymorphic DNA (RAPD) primer-mediated asymmetric-PCR (RM-PCR) was developed to screen differential seqeuences between *T. controversa and T. caries*. By employing RM-PCR format, a 1,322-bp DNA fragment (PR32) was selected from 18T controversa strain and 29T caries strain. The PR 32 genes were specific to *T. controversa* and they almost did not show any homology to *T. caries* or other fungi in the available database. When the primers designed from PR32, positive amplification from all 18 stains of *T. controversa* occurred, but not with any of the 29 strains of *T. caries* by standard PCR assay. SYBR Green I and TaqMan probe real-time PCR formats were established based on PR32 for rapid and precise identification of *T. controversa*. TaqMan real-time PCR was able to detect *T. controversa* in asymptomatic tissues successfully (Yuan et al. 2009).

It is difficult to identify obligate fungal pathogens based on the morphological characteristics of asexual spores/sporulating structures. Preparation of pure DNA from these obligate pathogens is problematical and amplification of impure DNA may lead to wrong interpretation of the results obtained. An in situ PCR technique was developed to overcome the problems posed by these fungal pathogens which have to be, otherwise, identified based on the morphological characteristics. The in situ PCR technique links PCR amplification to the light microscope image. The amplified tissue is stained, thus confirming which morphotype has been amplified. The PCR product is then sequenced. *Blumeria graminis* f.sp. *hordei* causing the powery mildew disease of barley was identified by this protocol. Conidia and mycelia were tested using primers derived from the sequences of the gene encoding the catalytic subunit protein kinase A (*bka 1*). This technique allows positive confirmation of the origin of genes cloned from obligate fungal pathogens and it could be adapted for testing samples containing two or more fungal species (Bindslev et al. 2002).

A major advantage of the PCR-based assays is the non-requirement of culturing the pathogens in artificial media. The obligate pathogens which are yet to be cultured and those pathogens which grow very slowly, are amenable for detection by PCR assays. Differences on the sequence of nucleotides of ITS regions of rDNA and PCR using species-specific primers have been used very effectively for detection or identification of powdery mildew pathogens (Takamatsu and Kano 2001). Whole-cell DNA of *Oidium neolycopersici*, incitant of tomato powdery mildew disease, was used for amplifying the nuclear rDNA region spanning the ITS1, ITS2,

and 5.8S rRNA by PCR. The primers ITS 5 and P3 were employed for the first amplification and the nested primer set ITS1 and P3 was used for the second amplification. The ITS sequences of North American anamorphs were identical with those of three Japanese and four European isolates of *O. neolycopersici*. This powdery mildew pathogen was shown to be distinct and it was neither identical nor closely related to any known polyphagous species of Erysiphaceae (Kiss et al. 2005). Powdery mildew disease of sunflower caused by *Podosphaera xanthii* (= *Sphaerotheca fuligena*), *Golovinomyces* (= *Erysiphe*) *cichoracearum* and *Leveillula taurica* may occur in severe forms during cool and dry seasons in several countries. Based on the sequence variation in the ITS region, species-specific primer pairs S1/S2, G1/G2 and L1/L2 for *P. xanthii*, *G. cichoracearum* and *L. taurica* respectively, were designed and applied for their detection and differentiation. The PCR assay was found to be accurate, sensitive and rapid and it required less labor and time compared with microscope examination or spore trapping methods (Chen et al. 2008) (Appendix 19).

Sugar beet crops were repoted to be infected by *Microsphaera betae*, later renamed *Erysiphe betae* in Europe and by *Erysiphe polygoni* in North America, creating confusion over the correct taxonomic identity of the pathogen causing powdery mildew diseases in these two well separated geographical locations. The isolates of the pathogen in UK and USA were investigated for polymorphisms in the rDNA ITS regions to determine, if the same species caused the disease in both countries. After examining a total of 18 isolates, 23 ITS sequences were obtained. The sequences of 15 isolates were identical indicating that the fungi were conspecific. The ITS sequences of UK and North American isolates were more closely related to *E. heraclei* than to *E. polygoni.* Hence, it was proposed that the species name *E. betae* be used for the powdery mildew pathogen of sugar beet. No evidence was found for the involvement of another pathogen species in beet powdery mildew disease (Francis et al. 2007).

Specific and sensitive PCR assay was developed for the detection of *Puccinia striiformis* f.sp. *tritici* (Pst), causing stripe rust disease in wheat (*Triticum aestivum*). The primer pair PSF/PSR was designed based on the ITS sequences. The PCR products were amplified with the universal primers ITS1 and ITS4 and they were cloned into pGEM-T Easy vectors and sequenced. The ITS sequences of Pst was compared with those of *Puccinia triticina*, *P. graminis* f.sp. *tritici*, *Blumeria graminis* f.sp. *tritici*, *Fusarium graminearum*, *Rhizoctonia cerealis* and *Bipolaris sorokiniana* that infect wheat foliage. A PCR product (169-bp) unique to Pst was amplified from the DNA of all Pst isolates, but not from the DNA of other foliar pathogens mentioned above (Table 2.6). Pst was detected from the asymptomatic wheat leaves inoculated with Pst under greenhouse conditions and also in leaves sampled around stripe rust foci of infection in wheat fields. The detection limit of the PCR assay was 0.1 pg, indicating the high sensitivity level of the test (Zhao et al. 2007). A later study, indicated the need for a specific and rapid detection of Pst, since Pst infection in the overwintering regions showed no visible symptom of infection. In order to detect Pst in the dormant stage in young wheat plants in the overwintering regions, the possibility of using *P. striiformis* repeat (PSR) sequence

Table 2.6 Specificity of detection of *Puccinia striiformis* f.sp. *tritici* using universal and specific primers in PCR assay (Zhao et al. 2007)

Fungi colonizing wheat leaves	Isolate	Primers	
		Universal ITS1/ITS4	PSF/ PSR
Puccinia striiformis f.sp. *tritici* (Pst)	Su11	+	+
Pst	Hy46	+	+
Pst	CYR-23	+	+
Pst	CYR-31	+	+
Pst	CYR-32	+	+
P. graminis f.sp. *tritici*	Pgt	+	_
P. triticina	Pt	+	_
Blumeria graminis f.sp. *tritici*	Bgt	+	_
Fusarium graminearum	Fg	+	_
Rhizoctonia cerealis	Rc	+	_
Bipolaris sorokiniana	Bs	+	_

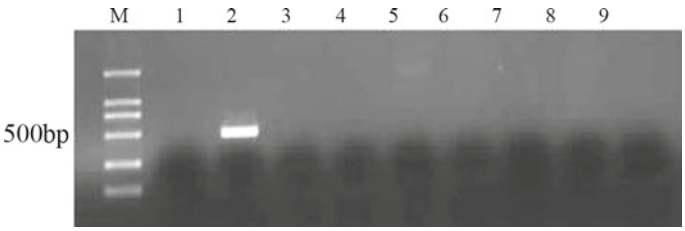

Fig. 2.7 Specificity of PCR for the detection of *Puccinia striiformis*. Note the presence of pathogen-specific band only in lane 2. Lane M: DNA ladder; Lane 1: healthy wheat leaves; Lane 2: *P. striiformis*; Lane 3: *P. recondita*; Lane 4: *P. graminis*; Lane 5: *Bipolaris sorokiniana*; Lane 6: *Fusarium graminearum*; Lane 7: *Rhizoctonia cerealis*; Lane 8: *Erysiphe graminis* and Lane 9: sterile distilled water (without template DNA) (Courtesy of Wang et al. 2008 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

for the development of genome-specific primers was explored. All isolates of Pst yielded a distinct band of a fragment of 470-bp amplified by the primers PST1 and PST2 whereas the DNA of other wheat pathogens tested was not amplified by these primers. The detection limit of the PCR was 10 pg of DNA. The presence of Pst in wheat leaves could be detected before any visible symptom appeared (Fig. 2.7). The pathogen could be detected in the dormant stage also by the PCR assay in leaf samples taken during winter season, indicating the usefulness of the PCR for detection of Pst in latent infected leaves of overwintering wheat plants that may serve as sources of infection (Wang et al. 2008) (Appendix 20).

The symptoms induced by *Peronospora tabacina*, causing tobacco downy mildew disease, are often non-specific under field conditions, making pathogen identification and disease diagnosis difficult. Two primers 160 2A and 160 2B that could amplify DNA samples extracted from tobacco plants exhibiting symptoms, were designed. All tobacco plants showing symptoms and those suspected to be

infected by *P. tabacina* were PCR-positive. On the other hand, the pathogen was detected only in 6–50% of the samples examined by light microscopy and yet the results were inconclusive. By eliminating sonication during DNA extraction, it was easier to perform the PCR, since fragmentation of DNA was prevented (Caiazzo et al. 2006). In order to detect and differentiate *P. tabacina* from other pathogens infecting tobacco, the primers ITS4 and ITS5 were employed, followed by sequencing and digestion with restriction enzymes. A specific primer PTAB was employed along with ITS4 to amplify a 762-bp region of DNA, unique for *P. tabacina*. The PTAB/ITS4 primer pair did not amplify host DNA or other 12 fungal pathogens including related *Peronospora* spp. capable of infecting tobacco. The primer pair amplified only the DNA of *P. tabacina* with a detection limit of 0.012 ng. As the presence of this pathogen can be detected in fresh, air-dried and cured tobacco leaves, the PCR protocol using the primer PTAB has the potential for application in regulatory and epidemiological investigations (Ristaino et al. 2007).

Several variants of PCR assay have been developed to enhance the specificity and sensitivity of detection as well as to quantify the pathogen population in the plants and also in their environments.

Magnetic Capture-Hybridization (MCH)-PCR Assay

Overcoming the problem of inhibition of PCR amplification by inhibitors is a major impediment to many applications, compromising both assay sensitivity and reliability. In order to reduce the adverse effects of PCR inhibitors coextracted with DNA from lignified apple tissues, a magnetic capture-hybridization (MCH)-PCR format was developed for the detection of *Nectria galligena*, causative agent of Nectria canker disease of apple and pear. The sequences of ITS1 region of the rRNA repeats were used as target. The trapping reagent used to coat the magnetic beads was an 81-bp ss-DNA oligonucleotide biotin-labeled on the 5′-terminal and designed to be complementary to part of the rRNA gene ITS1 region of the pathogen. Hybridization was performed in a total DNA extract of the woody tissue and magnetic recovery of the bead-oligomer-template conjugate separated target template from other DNA species and inhibitory compounds. After the magnetic capture-hybridization, PCR amplification was carried out using species-specific primers Ch1 and Ch2. The lower limit of visual detection on ethidium bromide-stained agarose gels was ~10–100 fg genomic DNA. The sensitivity of MCH-PCR was increased by 10- to 100-fold compared to conventional PCR assay. Further, MCH-PCR protocol could be effectively applied for studying disease aetiology and asymptomatic spread of the pathogen within the tree (Langrell and Barbara 2001).

Arbitrarily Primed (AP)-PCR

Verticillium dahliae and *V. albo-atrum* causing wilt diseases of several economically important crops like potatoes, were detected by employing the arbitrarily primed (AP)-PCR format. Two 15–16-mer oligonucleotide primers were used in

AP-PCR analysis to obtain the genome polymorphic patterns from the total DNA extracted from *V. dahliae*. A 350-bp fragment (designated MGC), unique for the recognition of *Verticillium* spp. proved specific for *V. dahliae* in Southern blot. This investigation points out the possibility of using such unique fragment for the detection of fungal pathogens and diagnosis of the diseases caused by them. *Colletotrichum acutatum*, the incitant of anthracnose disease, can survive in or on flowers, mummified fruits attached to alomond trees and blighted twigs during winter and these infected plant tissues may be responsible for its perennial occurrence in woody plants. Detection of this pathogen in both apparently healthy and infected plants was considered to be essential for the development of effective disease management strategies. Naturally diseased blueberry (*Vaccinium* spp.) bushes and their apparently healthy neighboring bushes were tested by conventional isolation and molecular methods. Arbitrarily primed (AP)-PCR technique was employed, using the primers $(CAG)_5$ and $(GACAC)_3$ for amplifying the pathogen-specific sequences. The isolates of *C. acutatum* obtained from diseased and symptomless bushes revealed the presence of isolates genetically identical, regardless of their origin. This implies that genetic variation of *C. acutatum* did not by itself predict the presence or absence of symptoms on each blueberry bush (Yoshida et al. 2007) (Appendix 21).

Polymerase Chain Reaction (PCR)-ELISA

Didymella bryoniae, causal agent of gummy stem blight disease of cucurbits was frequently isolated from infected cucurbits along with other *Phoma* spp. A PCR-ELISA format employing primers specific to *D. bryoniae* and *Phoma* was eavaluated using microplates. Primers were modified by addition of a fluorescein and a biotin label to the 5 ends of the forward and reverse primers respectively. PCR products were detected in ELISA, using horseradish peroxidase-conjugated antifluorescein antibody and three substrates that yielded three colored products. PCR-ELISA format successfully detected *D. bryoniae* (in 45 of 46 samples) and *Phoma* isolates (13) tested, providing results comparable with gel electrophoresis. One isolate of *D. bryoniae* that could not be detected by PCR-ELISA format, had a large DNA fragment, as visualized in agar gels. When "blind" fungal samples were tested, all the seven isolates of *D. bryoniae* and *Phoma* were detected and differentiated by PCR-ELISA protocol which was found to be simple, specific, rapid and convenient. However, its sensitivity was less, when compared to resolution by gel electrophoresis technique (Somai et al. 2002).

Reverse Transcription (RT)-PCR

Reverse transcription (RT)-PCR is an elegant diagnostic technique which detects target mRNA. It is also possible to quantify pathogen population in vivo. In contrast to DNA, mRNA is degraded rapidly in dead cells and most mRNA species have half-lives of only a few minutes (Ingle and Kushner 1996). Detection of mRNA by RT-PCR, as against

DNA detection methods, is considered as a better indicator of cell viability (Sheridan et al. 1998). RT-PCR has been applied to detect and quantify the expression of fungal pathogen genes in pathogenesis (disease development) or host plant genes leading to induction of resistance to diseases (McMaugh and Lyon 2003; Schenk et al. 2003).

With the aim of detecting viable populations of *Mycosphaerella graminicola* in wheat presymptomatically, a RT-PCR procedure was developed, using a specific primer set E1/STS P2 R, based on the sequences of ß-tubulin genes. The primer set amplified one single fragment only from the total RNA of the pathogen and infected wheat leaves, but not from those of healthy leaves or from five other common fungal pathogens infecting wheat. RT-PCR could detect *M. graminicola* at least 4 days before symptom expression in inoculated plants. After the appearance of symptoms of infection on the leaves, the increase in the band intensity of the amplified RT-PCR products in the gel was in general agreement with the visual disease intensity assessment. Presymptomatic detection is valuable because *M. graminicola* has a latent period of at least 4 weeks in northern Germany (Verreet et al. 2000). The ß-tubulin genes are among the most conserved genes with high copy numbers, making them to be suitable targets for reliable detection. The efficacy of two formats of RT-PCR was found to be less (100 pg total RNA) than that of one-step RT-PCR (5 ng total RNA) (Guo et al. 2005).

A RT-PCR format was applied for the detection of the powdery mildew pathogen *Oidium neolycopersici.* Single germinated conidia were individually transferred to 15 µl of PCR solution in a 200 µl microfuge tube for amplification of the entire 5.8S rDNA and its adjacent ITS sequences. The sensitivity level of the test was enhanced by adopting nested-PCR protocol to amplify the target nucleotide sequences. The transcripts expressed in single conidium were amplified by RT-PCR. *O. neolycopersici* or *Erysiphe trifolii* pathogenic and nonpathogenic respectively to tomato were discriminated and identified by the RT-PCR format. This investigation provided a reliable procedure, not only for the detection of the fungal pathogen, but also for monitoring gene expression in germinating conidia of the powdery mildew pathogen on tomato leaf surface (Matsuda et al. 2005).

The cherry disease, called as Amasya cherry disease (ACD) occurring in Turkey and as cherry chlorotic rusty spot (CCRS) from Italy, is suspected to be due to a fungal pathogen. However, association of multiple species of ds-RNAs, presumably of viral origin, has been reported (Coutts et al. 2004; Kozlakidis et al. 2006). The RT-PCR procedure for the detection of a mycoviral-like ds-RNA of 5.3 kb associated with ACD was developed. The primer set ZK14 and ZK17 based on the sequence of this fragment from RNA derived from all leaves of the cherry varieties suspected to be affected by ACD. No amplicons were detected in samples from healthy trees (Kozlakidis et al. 2007).

Nested-Polymerase Chain Reaction

The sensitivity of detection of fungal pathogens in plant tissues can be appreciably enhanced by nested-PCR assay. Both conventional and single-closed tube nested PCR format were developed for the detection of *Phytophthora nicotianae*,

causative agent of tobacco black shank disease. Two new specific primers designed from ITS1 and ITS2 regions, internal to the nucleotide sequence flanked by universal primers ITS4 and ITS6 were used. Morphologically characterized isolates (36) of *P. nicotianae* were tested. A positive reaction was shown by amplification of 737-bp product from the DNA of all isolates of *P. nicotianae* and two *P. nicotianae/cactorum* hybrids. No amplification occurred when other *Phytophthora* spp. and genera were tested, indicating the specificity of the assay. Nested-PCR format was at least 1,000-fold more sensitive than conventional PCR assay. Furthermore, samples from different infection sites, origins, crops, samples from nutrient solutions, water and rockwool used in hydroponic cultures were also analysed to validate the nested-PCR format (Grote et al. 2002).

Pyrenochaeta lycopersici causes corky root disease in tomato. The genomic DNA of *P. lycopersici* was detected by amplifying the target pathogen DNA, using the universal primers ITS4 and ITS5. However, a nested-PCR format was needed to detect this pathogen in the root tissues. Specific primers were employed for the second amplification. Specific PCR products from isolates belonging to Type 1 and Type 2 were amplified enabling the identification of *P. lycopersici* isolates with certainty (Infantino and Pucci 2005). Quarantine regulations demand a rapid and reliable detection of *Phytophthora fragariae* var. *fragariae* to prevent its introduction and further spread through infected plant materials into new locations. Nested-PCR protocol developed by Bonants et al. (2004) detected 100 ag (10^{-16} g) of pure pathogen DNA which is equivalent to ~1/60 part of one nucleus and this level of sensitivity was possible, because rDNA is a multy copy gene. However, in practice it was possible to detect between 5 and 10 zoospores of *P. fragariae* var. *fragariae* consistently by employing the nested-PCR format.

The occurrence of several 'new' species of *Phytophthora* during the past decade was reported, because of the availability of improved detection methods and possibly due to increased movement of infected/asymptomatic plant materials across the continents. PCR-based diagnostics (PCRDs) based on the ITS regions and sequences-characterized amplified region (SCAR) have been instrumental in rapid and precise detection, identification and quantification of fungal pathogens. However, ITS sequences are not always sufficiently variable to separate closely related taxa. A PCR-based 'molecular tool box' based on a region of the ras-related protein gene *Ypt1* was developed for the detection and identification of 15 *Phytophthora* spp. infecting forest tree species. This gene was useful to develop a pair of *Phytophthora* genus-specific primers (Yph 1F/Yph2R), as well as a multiplex-real-time approach to detect and quantify *P. ramorum*, P. *kernoviae*, *P. citricola* and *P. quercina* in naturally and artificially infected leaves (Schena et al. 2006). Amplification with *Phytophthora* genus-specific primers before amplification with various species-specific primers (nested-PCR) increased the sensitivity of detection over amplification with species-specific primers only. The detection limits ranged between 100 and 10 pg target DNA in the latter, compared with 100 fg in the nested format. It is possible to detect *Phytophthora* spp. in leaves using a single-round amplification, but nested-PCR was required for detecting these pathogens in water and soil samples (Schena et al. 2008).

Phytophthora nicotianae infecting tobacco was detected by employing the primers designed based on the sequences of a Ras-related protein (*Ypt1*) gene in a PCR assay. The specificity of the primers was evaluated by testing 115 isolates representing 26 species of *Phytophthora* and 29 other fungal plant pathogenic species. A specific 389-bp amplicon was obtained ony from the *P. nicotianae* isolates, but not from other pathogenic fungi tested. The detection limit of the PCR assay using the species-specific Pn primers was one ng of genomic DNA. In the nested PCR format, the primer pair Ypt1F/Ypt1R was used for the first round amplification and the primer pair Pn1/Pn2 was employed for amplifying the PCR product of the first round amplification. The sensitivity of the nested PCR procedure was enhanced by 100-fold. The standard PCR format was successful in detecting *P. nicotianae* in naturally infected tobacco tissues as well as in the soil samples. The standard and nested PCR formats may provide a simple method for diagnosis of the infection of tobacco by *P. nicotianae* and also for monitoring pathogen population facilitating effective disease management efforts (Meng and Wang 2010).

Two major eucalypt species *Eucalyptus globus* and *E. nitens* suffer severely due to Mycosphaerella leaf disease (MLD) in the temperate regions. For the development of sustainable disease management system, accurate, rapid detection and unambiguous identification of *Mycosphaerella* spp. causing MLD is essential. Hence, a nested-PCR approach, using specific primers for detection and identification of the causative agents was applied. Primer design was based on sequence alignment and phylogenetic analysis of 16S nonredundant sequences from the nuclear rDNA ITS regions of *Mycosphaerella* and related species. Primers were designed to differentiate two taxon groups, *M. grandis* and *M. parva* and *M. vespa*, *M. ambiphylla* and *M. molleriana.* In addition, *M. cryptica*, *M. nubilosa* and *M. tasmaniensis* were distinct and could be differentiated by species-specific primers. An internal amplification control was included in the test to highlight negative results, due to inhibition of PCR. The nested-PCR assay employed in DNA extracted from leaf or stem samples either as multiple or single lesions, detected and identified up to five *Mycosphaerella* spp. or taxon groups in both positively identified and in young (putative) MLD lesions. The nested-PCR assay identified *Mycospherella* spp. in 2 days, 1–5 months earlier than by classical isolation methods. This assay protocol has the potential for use in ecological, epidemiological and genetic studies (Glen et al. 2007).

The genus *Phaeoacremonium* includes some plant- and human-infecting isolates grouped with the name of *Phialophora parasitica*. Later, *Phaeoacremonium viticola* was described (Dupont et al. 2000). The DNA phylogenetic analysis based on ITS1/5.8S/ITS2 and especially of ß-tubulin, actin and calmodulin gene regions identified *P. australiensis*, *P. krajdenii*, *P. scolyti*, *P. subulatum* and *P. venezuelense* that develop on grapevines in addition to *P. viticola* (Mostert et al. 2005). Species-specific primers based on ITS region of rDNA were employed to detect and identify *Phaeomonium aleophilum* and *Phaemoniella chlamydospora* associated with grapevine decline disease (Whiteman et al. 2002). Primers Pm1 and Pm2 were designed from the sequences of the rDNA ITS1 and ITS2 respectively. They yielded a single amplicon of 415-bp for nine species of *Phaeoacremonium* that may

occur on grapevines. A nested-PCR (using general fungal primers ITS1/ITS4 in the primary reaction) was developed to detect *Phaeoacremonium* directly in grapevine wood. Identification of *Phaeoacremonium* spp. was achieved by digesting PCR amplicons with restriction enzymes *Bss* KI, *Eco* O1091 and *Hha* I. Different species were identified by the RFLP patterns, except for *P. viticola* and *P. angustius*. A species-specific PCR amplification of partial ß-tubulin gene using the primer pair differentiated *P. angustius* and *P. viticola* (Aroca and Raposo 2007).

Nested-PCR format for the detection of *Botryosphaeria dothidea*, causative agent of panicle and shoot blight of pistachio, was developed based on microsatellite regions. Primer pairs specific to *B. dothidea* were designed using the sequence of a species-specific 1330-bp DNA fragment amplified by a microsatellite primer T3B. The external and internal primer pair EBdF+EBdR and IBdF+IBdR amplified a 701-bp and a 627-bp fragment respectively from *B. dothidea*, but not from any other fungi associated with pistachio. The nested-PCR format was able to detect specific fragments in 1 fg DNA of *B. dothidea* or in the DNA of two conidia, showing its high level of sensitivity. Visible infection in leaves of *B. dothidea* could be confirmed by nested-PCR assay. This study showed that microsatellite regions could be advantageously used for developing highly sensitive PCR detection systems for other fungal pathogens infecting various crops (Ma et al. 2003).

Colletotrichum acutatum causes one of the destructive diseases of strawberry and other fruit crops. A nested PCR format was developed for the detection of this pathogen in symptomless leaves of inoculated strawberry plants. The inoculated leaves were frozen for 3 h, incubated for 2 days at 27°C, immersed in Tween-20 solution, sonicated for 30 min and finally the suspension was agitated for 1 min. The assay detected as little as 1 fg of DNA extracted from the pathogen mycelium and as few as 1.5 conidia/ml after sonication. When the extracts of inoculated symptomless leaves were tested, the nested-PCR protocol detected *C. acutatum* in the composite leaf samples containing one inoculated out of 50 uninfected leaves. The pathogen could be detected in field-collected asymptomatic strawberry leaves, revealing the potential of the protocol developed in this study for reliable diagnosis of *C. acutatum* under field conditions (Pérez-Hernández et al. 2008).

Corynespora cassiicola causes the Corynespora leaf fall disease of rubber (*Hevea brasiliensis*), a major factor limiting rubber latex production in Asia and Africa. The isolates (16) of *C. cassiicola* included in this investigation were considered to belong to race 1, based on the pathogenicity tests. Initial PCR assay employed the species-specific primer set CCF/CCR-2 which amplified a fragment of 272-bp from ITS1-5.8S-ITS2 region. A nested PCR protocol was developed to improve the sensitivity of detection of *C. cassiicola* by using diluted (1:100) amplified product from the initial PCR as template and species-specific oligonucleotide primers CCF and CCR-1. This procedure resulted in the amplification of the expected product of 152-bp. The detection limit of the nested PCR assay was 168 fg, showing a 100-fold enhancement of sensitivity over the initial PCR assay that could detect *C. cassiicola* only at a concentration of 16.8 pg of pathogen DNA. Further, *C. cassiicola* could be detected from artificially inoculated rubber trees at 3 days after inoculation. As the nested PCR exhibited high levels of sensitivity,

specificity and reliability, it might be useful for screening and certification of young rubber plants for distribution to commercial growers (Qi et al. 2009).

As the isolation-based methods are time-consuming, a nested PCR format was developed to accelerate and simplify the process of detection *of Puccinia striiformis* f.sp. *tritici* (*Pst*) in infected wheat plants. Specific primer pair Psta/Psts was designed based on the genome-specific sequence of *Pst*. In the nested PCR, the detection limit was 2 pg DNA in the first round PCRwith the primer pair Psta/Psts. The second round PCR was performed using the amplified product from the first PCR as template and Nesta/Nests as the primer pair. An amplification signal could be recognized at 2 fg of *Pst* DNA. The sensitivity of detection of *Pst* by the nested PCR format was enhanced by 1,000-fold. The pathogen was detected even before symptom appearance by testing the extracts from asymptomatic leaves of stripe rust-infected wheat plants. The nested PCR assay has the potential for detecting *Pst* in latently infected leaves of overwintering wheat plants (Xiaojie et al. 2009).

In order to improve the *in planta* detection of *Peronospora arborescens*, an obligate oomycete infecting cultivated opium poppy, a sensitive nested PCR format was developed. Two primer pairs employed in the nested PCR assay increased the sensitivity by 100- to 1,000-fold over the detection limit of single PCR using the same primers. The new format allowed amplification of 5–0.5 fg of *P. arborescens* DNA mixed with *Papaver somniferum* (host plant) DNA. Further, the pathogen DNA could be specifically amplified from 96-year old herbarium specimens of *Papaver* spp. The pathogen was detected in symptomatic and asymptomatic plants of cultivated opium poppy and wild *Papaver* spp. *P. arborescens* was detected also in the seeds of commercial opium poppy seeds samples with a high frequency in Spain, indicating a possible threat for rapid pathogen spread (Montes-Borrego et al. 2009).

Multiplex-PCR Assay

There is no need for isolation of the fungal pathogen from the infected plant tissues, when the multiplex-PCR assay is performed. This format provides the advantage of acceleration of detection and identification process. A one-tube PCR multiplex format was developed, using the sequences of a repetitive satellite DNA fragment of *Phytophthora infestans* for designing the specific primers. These primers were effective in detecting 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 using sequences of ITS2 region of DNA were employed to detect *P. infestans* and *P. erythroseptica*, causing pink rot disease of potato tubers, even before the development of any visible symptoms (Tooley et al. 1998).

Hops (*Humuls lupulus*) are severely infected by many fungal pathogens including *Pseudoperonospora humuli*, causing downy mildew and *Sphaerotheca humuli*, causing powdery mildew disease. As the infection by these pathogens remain symptomless, detection by visual observations is very difficult. Specific PCR assays were developed to detect *P. humuli* and *S. humuli* in naturally infected hop

plants. Specific PCR primer combinations P1+P2 and S1+S2 amplified specific ITS sequences from *P. humuli* and *S. humuli* respectively and did not cross-react with host plant DNA or DNA from other fungi tested. A multiplex-PCR method was developed for the detection of two or more fungal pathogens, in addition to *P. humuli* and *S. humuli*. PCR primers R1 and R2 amplified a fragment of 305-bp nuclear DNA (mainly ITS1) region from hop plants, a fragment of 297-bp nuclear rDNA from *P. humuli*, a fragment of 248-bp nuclear rDNA from *S. humuli*, a fragment of 204-bp nuclear rDNA from *Verticillium albo-atrum* and a fragment of 222-bp nuclear rDNA region from *Fusarium sambucinum*. Similarly R3 and R4 primers amplified 397-bp, 598-bp, 312-bp, 331-bp and 317-bp fragments respectively from the hop, *S. humuli*, *P. humuli*, *V. albo-atrum* and *F. sambucinum*. By using PCR primer combinations R1 + R2, R3 + R4, these four pathogens were detected and differentiated rapidly and precisely (Patzak 2003).

A rapid multiplex-PCR was developed, using primer pairs S1/S2 and G1/G2 based on ITS sequences specific respectively for *Podosphaera xanthii* and *Golovinomyces cichoracearum*, causing powdery mildew disease of sunflower. The amplicons of 454-bp and 391-bp were amplified by these primer pairs. When the multiplex-PCR amplification with these primers was performed for fungal DNA samples from infected sunflowers, *P. xanthii* and *G. cichoracearum* were successfully detected and differentiated (Chen et al. 2008).

Detection of *Verticilliun albo-atrum* and differentiation of its two pathotypes infecting hop was possible by employing sequence-characterized amplified region (SCAR) primer pairs in multiplex PCR format. Three pairs of SCAR primers viz., 9-21-For/9-21-Rev, 11-For/11-Rev and 9-21For/9-21-Rev were very effective in the diagnosis of hop PG1 and PG2 pathotypes of hop. The amplified PCR products corresponded to the SCAR markers, indicating that the specificity of the primers remained unlatered by the multiplex reaction. The sensitivity and specificity of diagnosis of the pathotypes was improved by the simultaneous amplification of two specific loci for PG2 and one locus for PG1, making pathotype screening by multiplex-PCR assay more reliable (Radišek et al. 2004).

Detection of infection of trees by wood decay fungi is mainly based on visual tree assessment (VTA) consisting of visual inspection of signs and symptoms linked to the presence of imperfections in the structure of the trees. But the VTA approach is useful for the diagnosis of decay at an advanced stage, but not in the early stages. Multicopy arrangement and highly conserved priming sites, typical of both nuclear and mitochondrial rDNA, permit amplification of several fungi. The amplification of rDNA genes with universal fungal primers followed by restriction endonuclease digestion (RFLP) has been demonstrated to be suitable for taxon-specific identification of decay fungi (Adair et al. 2002). Simultaneous application of taxon-specific primers in multiplex-PCR reaction saves time and efforts without compromising specificity of the analysis. Eleven taxon-specific primers were designed for PCR amplification of either nuclear or mtrDNA regions of 11 fungi including *Armillaria* spp. *Ganoderma* spp. *inonotus/ Phellinus* group. Multiplex reactions were developed and optimized to detect fungal DNA and identify each taxon with a sensitivity of at least 1 pg target

DNA in the template. This multiplex-PCR protocol correctly identified the causative agents of decay in 82% of tested wood samples and it has the potential for early detection of wood decay fungi which is crucial for assessment of tree stability in urban landscapes (Guglielmo et al. 2007).

Aspergillus flavus is a prominent contaminant of food material and also causes the aflaroot disease in peanut plants. All strains of *A. flavus* do not produce the mycotoxin aflatoxin that is harmful to humans and animals consuming contaminated food and feed. A multiplex RT-PCR method was developed to discriminate aflatoxin-producing strains from the nonproducers. Five genes of the aflatoxin gene cluster of *A. flavus*, two regulaltory (*aflR* and *aflS*) and three structural (*aflD*, *aflO* and *aflQ*) were targeted with specific primers to highlight their expression in mycelia cultivated under inducing conditions for aflatoxin production. A good correlation was noted between expression of aflatoxin genes analyzed by multiplex RT-PCR and aflatoxin production. This investigation appears to be the first in applying a combination of mutiplex PCR and RT-PCR approaches to screen for differential ability of toxin production by strains of a fungal pathogen (Degola et al. 2007).

Real-Time PCR Assay

Quantification of PCR amplicon by image analysis, after separation in agarose gel, is somewhat cumbersome and time-consuming, especially if hundreds of samples have to be analysed. Application of real-time PCR is effective in overcoming this problem and this format quantifies diagnostic amplicons on-line with a fluorescence detection system. An additional dual-labeled flourigenic probe is used for hybridizing to the target DNA within the region defined by the two fluorescent dyes, a reporter (6-carboxy-fluorescein (FSM)) at the 5′ end and a quencher (6-carboxy-tetramethylrhodamine (TAMRA)) at the 3′ end, which is, in addition, blocked by a phosphate group in order to prevent PCR-derived elongation. Both fluorescent dyes are excited with an argon-ion-laser at 488 nm. The proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer. During the extension phase of PCR, however, the 5′ nuclease activity of the *Taq* polymerase cleaves the reporter dye from the fluorigenic probe. The separation of the reporter from the quencher results in an increase in fluorescence. Based on the measurable light emission of the reporter dye, the synthesis of amplicons can continuously be monitored during thermocycling and no post-PCR handling is required for the product quantification.

A specific Ct-value is worked out for each sample. The Ct value may be defined as that number of cycle at which a statistically significant increase in the reporter fluorescence can first be detected. The Ct-values are then used to calculate the starting copy number of the target for each sample. This calculation is done automatically by comparing the Ct-values of unknown samples with the Ct-values of standards with known amounts of target copies. The chief advantage of real-time PCR is that amplification products can be monitored, as they are accumulated in the

long-linear phase of amplification. In addition, the rapidity and greater accuracy of real-time PCR are the other advantages of real-time PCR over conventional PCR assay (Böhm et al. 1999; Schaad et al. 2003; Vandemark and Barker 2003).

The real-time PCR used to detect and study microbial plant pathogens may be either amplicon sequence non-specific (SYBR Green) or sequence-specific (TaqMan, Molecular Beacon or Scorpion PCR) (Mumford et al. 2006; Cooke et al. 2007). SYBR Green is a non-specific dye that fluoresces, when intercalated into double-stranded DNA. On the other hand, amplicon sequence-specific methods are based on the labeling of primers or probes with fluorogenic molecules that allow the detection of a specific amplified target fragment. Real-time PCR performed via specific or non-specific methods have been demonstrated to be more sensitive, specific and rapid compared with conventional PCR assay, in addition to reduction in the risk of false positives and absence of the need for post-amplification steps. Real-time PCR technique promotes quantitative and multiplex analyses (Schena et al. 2006).

The real-time quantitative PCR was applied for the detection of *Phytophthora infestans* and *P. citricola* in potato and forest tree species respectively. DNA was isolated from stem tissues of potato field plants, exhibiting symptoms of late blight disease. By employing the *P. infestans*-specific oligonucleotide primer pair, the diagnostic amplicon was obtained by PCR assay. Based on the primers P3, P4 and fluorogenic probe F2, the initial amount of pathogen template DNA within the infected samples was analyzed by real-time PCR using 45 cylces. Stem samples of naturally infected field plants yielded a much higher starting copy number of pathogen target DNA/mg (wet weight) of host tissue than samples from artificially inoculated tubers under the conditions of experimentation. Likewise, DNA of *P. citricola*, causing root rot disease in beech and oak seedlings was quantified by real-time PCR assay which yielded slightly higher amounts of starting template DNA/mg of host tissue in comparison with image analysis (Böhm et al. 1999).

Phytophthora capsici, causing root rot disease of pepper, was detected by using specific primers based on ITS region of rDNA in PCR in artificially inoculated and naturally infected plants. With a view to estimating pathogen DNA quantitatively, a real-time PCR format was developed. Using SYBR Green dye (ds-DNA-binding dye (DNA quantity is reflected by the dye and hence fluoresces)) and specific primers for *P. capsici*, the pathogen DNA was quantified. 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. The stem tissues, among the plant tissues tested, contained maximum pathogen biomass (Silvar et al. 2005a, b). A conventional PCR and a SYBR Green real-time PCR assays were employed for detecting and quantifying *P. cryptogea*, causing serious root rot disease of gerbera, an important cut-flower crop in Europe. A conventional primer pair Cryp 1 and Cryp 2 was designed from the *Ypt1* gene of *P. cryptogea*. The highly polymorphic nature of the *Ypt1* gene sequences obtained from different species of *Phytophthora* enables the differentiation of closely related species that have identical ITS regions. The primer pair Cryp1/Cryp2 amplified a 369-bp product from the DNA of 17 isolates of *P. cryptogea*, but not from the DNA of 34 other *Phytophthora* spp., water molds,

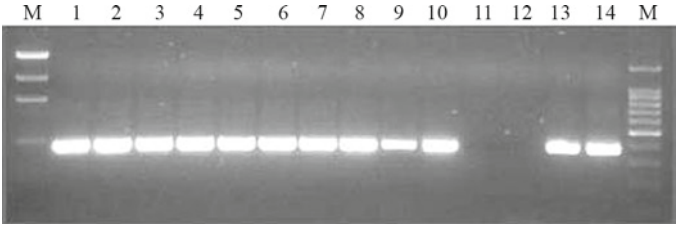

Fig. 2.8 Detection of real-time PCR products following agarose gel electrophoresis of extracts from symptomless gerbera roots collected 14 days after inoculation with *Phytophthora cryptogea.* Lane 1–7: tenfold dilution of plasmid DNA in the range of 160 pg–160 ag; Lane 8: DNA from symptomless gerbera roots; Lane 9: DNA extracted from zoospores present in the nutrient solution collected 7 days after inoculation; Lane 10: DNA from roots artificially inoculated plants; Lane 11: uninoculated roots; Lane 12: no DNA (control); Lane 13: DNA from symptomatic roots 21 days after inoculation; Lane 14: DNA from naturally infected roots; Lane M: DNA ladder; (Courtesy of Minerdi et al. 2008 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

true fungi and bacteria tested. After adapting this primer pair to real-time PCR format, the efficiency of conventional and real-time PCR formats was assessed. The PCR assay detected *P. crytpogea* in naturally infected gerbera plants at 21 days after inoculation. The detection limit was 5×10^3 zoospores or 16 fg of DNA of the pathogen. On the other hand, real-time PCR was more sensitive (100-fold) with a detection limit of 50 zoospores and 160 ag of pathogen DNA. The presence of *P. cryptogea* in symptomless roots was also detected by real-time PCR assay, confirming its potential for wider application (Fig. 2.8) (Minerdi et al. 2008).

Leaves and stems of potato plants generally do not exhibit symptoms of infection by *Phytophthora erythroseptica* causing pink rot disease of potato tubers. Molecular methods have been employed for the detection of *P. erythroseptica* in the tubers. As no precise detection procedure was available, both standard PCR and real-time PCR assays were developed to detect the pathogen in the stem and leaves of potato plants. The primers PERY2 and ITS4 (Applied Biosystems, USA) for standard PCR format and 99F and 177R primers and the probe 133T for the real-time PCR assay were used. About 95% of samples were positive for real-time PCR assay, while standard PCR assay was successful in detecting *P. erythroseptica* only in 45% of the samples tested. The real-time PCR was found to be significantly more sensitive than the standard PCR format. Further, the presence of *P. erythroseptica* in aerial tubers in plants growing from artificially inoculated and naturally infected tubers. Some progeny tubers and stolons also showed infection by this pathogen. *P. erythrosepitca* was also detected in some progeny tubers, stolons and in a few samples of debris taken from naturally senesced above-ground potato tissues after harvest by employing the real-time RT-PCR assay (Nanayakkara et al. 2009).

Sudden oak death disease caused by *Phytophthora ramorum* is perceived as a serious threat to forest ecosystems, if proper management practices are not implemented. A single round TaqMan PCR assay was developed for the detection of *P. ramorum* within 2 h under field conditions. Specific primers *Pram*-114F and

Pram-190R and probe (*Pram* probe) and generic 5.8S TaqMan primers (5.8SF and 5.8SR) and probe (5.8S probe) were designed based on ITS sequences. The target DNAs were extracted from healthy and infected plants outside the laboratory under field conditions at disease outbreak sites several hundred miles away from the diagnostic laboratory. The results were similar to those of real-time PCR assay performed inside the laboratory (Tomlinson et al. 2005). *P. ramorum* was detected by using 5 (prime) fluorigenic exonuclease (TaqMan) chemistry with detection limit of 15 fg, when used in a nested design or 50 fg when used in a single-round of PCR. The absence of amplification of DNA of 17 other *Phytophthora* spp. tested, revealed the high level of specificity of this assay. In addition, the nested methods were shown to have higher degree of sensitivity compared to non-nested methods. The host substrate had appreciable influence on the outcome of the assays. Under field conditions, the nested TaqMan protocol detected *P. ramorum* in 255 of 874 plants tested as against a few positive reactions provided by a single round TaqMan method (Hayden et al. 2006).

A real-time PCR was developed to detect *P. ramorum* based on mitochondrial (mt) DNA sequence with an ABI Prism 7700 (TaqMan) Sequence Detection System. In addition, specific primers and probes were designed for the detection of newly described *P. pseudosyringae* which causes similar symptoms as *P. ramorum*. A multiplex assay employing the species-specific primer-probe systems detected successfully both pathogens. The lower limit of detection of *P. ramorum* DNA was 1 fg of genomic DNA. Using a three-way multiplex format, the DNAs of *P. ramorum*, *P. pseudosyringae* and host plant were detected in a single tube. The multiplex assay was able to detect *P. ramorum* in infected rhododendron field samples and also samples from several host plant species. The assay format developed in this study has been found to be highly sensitive and specific with several advantages over conventional PCR assay (Tooley et al. 2006). An on-site real-time PCR assay was developed for the detection of *P. ramorum*, using TaqMan PCR format suitable for locations where eradication of infected *Parrotia persica* was undertaken as a disease management strategy to prevent the spread of the disease (Hughes et al. 2006). An intra-laboratory procedure was developed for validating a real-time protocol for the detection of *P. ramorum*. The real-time PCR method developed by Ivors et al. (2002) was considered to be suitable for rapid screening for the detection of *P. ramorum* in the tissues of *Rhododendron* spp., *Viburnum* spp. and *Pieris* spp. The limit of detection was 50 fg and at this level of sensitivity it would be possible to detect the pathogen in the asymptomatic plant tissues (Chandelier et al. 2006).

Molecular assays available then, although sensitive and rapid in detecting fungal pathogens, failed to reliably distinguish *Phytophthora ramorum* and closely related species. To overcome this limitation and to provide additional assays to increase confidence, ITS, ß-tubulin, and elicitin gene regions were sequenced and searched for polymorphisms in a collection of *Phytophthora* spp. Three different technologies viz., SYBR Green, TaqMan and molecular beacons were compared for their efficacy. These assays detected and differentiated *P. ramorum* from the 65 species of *Phytophthora* tested. The pathogen could be detected in DNA extracts from

infected plant samples. Likewise, *P. ramorum* was also detected by all three assays in the environmental samples from which the pathogen was isolated in PARP-V8 medium. The assays based on detection of the ITS and elicitin regions using TaqMan tended to have lower cycle threshold values than those using ß-tubulin gene and appeared to be more sensitive (Bilodeau et al. 2007).

Soybean sudden death syndrome (SDS) is due to *Fusarium solani* f.sp. *glycines* which is difficult to detect and quantify, because of its variable phenotypic characterisitics and slow-growing nature. Real-time quantitative PCR (QPCR) assay was developed for both absolute and relative quantification of *F. solani* f.sp. *glycines.* The mitochondrial small-subunit rRNA gene sequences form the basis of primer design for performing QPCR assay in a 96-well plate format. By applying the absolute QPCR assay, as low as 9.0×10^{-5} ng of DNA of *F. solani* f.sp. *glycines* could be detected in soybean plants with or without SDS foliar symptoms (Gao et al. 2004). 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* were the economically important pathogens that were detected by this real-time PCR format (Schroeder et al. 2006).

Aphanomyces euteiches, causal agent of pea root rot disease, was detected in susceptible and resistant pea germplasm entries by employing a real-time PCR format. Relative contents of pathogen DNA were determined in susceptible and resistant pea population at 7, 10 and 14 days after inoculation. At all sampling intervals, the DNA contents of *A. euteichus* were greater in susceptible plants than in resistant plants. The earliest significant correlation between disease severity and pathogen DNA contents was observed at 14 days after inoculation. The results suggested that management practices have to be directed at the early stages of pathogen life cycle, before the formation of sexual spores (oospores) which are resistant to chemicals and adverse environmental conditions (Vandemark and Ariss 2007).

The primer pair VertBt-F/VertBt-R designed based on the sequence of the ß-tubulin gene was used in a real-time quantitative PCR assay for the detection and quantification of *Verticillium dahliae*, causing potato early dying (PED) or wilt disease. The efficacy of this primer pair was found to be greater (>95%) than monoplex QPCR and duplex methods, using the primers PotAct-F/PotAct-R designed from the sequences of actin gene. The pathogen DNA as few as 148 fg (equivalent of 5 nuclei) could be detected and quantified. This QPCR protocol was successfully applied for the detection of *V. dahliae* in naturally-infected, air-dried potato stems and fresh stems of inoculated plants. Conventional isolation method failed to detect the pathogen in 10% of stem samples which were QPCR-positive, indicating the reliability and sensitivity of this PCR format. The response of potato breeding lines to infection by *V. dahliae* may be assessed by QPCR assay more reliably, because of the rapidity and sensitivity of the technique (Attallah and Stevenson 2006; Attallah et al. 2007).

Green fluorescent protein (GFP) transformation approach has been adopted in analyzing various fungal/fungus-like pathogen–host interactions. The principal advantage of this method is that infection and colonization of plants by

Table 2.7 Detection of DNA of *Verticillium* spp. in *Brassica napus* seedlings inoculated with *V. longisporum* (VL) or *V. dahliae* (VD) by real-time PCR (Eynck et al. 2007)

Days after	Hypocotyl		Leaves	
Inoculation	VL[a]	VD[a]	VL[a]	VD[a]
7	2.88 (±0.89)	0.53 (±0.18)	0.00 (±0.00)	0.00 (±0.00)
14	9.94 (±2.79)	0.18 (±0.09)	0.01 (±0.01)	0.00 (±0.00)
21	3.95 (±1.65)	0.05 (±0.02)	0.51 (±0.29)	0.02 (±0.02)
28	10.41 (±1.52)	2.62 (±1.18)	0.59 (± 0.26)	0.01 (±0.01)
35	25.58 (±6.79)	0.24 (±0.12)	6.89 (±5.37)	0.06 (±0.06)

[a]ng DNA/g fresh weight; ± Standard error
Values are means of the amount of fungal DNA in hypocotyls and leaves

GFP-expressing pathogen can be followed by the fluorescence microscopy in intact plant tissues or tissue sections without the need for cofactors or substrates (Bolwerk et al. 2005). *V. dahliae* and *V. longisporum* were stably transformed via *Agrobacterium tumefaciens*-mediated transformation technique. The interaction of *V. dahliae* with *Brassica napus* (oilseed rape) differed entirely from that of *V. longisporum*. *V. dahliae* was infrequently able to penetrate and colonize the root tissue, whereas *V. longisporum* rapidly spread into the vascular system after penetration of root tissues. This histological and real-time PCR analyses showed that *B. napus* was not a suitable host for *V.dahliae* (Table 2.7). Non-host resistance against *V. dahliae* appeared to restrict systemic spread rather than inhibition of penetration of root tissues (Eynck et al. 2007).

Verticillium dahliae infects pepper (*Capsicum annuum*) causing serious losses. Infected plants have to be detected in the early stages of infection, when no visible symptoms can be recognized. Real-time PCR assay was applied for early detection of infection and quantification of pathogen population. A sequence characterized amplified region (SCAR) was used to design the primers VDS1 and VDS2. Pathogen DNA was detected in the roots and hypocotyls of infected plants by real-time PCR assay. Quantification of pathogen DNA in the infected root tissues revealed that higher amounts of pathogen biomass were present in the susceptible cultivars compared to resistant cultivars. The real-time PCR assay was able to detect *V. dahliae* assay in pepper plants even before symptom expression (Gayoso et al. 2007).

Olive (*Olea europaea*) trees are seriously damged by *Verticillium dahliae*. Based on the type of symptoms induced the isolates are divided into defoliating (D) and non-defoliating (ND) pathotypes. The real-time quantitative (Q) PCR assay was applied to detect and quantify the pathogen DNA in the susceptible cv. Amfissis and tolerant cvs. Kalamon and Koroneiki. The percentages of detection of *V. dahliae* in the stems and roots of olive plants by real-time QPCR were correlated to the percentages of isolation of *V. dahliae* in the infected tissues. QPCR assay demonstrated the presence of a higher DNA amounts of D and ND pathotypes in susceptible than in tolerant cultivars. The D pathotype was present in three-, seven- and ninefold greater

than the ND pathotype in the stem, roots and shoots of cv. Amfissis respectively. The results of QPCR assay with symptom development and isolation of *V. dahliae* showed that D pathotype had higher level of virulence compared to the ND pathotype against cv. Amfissis. However, the tolerant cultivars showed similar responses to both pathotypes. Based on the amounts of *V. dahliae* biomass present, the levels of resistance of olive cultivars could be assessed (Marakakis et al. 2009).

Sclerotinia sclerotiorum, causing stem rot disease of oilseed rape (SROR) is a devastating pathogen with a wide host range of about 400 plant species. A pair of primers (SsF/SsR) was designed based on the sequences of a DNA region amplified by a microsatellite primer M 13. The primer generated a 252-bp DNA fragment from each of the 65 isolates of *S. sclerotiorum* from oilseed rapes collected at different locations and in different years, but not from any other fungal species (21) tested, indicating the specificity of the primer pair. For rapid detection of early infection on petals of oilseed rape, a real-time PCR assay was developed using this pair of primers used in the standard PCR format. Sensitivity tests revealed that the target fragment from 5 pg of pathogen DNA spiked with 2 ng of oilseed rape DNA. The real-time PCR could detect *S. sclerotiorum* in petals containing 0.0252–0.1111 ng/mg of petals. In order to eliminate PCR inhibitors, 2% polyvinyl pyrrolidone (PVPP) was added to the extraction buffer and the extracts were further purified using a commercial UNIQ gel extraction kit. The cost of purification was $0.18 only. This procedure provided a high quality template DNA for PCR amplification of the target DNA fragment (Yin et al. 2009).

Sheath blight disease caused by *Rhizoctonia solani* is one of the major diseases accounting for appreciable loss in yield and quality in rice. Visual examination in the early stages of infection does not provide reliable estimation of disease severity. Hence, a real-time, quantitative (Q) PCR format was developed to detect and quantify *R. solani* AG-1- IA DNA from infected rice plants. A specific primer based on ITS region of rDNA of the pathogen was designed. This protocol could detect quantities as low as 1 pg of pathogen DNA enabling reliable and specific detection of *R. solani* and quantification of fungal DNA and evaluation of levels of resistance of rice cultivars to sheath blight disease (Sayler and Yang 2007). Rhizoctonia root rot is one of the serious limiting factors affecting dryland cereal productions systems of the Pacific Northwest. A SYBR Green I-based real-time quantitative (Q)-PCR assay was developed for detection, identification and quantification of *Rhizoctonia solani* and *R. oryzae*, the principal causal agents of the disease. Primers specific to ITS1 and ITS2 of the nuclear rDNA of *R. solani* and *R. cerealis* were designed. The assays specifically detected *R. solani* AG-2-1, AG-8 and AG-10, three geneotypes of *R. oryzae* and an AG-I-like binucleate *Rhizoctonia* spp. The results were reproducible quantitatively at or below a cycle threshold (Ct) of 33 or 2–10 fg of mycelial DNA from cultured fungi, 200–500 fg of pathogen DNA from root extracts and 20–50 fg of pathogen DNA from soil extracts. The pathogens could be detected in all types of extracts at about 100-fold below the concentration required for quantification (Okubara et al. 2008).

Scorpion-PCR is based on a unimolecular mechanism in which the hybridization reaction occurs within the same strand. Unimolecular rearrangement is important

as the reaction is instantaneous and occurs prior to any competing or side reactions, such as target amplicon re-annealing or inappropriate target folding. This leads to stronger signals, a more reliable probe design, shorter reaction times and better discrimination (Thelwell et al. 2000). Primers designed based on the ITS regions of rDNA genes of *Rosellinia necatrix* were screened against two isolates of *R. necatrix* and six other *Rosellinia* spp. A single specific product from *R. necatrix* was obtained due to amplification by most of the primers. Two primer pairs (R2–R8 and R10–R7) confirmed their specificity, when tested against 72 isolates of *R. necatrix*. The R10 primer was modified to obtain a Scorpion primer for detecting a specific 112-bp amplicon by fluorescence emitted from a fluorophore in a self-probing PCR assay. This PCR format specifically recognized the target sequence of *R. necatrix* over a large number of other fungal species tested. The detection limit of Scorpion PCR was 1 pg/µl, whereas the nested-Scorpion PCR had a detection limit of 1 fg/µl which is ten times more sensitive than conventional PCR. A protocol for the extraction of DNA from the soil, suitable for PCR assays was also developed in this investigation. *R. necatrix* could be detected in the inoculated soil within 6 h (Schena et al. 2002).

Fuscoporia torulosa (= *Phellinus torulosus*) causes rotting of heartwood in roots and lower stems, killing cortical tissues of *Quercus ilex* and other forest tree species. Visual symptoms appear only in the late stages of infection. Isolation of the fungus and its identification by morphological characteristics are problematic. Hence, a reliable, rapid, sensitive and specific diagnostic method was considered essential to contain the disease spread and consequent losses due to *F. torulosa*. Scorpion-PCR assay was developed to detect and identify the pathogen *in planta*, using the primers designed on a highly polymorphic portion of the ITS region of the pathogen DNA. The specificity of primers and probe was assessed by means of both BLAST analyses and using genomic DNA from 131 *F. torulosa* isolates and 43 other fungi and oomycetes from different hosts and geographic areas. A pair of primers amplifying a 150-bp fragment (P1–P2) was employed to develop a real-time PCR detection method based on Scropion-PCR. The Scorpion-PCR reactions enhanced fluorescence from all isolates of *F. torulosa* indicating positive reactions, with no cross-reaction with nontarget microorganisms tested and with DNA from non-infected wood samples. This protocol detected amplification products up to 1 pg of target DNA, enabling the detection of *F. torulosa* in naturally infected plant tissues. Scorpion-PCR protocol was more sensitive and reliable with real-time PCR compared to conventional methods of isolation, since this technique detected the pathogen in 11 of 15 plants without fruit bodies, while the pathogen could be isolated from 5 samples only. Detection by Scorpion-PCR could be completed within 6 h as against several days required for isolation method (Campanile et al. 2008).

Analysis of historical samples preserved in archives is considered to be important, since such investigations provide evidence for the existence of infectious diseases affecting humans, animals and plants (Zink et al. 2002; May and Ristaino 2004). Wheat samples containing *Phaeospora nodorum* and *Mycosphaerella graminicola*, maintained in wheat archives since 1843 at Rothamsted were examined using PCR assays to understand the long-term dynamics of these pathogens.

Quantitative real-time PCR assays were employed to assess the amounts of *M. graminicola*, *P. nodorum* and wheat DNA present in a set of samples covering a 160-year period of wheat production. *M. graminicola* was present in high populations in the mid-ninetenth century, whereas *P. nodorum* DNA was more predominantly found for much of the twentieth century with a peak around 1970. The ratio of the DNA of *M. graminicola* and *P. nodorum* correlated well with the ratio of severity of the two Septoria diseases, determined from the survey data during 1970–2003 (Bearchell et al. 2005).

Barley net blotch pathogen *Pyrenophora teres* occurs in two forms, *P. teres* f. *teres* (PTT) and *P. teres* f. *maculata* (PTM), causing net blotch and spot type of symptoms respectively. In order to monitor and quantify their occurrence during barley growing season, a diagnostic system based on real-time PCR format was developed. TaqMan MGB (Minor Groove Binder) primers and probes were designed on the *RacB* gene sequences that exhibited high specificity for the two forms of the pathogen. The procedure was optimized on pure fungal DNA and on plasmid standard dilutions. Unknown samples were quantified by comparing Ct values with those obtained from plasmid standard dilutions, the detection limit being five gene copies/reaction. Quantitative data could be reliably obtained over a range of six orders of magnitude. Correlation ($R^2 = 0.52$) was observed between the Ct values and size of lesion areas in the early stages of infection. PTT form was found to be predominant on 20 barley cultivars during 2003 and 2004. The protocol has the potential for use in monitoring the dynamics of the two forms of *P. teres* during the growing seasons (Leisova et al. 2006).

Rhynchosporium secalis causes an economically important leaf blotch disease of barley in the United Kingdom. By employing the standard PCR assay, symptomless colonization of barley leaves by *R. secalis* could be demonstrated. A multiplex and real-time PCR assays were employed to detect and quantify the levels of colonization and infection of winter barley by *R. secalis*. The multiplex PCR assay was useful for detecting *R. secalis* in barley samples and for simultaneously checking the presence of PCR inhibitors. However, this assay could not be used for quantification of pathogen DNA. The sensitivity and specificity of PCR assays using different target genes were compared with assessment by visual examination. Three different real-time PCR assays (ß-tubulin TaqMan probe, cytochrome *b* SYBR Green I, and cytochrome *b* LNA probe assay) were highly specific in detecting *R. secalis*, as there was no fluorescence signal when other fungi were tested. The cytochrome *b* LNA probe assay was the most sensitive and it could detect as little as 0.1 pg of *R. secalis* DNA in the presence of 50 ng of barley leaf DNA. The detection thresholds for the cytochrome *b* SYBR Green I and ß-tubulin TaqMan probe assays were 1.0 and 3.0 pg of pathogen DNA respectively in the presence of 50 ng of barley DNA. The pathogen DNA contents were ~tenfold greater in susceptible cultivar. However, resistance ratings did not correlate well with levels of disease assessed by visual examination or pathogen biomass determined by real-time PCR assays (Fountaine et al. 2007).

Crown rust of oat caused by *Puccinia coronata* f.sp. *avenae* accounts for serious losses. A quantitative method was developed for detecting and quantifying the

fungal DNA (FDNA) in oat varieties showing different levels of resistance to the crown rust disease. This method includes simple inoculation application, quantitative sampling of inoculated areas, a closed tube DNA extraction method to restrict loss of plant tissue and real-time PCR assay using a pathogen-specific TaqMan primers/probe set which amplifes a 75-bp fragment only from *P. coronata* (PC) isolates. By employing this procedure, the template DNAs from *P. graminis* f.sp. *avenae* and *P. triticina* did not produce any amplification product, indicating the specificity of the TaqMan assay. This protocol could be applied not only for detecting the pathogen, but also for quantifying the FDNA which was correlated to the levels of resistance to the crown rust disease (Jackson et al. 2006) (Appendix 22).

Gremmeniella abietina and a *Phomopsis* sp. were frequently isolated from Norway spruce seedlings. The ITS rDNA sequence analysis and random amplified microsatellites profiling indicated that *G. abietina* strains belonged to the large tree type (LTT) ecotype of the European race of *G. abietina* var. *abietina. Phomopsis* sp. based on ITS rDNA sequence analysis was found to be different from other characterized *Phomopsis* spp. Pathogenicity tests showed that *G. abietina* alone was pathogenic. Real-time PCR assay was employed to detect and quantify *G. abietina* in Norway spruce seedlings exhibiting dieback symptoms. *Phomopsis* sp. might be a secondary colonizer of weakened twigs of nursery stock (Børja et al. 2006). *Phoma sclerotioides*, causative agent of alfalfa brown root rot disease, has also been recovered from the roots of winter wheat and perennial ryegrass plants, in addition to alfalfa. The ITS1, 5.8S and ITS2 of the rDNA of the isolates from alfalfa and wheat were identical and matched the sequences of *P. sclerotioides*. A real-time PCR with greater sensitivity of detection, compared with conventional PCR assay was developed. Alfalfa isolates of *P. sclerotioides* were pathogenic to wheat. The pathogen was detected from plant tissues and also from the soil (Larsen et al. 2007).

Phoma tracheiphila, causing a tracheomycotic disease of citrus called 'mal secco' induces the dieback of twigs and branches. This pathogen is of quarantine significance and the principal preventive measures that could be applied, continues to be early diagnosis to prevent the introduction and to limit the spread of the disease. *P. tracheiphila* infection frequently remains latent and an apparently healthy plant may exhibit all the symptoms suddenly and collapse emphasizing the need for rapid detection of the pathogens in symptomless plants. A specific primer pair and a dual-labeled fluorogenic probe were used in real-time PCR with the Cepheid Smart Cycler II System to detect the pathogen in the citrus plants. Conventional and real-time PCR formats successfully detected the pathogen in woody samples of naturally infected lemon and artificially inoculated sour orange seedlings. However, real-time PCR was about 10- to 20-fold more sensitive than the conventional PCR assay. In addition to detecting the pathogen, real-time PCR procedure may be applied for quarantine monitoring of pathogen biomass in infected plant tissues. Real-time PCR assay could detect *P. tracheiphila* in symptomless sections of lemon twigs from infected plants. Furthermore, this protocol is faster than the conventional PCR assay, because PAGE analysis of mycelial proteins requiring about 10 days is not required, thus saving considerable time (Licciardello et al. 2006).

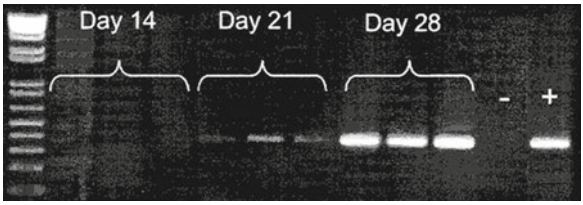

Fig. 2.9 Detection of *Phoma tracheiphila* in artificially inoculated *Citrus aurantium* seedlings by standard PCR assay. Note the presence of the amplification product of 378-bp detectable at 28 days after inoculation (Courtesy of Demontis et al. 2008 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

Rapid and reliable techniques for *in planta* specific detection and identification and absolute quantification of *Phoma tracheiphila* viz., the SYBR® Green I detection dye and a TaqMan hybridization probe were employed. These assays were tested on plant material from sour orange inoculated with *P. tracheiphila* and the results were compared with the classical isolation and plating method. The primers and hybridization probe were designed based on the sequences of the ITS nuclear rRNA genes. Detection and quantification of the pathogen was possible by both technologies, the detection limit being ten copies of the cloned target sequence and 15 pg of genomic DNA extracted from fungal spores. The presence of non-target fungal DNA did not affect the specificity of the assay, but reduced the sensitivity by tenfold. Real-time PCR was much faster and easier to perform compared to isolation method, in addition to the advantage of quantification of the pathogen biomass in the infected plant tissues (Fig. 2.9) (Demontis et al. 2008).

Tomato suffers heavily from the leaf mold disease caused by *Cladosporium fulvum* all over the world. Hence, a reliable, sensitive and rapid detection technique is necessary for effective management of this disease. The microsatellite DNA sequences are well distributed across genomes of the target pathogens and primers developed based on such sequences may be expected to be more sensitive than those developed from single copy genes such as ß-tubulin. The comparative efficacy of the PCR primers designed based on sequences of ß-tubulin gene, ITS regions and a microsatellite region of *C. fulvum* was assessed. The PCR primer pair CfF1/CfR1 from microsatellite DNA was 100-fold more sensitive than the primer pair CfF3/CfR3 from ß-tubulin sequences. The intensity of the band amplified by CfF1/CfR1 was stronger than that amplified by CfF2/CfR2 primer pair designed by using ITS sequence (Fig. 2.10). The most sensitive primer pair CfF1/CfR1 was used to develop a real-time PCR to detect *C. fulvum* in tomato leaves. The amount of *C.fulvum* DNA detected in tomato leaves ranged from 0.28 to 20.78 pg (Yan et al. 2008).

Anthracnose or crown rot disease of strawberry may be due to *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* in many countries. Three real-time PCR assays were developed for the specific detection, quantification and discrimination of *Colletotrichum* spp., *C. acutatum* and *C. gloeosporioides*, employing the primers and probes based on the sequences of most divergent area of ITS1, ITS2 and 5.8S rRNA gene region. A protocol for the extraction of DNA from plant

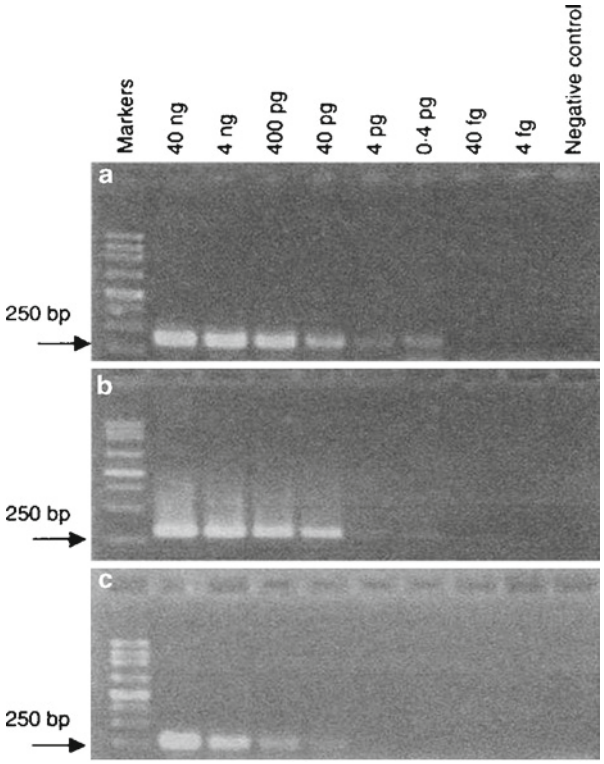

Fig. 2.10 Comparative sensitivity of primer pairs designed based on the sequences of three regions for the detection of *Cladosporium fulvum*. (**a**) CfF1/CfR1: sequences of microsatellite region, (**b**) CfF2/CfR2: sequences of ITS region and (**c**) ß-tubulin gene (Courtesy of Yan et al. 2008; Society of Applied Microbiology/Wiley-Blackwell, Oxford, UK)

materials was optimized for use in conjunction with the new real-time PCR assays. The real-time PCR formats allowed detection of these pathogens directly from plant materials. The sequences selected for designing primers and probes showed identity with homologous sequences from desired target organisms. The new assays were 10–100 times more sensitive than conventional PCR assay. The real-time PCR assays showed higher levels of sensitivity compared with ELISA method also, as evidenced by the positive results obtained in samples that were ELISA-negative. The development of *C. acutatum* was monitored using artificially infected strawberry crowns from two strawberry cultivars. The pathogen populations varied significantly by month ($p < 0.001$), but not by cultivar ($P = 0.394$). Diagnosis based on real-time PCR assays developed in this investigation provides rapid, sensitive and accurate results in 1–2 days, allowing high-throughput and inexpensive screening of plant samples directly (Garrido et al. 2009) (Appendix 23).

Puccinia horiana infecting *Chrysanthemum* × *morifolium* cultivars grown for cut- flowers, is one the most important pathogens of quarantine importance.

Standard, nested and real-time PCR formats were employed for the detection of *P. horiana* by using highly specific primer pairs that could amplify selected regions in the ITS1 and ITS2 of the nuclear rDNA. The relative sensitivities of the three PCR formats were reflected by the detection of 10 pg, 10 fg and 5 fg genomic DNA of the pathogen respectively. When the cloned target DNA was used as template, the detection limits for standard, nested and real-time PCR formats were 5,000, 50 and 5 target copies respectively. Presence of host plant DNA did not affect the detection limits of the assays performed. SYBR Green I technology enabled reliable real-time PCR signal detection, because of high specificity of the primers. The lowest proportion of infected plant material that could be detected in a mixture containing infected and healthy plant material was 0.001%. The detection limit of real-time PCR assay was as few as eight basidiospores of the pathogen. As the real-time PCR format is highly sensitive and specific giving reliable results, it may be useful for disease diagnosis as well as for disease forecasting programs (Alaei et al. 2009).

Anthracnose crown rot disease of strawberry may be due to *Colletotrichum fragariae* and *C. gloeosporioides* in many countries and *C. acutatum* may also be responsible in some geographical locations as California. Three real-time PCR (TaqMan®) assays were developed for the specific detection, quantification and discrimination of *Colletotrichum* spp., *C. acutatum* and *C. gloeosporioides*, employing the most divergent area of ITS1 and ITS2 and 5.8S rRNA gene region. A protocol for the extraction of DNA from the plant materials was optimized for use in conjunction with the new real-time PCR assays. These formats allowed detection of these pathogens directly from plant materials. The sequence selected for designing primers and probes showed identity with homologous sequences from desired target organisms. The new assays were 10–100 times more sensitive than conventional PCR procedure. The real-time PCR assays showed higher levels of sensitivity compared with ELISA method, because positive results were obtained using these assays in samples that were ELISA-negative. The development of *C. acutatum* was monitored using artificially infected strawberry crowns from two strawberry cultivars. The pathogen population varied significantly by month ($P < 0.001$), but not by cultivar ($P = 0.394$). Diagnosis based on real-time PCR provides a rapid, sensitive and accurate results in 1–2 days, allowing high throughput and inexpensive screening of plant samples directly (Garrido et al. 2009) (Appendix 23).

Aspergillus flavus infects peanut (groundnut) roots and kernels causing aflaroot disease. Infection of immature pods and later kernels during storage is more important, because of the presence of the mycotoxin produced by *A. flavus* and other species of *Aspergillus*. Further, *A. flavus* is an opportunistic human pathogen causing pulmonary mycosis in immunosuppressed individuals after inhalation of aerosolized spores. A real-time quantitative PCR (QPCR) assay was developed using three primers and probe sets Asp1S/ITS4 (forward primer pair), AflR2/AflR3 (reverse primer) and probe AflP (probe). The selected primers and probe set amplified all nine *A. flavus* isolates tested, including an aflatoxin-producing strains. The primers did not amplify DNA from 39 other fungal species including 18 other *Aspergillus* spp. and six *Penicillium* spp. tested. However, cross-reactivity with

A. oryzae was recorded. The assay was shown to be rapid giving the results within 1 h after DNA extraction (Cruz and Buttner 2008).

Grapevine downy mildew disease caused by a strictly biotrophic oomycete *Plasmopara viticola* has a worldwide distribution. A rapid high-throughput multiplex real-time quantitative PCR assay with TaqMan chemistry was developed. This method allows simultaneous amplification, but independent detection of pathogen DNA and host DNA is also possible, by using species-specific primers and TaqMan probes that are labeled with different fluorescent dyes. Inclusion of host DNA in the test provides an endogenous reference and allows normalization for variations caused by sample-to-sample differences in DNA extraction. This PCR assay has a detectable quantitative limit of 0.1 pg providing high precision, reliability and reproducibility (Valsesia et al. 2005).

Duplex real-time PCR assays were employed for the detection of obligate plant pathogens like the rust pathogens also. The sequence similarities and differences in the ITS1 region were useful in discriminating the DNA of these pathogens. Variable ITS1 region of nuclear rDNA gene was selected to differentiate rust pathogens *Puccinia graminis*, *P. striiformis*, *P. triticina* and *P. recondita.* The conserved 28S region as an internal control was used as to design the primer/probe sets. Species-specific ITS1 primers/probe sets were highly specific and capable of detecting as low as <1 pg of 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 speicies (Fig. 2.11). The rust fungi infecting pature grasses could be reliably identified by this PCR format. This report appears to be the first to describe detection and discrimination of four rust pathogens using real-time PCR assay (Barnes and Szabo 2007).

Bacterial DNA contamination of rust fungal DNA has been a problem during pathogen DNA sequencing. A quantitative real-time PCR (qPCR) format was developed to quantify bacterial DNA within rust fungus, *Puccinia graminis* DNA samples and the results were compared with those of conventional isolation method expressed in terms of CFU counts. Relatively higher values for bacterial contamination than CFU counts were obtained with qPCR assessments. However, the ranking of samples from low to high for bacterial contamination was consistent between the methods. When the known quantities of *Escherichia coli* DNA were spiked to *P. graminis* DNA, the contaminant was reliably quantified at ≥1.0% of total sample DNA. However, when the bacterial DNA contamination was <1%, the fungal DNA was also amplified occasionally. To overcome this problem, spiking the fungal samples with a known concentration of *E. coli* DNA was followed to eliminate the possibility of amplification of fungal DNA (Barnes and Szabo 2008).

Kinetic-PCR (kPCR) Assay

Lack of culturability of biotrophic pathogens demands certain additional efforts for their detection and differentiation.The kinetic-PCR (kPCR) has been successfully applied for the detection and quantification of *Melampsora* spp., causing poplar

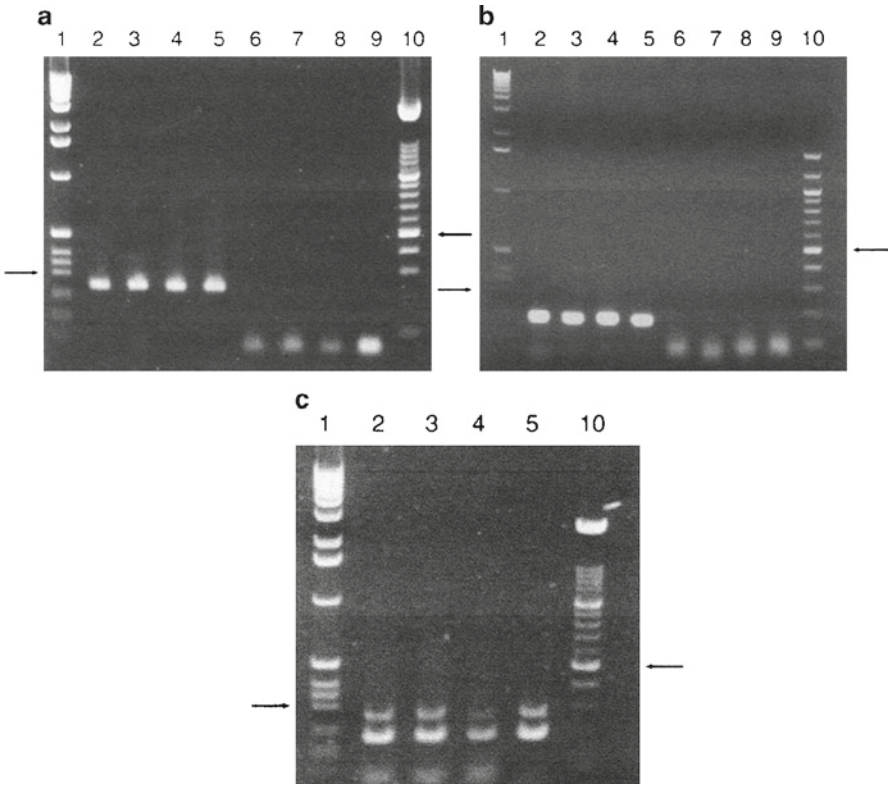

Fig. 2.11 Detection of cereal rust pathogens using PCR assay and employing different primer pairs (**a**) ITS1 region using ITS1rustFiod/ITS1rustR3c primer pair, (**b**) 28S region using StdLSUF5a/StdLSUR2 primer pair, (**c**) Duplex assay using both primer pairs. Lane 1: 1 kb MW marker; Lane 2: *Puccinia graminis*; Lane 3: *P. recondita*; Lane 4: *P. striiformis*; Lane 5: *P. triticina*; Lane 6: *Ustilago maydis*; Lane 7: *Fusarium graminearum*; Lane 8: *Triticum aestivum*; Lane 9: no template (control); Lane 10: 100 bp MW marker (Courtesy of Barnes and Szabo 2007; American Phytopathological Society, MN, USA)

rust disease in North America and Europe. The ability of kPCR to estimate the pathogen DNA precisely permits construction of growth curves that provide details of the pathogen infection that were not provided by the methods already available. Since DNA replication is intimately linked to cell division, quantification of pathogen DNA present in infected leaf tissues was determined by kPCR technique. Growth curves commencing from inoculation through the final stages of uredinial maturation, as well as pathogen monitorimg before symptoms are expressed, could be documented. The variations in the 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. kPCR procedure provides another advantage over conventional PCR by its ability to differentiate two rust pathogen species present in mixed infections. *Melampsora*

medusae f.sp. *deltoidae* and *M. larici-populina* were distinguished by using species-specific primers. Pathogen detection was not influenced significantly 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 different pathogens. This shows that kPCR provides the possibility of monitoring microorganisms in their environments – plants or soils (Boyle et al. 2005).

2.1.5.5 Amplified Fragment Length Polymorphism

Studies on population dynamics require more information over and above precise and rapid identification of the pathogen(s) concerned. Amplified fragment length polymorphism (AFLP)-fingerprinting technique was employed to detect and characterize pathogenic *Pythium* spp. and intraspecific populations. Diagnostic AFLP fingerprints of economically important pathogens *P. aphanidermatum*, *P. irregulare* and *P. ultimum* were determined, in addition to tentative fingerprints for six isolates of the described *Pythium* spp. (Table 2.8). Accurate identification of 29 isolates of the described *Pythium* spp. out of 48 blind samples confirmed the usefulness of the AFLP fingerprinting approach. Furthermore, five isolates of *P. ultimum*, misidentified based on morphological characteristics were assigned to their proper taxonomic

Table 2.8 Identification of *Pythium* spp. by AFLP fingerprints using selective primer combination (Garzón et al. 2005)

P. aphanidermatum[a]	*P. ultimum*[a]	*P. irregulare*[a]	Clade I	Clade II
55[b]	54	78	78	78
116	64	128	128	96
126	153	131	131	128
130	163	197	173	131
143	211	470	186	164
153	234		197	166
167	252		209	197
176	265		299	298
186	304		434	311
213	325		470	319
226	328			327
248	373			366
251	466			462
263				470
345				
373				
380				
406				
426				

[a]Species diagnostic fingerprints
[b]Values indicate fragment size in base pairs (+/–/bp)

positions, based on AFLP fingerprinting method (Garzón et al. 2005). *Pyrenophora teres*, causing net blotch disease in barley leaves was identified by AFLP fingerprinting technique. Specific primers were designed based on the sequences of AFLP fragments. The primers amplified the DNA from *P. teres* f. *teres* (net form), but not from the closely related *P. teres* f. *maculata* (spot form), indicating the specificity of detection and the potential of this protocol for distinguishing closely related pathogen species/strains. Form-specific PCR products were generated due to the high specificity of the primers (Leisova et al. 2005).

Eutypa lata, causing Eutypa dieback disease in grapevines could not be identified by applying a PCR-RFLP-based procedure (Rolshausen et al. 2004), because the universal ITS primers employed in that procedure are likely to amplify the DNA from other fungi, giving mulptiple PCR products. In order to rapidly detect the pathogen directly in the infected grapevine wood and to identify the pathogen precisely, sequence characterized amplified region (SCAR) primers were designed from the sequences of RAPD fragments. Primer pair Eut 02F3/Eut 02R2 derived from RAPD primer OPAM02 was tested with genomic DNA from 24 isolates of *E. lata* and 12 other fungi isolated from grapevine. DNA fragments of 643-bp were found in all *E. lata* isolates, but not in any other fungi tested, confirming the specificity of the primer used. The presence of the 643-bp PCR product was detected in grapevine canes inoculated with *E. lata*. Inconsistency was observed in PCR amplification of DNA extracted from infected canes following different protocols (Lardner et al. 2005).

Mango malformation disease occurring in tropical and subtropical areas is caused by the presumptive pathogens *Fuarium mangiferae*, *F. proliferatum*, *F. sacchari*, *F. sterilihyphosum* and *F. subglutinan*s. The isolates of *Fusarium* spp. were evaluated through analyses of AFLPs and DNA sequences of the genes encoding ß-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*). In the AFLP analysis, the Brazilian isolates formed a unique cluster. In addition, one small cluster was comprised of isolates of *F. sterilihyphosum* from Brazil and South Africa, whereas another cluster included isolates of *F. mangiferae* from Egypt, India, South Africa and USA (Lima et al. 2008). In a further study, AFLP and vegetative compatibility group (VCG) analyses were applied to identify genetic groups of causal agents of mango malformation disease. The isolates of *F. subglutinans* could be classified into six subgroups. *F. mangiferae* isolates were divided into two groups. Each AFLP group corresponded to a VCG group. By using AFLP banding patterns, isolates of *F. mangiferae* could be assigned to different VCGs. A correlation between AFLP and VCGs has been observed as in the case of several *formae speciales* of *F. oxysporum* (Lima et al. 2009).

2.1.5.6 Random Amplified Polymorphic DNA Technique

The random amplified polymorphic DNA (RAPD) technique is a PCR assay that uses arbitrary primers and it can be applied to differentiate races, strains and pathogenic or non-pathogenic isolates of fungi. The primers employed in this technique

are very short pieces (ten or fewer bases) of DNA from a known source. It is highly probable that these primers may be able to find some complementary sequences in the target DNA, producing a mixture of DNA fragments of various sizes. When the products from such a reaction are analysed by gel elelctrophoresis technique, distinct banding patterns are produced and some of these patterns may prove to be specific to certain species or varieties or strains. The patterns themselves may be useful for detection and diagnosis of some pathogenic fungi, but some of the bands in certain cases, may be cut out of a gel and sequenced to produce specific primers for more precise PCR analysis or probes for dot hybridization and other detection procedures.

The usefulness of RAPD technique for identification and classification of *Phytophthora* spp. infecting a wide range of plant species has been demonstrated. The fragments obtained from the products of RAPD-PCR amplification of *P. cinnamomi* DNA were tested for specific hybridization to *P. cinnamomi* DNA. The DNA fragments that hybridized specifically were cloned and could be employed for the detection of this pathogen (Dobrowolski and O'Brien 1993). Ten randomly chosen 10-mer primers were employed to study variations among 37 isolates of *P. citrophthora*, *P. parasitica*, *P. capsici*, *P. palmivora* and *P. meadii* from rubber and citrus trees and *P. colocasiae* from taro (yams). The RAPD profiles generally were similar within species of *Phytophthora* and were different between species. The pooled data from all primers showed that the isolates of each species clustered together forming six groups corresponding to the six morphological species included in this study. Further, the group corresponding to *P. citrophthora* could be subdivided into groups related to the host plant species susceptible to different subgroups and geographical locations from where they were collected. The RAPD analysis may be useful for confirmation and validation of classical taxonomic classification (Zheng and Ward 1998). The specificity of detection of *P. cactorum* infecting agricultural and ornamental crops as well as forest tree species was enhanced by designing a new pair of primers (PC1/PC2) derived from a specific RAPD generated fragment. These primers amplified a single product of ~450-bp only from *P. cactorum*, but not from the DNA of *P. pseudotsuge* or *P. idaei*. The detection limit for this assay was 6 pg of *P. cactorum* DNA extracted from the mycelium. The pathogen could also be detected in infected tissues of pear, potato, strawberry, tomato, pea and walnut trees (Causin et al. 2005) (Appendix 24).

RAPD analysis can provide markers to identify and differentiate microbial plant pathogens. Any band that appears to be unique to particular taxa may be labeled and tested for use as specific probes. Specific DNA bands were selected as probes from the RAPD profiles of 13 *formae speciales* of *Fusarium oxysporum*. The *formae specialis*-specific probe OPC18300c and OPC18520f were used to identify *Fusarium oxysporum* f.sp. *cucumerinum* (FOC) and *F. oxysporum* f.sp. *luffae* (FOL) infecting cucumber and *Luffa cylindrica* respectively by RAPD-PCR followed by dot blot hybridization. This procedure may be adopted for pathogen identification without the need for pathogenicity tests and the results can be obtained rapidly (Wang et al. 2001). *F. oxysporum* f.sp. *cucumerinum* (FOC) causes vascular wilt disease in cucumber as its unique host, whereas *F. oxysporum* f.sp.

radicis-cucumerinum (FORC) causes root rot and stem rot on multiple host plant species, in addition to cucumber (Vakalounakis 1996). A RAPD marker-based assay was developed to specifically identify and discriminate FOC and FORC from each other and other strains that are not pathogenic to cucumber. Based on RAPD markers that were identified, robust SCAR markers were developed that allowed specific detection of FOC and FORC, not only in infected plants, but also in the environmental samples like recirculating water and potting mix samples. There was no cross-reaction for FORC, but two cross-reactions for FOC marker were noted (Lievens et al. 2007).

Rhizoctonia solani has a wide host range and at least 13 distinct anastomosis groups (AGs) have been recognized in this pathogen species. *R. solani* AG-3 causes significant losses in potato in eastern Canada and the United States, necessitating rapid and reliable identification of this pathogen. By employing RAPD technique, specific genetic markers of AG-3 isolates were identified. RAPD amplification revealed the presence of a specific DNA fragment (2.6 kbp) in all AG-3 isolates. A PCR-based restriction mapping method was developed using the restriction enzyme *Xho*I, for the identification of AG-3 isolates. These virulent isolates could be rapidly detected both in infected plant tissues and infested soil samples (Bounou et al. 1999). *R. solani* AG-8 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). In addition, vegetatively compatible populations (VCPs) exist within each ZG with AG-8 (MacNish et al. 1997). RAPD-PCR technique was employed to determine relationships between four ZGs within AG-8. *R. solani* AG-8 isolates (79) representing four pectic isozyme groups (ZG 1-1, 1-2, 1-4 and 1-5) from different locations in southern Australia were analyzed by RAPD-PCR assay. Six primers were used to determine the relatedness of the isolates. The results indicate that AG-8 populations included four distinct groups that matched the four ZGs, lending support to the concept that ZGs are distinct intraspecific groups (MacNish and O'Brien 2005).

Detection and identification of *Elsinoe fawcetti* (*Ef*) and *E. australis* (*Ea*), causative agents of citrus scab and sweet orange scab diseases respectively, based on isolation and study of morphological characteristics, is difficult. Specific primer sets Efaw-1 for Ef, Eaut-1, Eaut-2, Eaut-e and Eaut for *Ea* and Ea Nat-1 and Ea Nat-2 for the natudaidai pathotypes with *Ea* were employed for their detection and differentiation by using RAPD products unique to each species or pathotype. Likewise, the *Ef* isolates from Korea, Australia and USA (Florida) were identified by using Efaw-1. Efaw-2 primer sets for identification of both sweet orange pathotype isolates from Korea. Efaw-1 and Efaw-2 primer sets were able to identify *Ef* present in lesions on leaves as well as on the fruits (Hyun et al. 2007).

2.1.5.7 DNA Sequence Analysis

Morphological characteristics of the test fungal pathogens and sequences of segments of DNA have been used to establish their identity, as in the case of *Phytophthora*

cinnamomi isolated from diseased roots and runners of cultivated cranberry (*Vaccinium macrocarpon*). Amplification of pathogen DNA containing the complete ITS1, 5.8S rRNA gene and ITS2 was carried out by PCR, using universal ITS primers 1 and 4. Amplified fragments were separated by agarose electrophoresis and purified using spin columns. After ligating to appropriate plasmid vector, sequencing was performed. All *P. cinnamomi* isolates (6) tested had identical ITS region sequences. The isolates identified as *P. cinnamomi* exhibited all morphological traits attributed to this species. Isolates tentatively identified as *P. megasperma* (*Phytophthora* taxon cranberry) also had identical ITS region sequences (Polashock et al. 2005).

Mango malformation disease (MMD) was reported first in India and later in several countries in Asia and Americas. *Fusarium subglutinans* and *F. mangiferae* have been shown to be primarily responsible for this disease. Based on the nuclear and mitochondrial DNA sequences, *F. mangiferae* was established as a new species and found to be phylogenetically related to *F. fujikuroi* and *F. sacchari* (Marasas et al. 2006). The combination of morphological and molecular characters such as conidial morphology and ITS rDNA sequence data was considered for the identification of *Pyricularia* spp. The potential of rDNA sequences in the analysis of anamorph–teleopmorph relationships at generic level or using sequence analysis of rDNA combined with PCR-fingerprinting to prove the connection between anamorph species and an ascomycete has been indicated. The ITS region containing ITS1 region, 5.8S gene and ITS2 region were amplified from all *Pyricularia* and related species. No sequence variation was detected within species and variation was low among species within the genus. Molecular studies suggested that *Pyricularia* spp. isolated from different hosts are genetically distinct. This study, based on the comparison of both morphological and molecular characters, concluded that conidial shape may be used as a primary character to distinguish *Pyricularia* spp. and *Dactylaria* spp. (Bussaban et al. 2005). Likewise, *Botryodiplodia theobromae*, causing gummosis disease of *Jatropha podagrica* in China was identified based on morphological characteristics. The rDNA sequence of the ITS region of the pathogen completely matched with the ITS sequence of *B. rhodina* registered in GenBank. The identity of the pathogen has to be conclusively established (Fu et al. 2007).

Botrytis cinerea has a wide range including several crops that show appreciable loss due to the gray mold disease induced by this pathogen. Another fungal species *B. mali* has also been reported to cause gray mold in apple along with *B. cinerea.* DNA sequence analyses of the ß-tubulin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) genes were used to examine the relationship between *Botrytis* isolates causing apple decay. The combined G3PDH and ß-tubulin sequence data support the view that *B. mali* is a unique species, phylogenetically distinct from *B. cinerea. B. mali* has been possibly misidentified and overlooked during the collection of isolates from different locations (O'Gorman et al. 2008). In the attempt to establish the cause(s) of perennial cankers and consequent dieback of grapevines, two *Botryosphaeria* spp., *Lasiodiplodia theobromae* and *Diplodia seriata* were isolated from infected wood. In addition to morphological and cultural

characteristics of these fungi, nucleotide sequences of three genes, the ITS region (ITS1-5.8S-ITS2), partial sequences of ß-tubulin gene and part of the translation elongation factor 1-α gene (EF 1-α) were determined. *L. theobromae* and *D. seriata* were found to be pathogenic on inoculation to rooted cuttings and green shoots of susceptible grapevine cultivars. *L. theobromae* isolates were more virulent than isolates of *D. seriata*, confirming that these two were the causative agents of die-back and canker formation of grapevines, while the sequence information was useful to identify these pathogens reliably and rapidly (Úrbes-Torres et al. 2008).

The anthracnose pathogen infecting Norway maple (*Acer platanoides*) was determined to be *Glomerella* sp., the teleomorph of *Colletotrichum acutatum*. In order to determine the taxonomic position of the pathogen isolated from Norway maple, morphological and molecular analysis was carried out. This pathogen was found to be phylogenetically related to the *C. acutatum* clade that includes *C. acutatum*, *C. lupine*, *C. phormii*, *C. acutatum* f.sp. *pineum* and *Glomerella miyabeana* based on the ITS and LSU DNA sequences. The consistent production of perithecia on the infected leaves and in the culture was an unusual feature that is not seen in other pathogens causing anthracnose diseases (LoBuglio and Pfister 2008).

Microsatellites or simple sequence repeats (SSRs) have been used as molecular markers in the recent years, as they are multiallelic, codominant and highly polymorphic. The microsatellites have been used to investigate the genetic structure and reproductive biology of fungal pathogens such as *Venturia inaequalis* (Tenzer et al. 1999), *Colletotrichum* spp. (Urena-Padilla et al. 2002) and *Phytophthora* spp. (Dobrowolski et al. 2003). Microsatellites specific for *Phytophthora ramorum* were employed to distinguish European and North American isolates of this pathogen (Prospero et al. 2004). Microsatellite markers (12) from the whole genome sequence data of *P. ramorum* were developed, optimized and used in conjunction with mtDNA *coxI* sequences to characterize 71 isolates and 80 isolates respectively from the United States and nine European countries. The combined microsatellite, sequencing and morphological analyses suggest that three clades represent distinct evolutionary lineages. All three clades were identified in some US nurseries, emphasizing the role of commercial plant trade in the movement of *P. ramorum* (Ivors et al. 2006).

The cost-effectiveness of diagnostic technique is a crucial determining factor for wide and routine application. Hence, microsatellite-based method may not be preferred, as the development of these markers is both expensive and laborious. A novel strategy was developed to reduce the cost of developing SSRs substantially (to one half of SSR-based technique). This method is based on anchored PCR in which microsatellite amplification is achieved with one primer complementary to the flanking sequence and one that is specific to the repeat motif (Hayden et al. 2004). Markers amplified by anchored PCR are designated sequence tagged microsatellites (STMs). They are generally developed using selectively amplified microsatellite (SAM) analysis and sequence tagged microsatellite profiling (Hayden et al. 2006). A rapid and cost-effective technique was developed for generating 168 sequence tagged satellites (STMs) in the barley scald pathogen, *Rhynchosporium secalis.* Anchored PCR was employed to directly generate a DNA library higly enriched for microsatellite clones

which was screened by colony PCR to identify uniquely sized SSR fragments. With this approach, it was possible to reduce the DNA sequencing efforts required to select SSR clones suitable for primer design. Furthermore, the need to design a specific flanking on only one side of the microsatellite repeat halved the amount of primer synthesis compared to conventional SSRs which require a pair of primers flanking the repeat. Sixty two STMs amplifying 66 loci, indicated a high level of polymorphism among a diverse set of 16 Australian isolates. The STMs developed in this study, reduced the cost of performing fluorescence-based microsatellite assays (Keipfer et al. 2006).

Barley net blotch diseases are caused by two forms of *Pyrenophora teres* (anamorph – *Drechslera teres*) which are differentiated by the types of lesions i.e., spot due to *P. teres* f. *maculata* and by net due to *P. teres* f. *teres* formed on leaves and leaf sheaths. The genetic distinctiveness of these two forms has been established. But it is difficult to differentiate them based on the symptoms and conidial morphology. Microsatellite (simple sequence repeat, SSR) markers (80) for two barley *P. teres* forms were developed (Keipfer et al. 2007), using a modified sequence tagged microsatellite profiling technique developed for organisms with small genomes, like *Rhynchosporium secalis* (Keipfer et al. 2006). The SSRs were evaluated using six randomly selected single-spore isolates of each *P. teres* form. A total of 67 SSR loci were amplified with 26 polymorphic loci revealed in one or both *P. teres* forms (Keiper et al. 2007). In a further study, nine SSRs were found to amplify loci in one *P. teres* form only, when tested on the same set of six field-sampled isolates derived from single spores of each *P. teres* form. Loci with different allele size ranges in each form or different numbers of loci in each form were identified. These SSRs have the potential for application as diagnostic markers to detect and differentiate *P. teres s*pot and net forms (Keiper et al. 2008).

2.1.5.8 Molecular Beacon Technology

Molecular beacon technology first developed by Tyagi and Kramer (1996) involves the use of single-stranded oligonucleotides that exist in a hairpin conformation as molecular beacons (probes). The stem portion consists of complementary sequences at the 5′ and 3′ terminals of molecule, while the loop portion contains probe sequences that are complementary to the target sequence. A fluorescent moiety (fluorophore) is attached to one end, whereas a quenching moiety (quencher) is attached at the opposite end. When the RT-PCR is carried out with primers that amplify specific genome sequences of interest, targets complementry to their respective molecular beacons are produced for subsequent detection. Once the beacon hybridizes with the target, the fluorophore and quencher are separated. As the hairpin structure is disrupted, fluorescence becomes detectable. The fluorescence intensity is continuously monitored during the entire period. When the beacon is not bound to the target, the hairpin structure positions the fluorophore and the quencher in close proximity. The fluorescence emitted from the fluorophore is quenched by Förster fluorescence resonance energy transfer (FRET) via the

quencher due to the close proximity to each other. Fluorescence signals are emitted only upon hybridization with the target nucleic acids, indicating positive reaction. In contrast, unhybridized molecular beacons do not fluoresce revealing the absence of target molecules and consequent negative reaction (Eun and Wong 2000).

The molecular beacon technique was demonstrated to be effective for detection of *Phytophthora fragariae* infecting strawberry. A Molecular Beacon™ with a central region complementary to the target amplicon and a 607-bp sequence labeled with a quencher at one end and a fluorescent dye at the other end, was employed to detect *P. fragariae* amplicons in a quantitative manner similar to that of TaqMan™. A linear relationship with dilutions from 100 attogram (ag) to 1 pg was observed. The probe detected amplicons in samples with as little as 100 ag of genomic cDNA and as few as 25 zoospores of *P. fragariae*, in addition to pathogen DNA in water samples. Both Molecular Beacon™ and TaqMan™ could provide "real-time" measurements in a closed-tube system on the ABI 7700 and quantitative determination of the pathogen DNA was possible. As this pathogen is under strict surveillance with 'nil tolerance', quantification of the pathogen population by this technique does not offer any additional advantage (Bonants et al. 2004).

2.1.5.9 Single-Strand Conformation Polymorphism Analysis

Evaluation of 11 reported diagnostic techniques for the detection of *Phytophthora ramorum* was taken up in 7 laboratories. The diagnostic protocols employed conventional (based on ITS and cytochrome oxidase gene *cox1* and *cox2* spacer regions) and real-time PCR (based on ITS and *cox1* and *cox2* spacer regions as well as ß-tubulin and elicitin genes). Single-strand conformation polymorphism (SSCP) analysis using an automated sequencer for data collection was also evaluated for identification of all *Phytophthora* spp. tested. Different protocols exhibited varying levels of specificity. However, with few exceptions, all assays correctly identified all isolates of *P. ramorum* and low levels of false negatives were noted for the mitochondrial *cox* spacer markers. Most of the real-time assays based on nuclear markers exhibited a diagnostic specificity between 96% and 100%. The SSCP analysis accurately identified *P. ramorum* and it could be applied for appropriate classification of a number of isolates to the species level. The SSCP analysis could identify 8 of 11 *Phytophthora* spp. other than *P. ramorum* (Martin et al. 2009).

2.1.5.10 Padlock Probes – Multiple Detection System

A detection technique that has the potential for simultaneous detection of several pathogens infecting the same plant species is preferred over other methods that can detect only one pathogen at a time. Generally, multiplex strategies involve either amplification with generic primers that target a genomic region containing species-specific information or multiple primer sets. Padlock probes (PLPs) have the ability to combine pathogen-specific molecular recognition and universal amplification,

thereby providing enhanced sensitivity and multiplexing capabilities without affecting the range of potential target pathogens. PLPs are long oligonucleotides of ~100 bases containing target complementary regions at their 5′ and 3′ ends. These regions can recognize adjacent sequences on the target DNA. The universal primer sites and a unique sequence identifier known as ZipCode are placed in between these segments. The end of the probes move into adjacent position and can be joined by enzymatic ligation. Only if both end segments recognized their target sequences correctly, a circular molelcule can be formed. Non-circularized probes are removed by exonuclease treatment, whereas the circularized probes may be amplified by using universal primers. Then the target-specific products can be detected by a universal complementary ZipCode (cZipCode) microarray. The PLPs have been shown to possess high level of specificity and multiplexing capacity. PLPs enable the development of flexible and extendable diagnostic systems, targeting diverse organisms.

A detection system was developed on PLP and microarray for ten economically important plant pathogens including oomycetes (*Phytophthora* spp. and *Pythium* spp.), fungi (*Rhizoctonia* sp., *Fusarium* spp. and *Verticillium* spp.) and a nematode (*Meloidogyne* sp.). Specific PLPs were designed and characterized to target ITS sequences of rRNA operons of these pathogens. The genomic DNA of the test fungal pathogen was fragmented by digestion using restriction enzymes *Eco*RI, *Hind*III and *Bam*HI and used as template. Cycled ligation was carried out in the reaction mixture containing *Taq* ligase. Amplification of ligated PLPs was followed by real-time PCR using an ABI Prism 7700 Sequence Detector System (Applied Biosystems) and the PCR Kit (Eurogentec). The pathogens detected were *Phytophthora cactorum*, *P. infestans*, *P. nicotianae*, *P. sojae*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Myrothecium roridum*, *Pythium ultimum* and *Meloidogyne hapla.* The detection limit of the assays was 5 pg of pathogen DNA (Szemes et al. 2005).

2.1.5.11 DNA Array Technology

DNA array technology was first developed to screen for human genetic disorders (Saiki et al. 1989; Kawasaki and Chehab 1994). DNA arrays and chips have been demonstrated to be powerful tools for gene expression profiling and it can be efficiently applied for the identification and differentiation of microorganisms, including plant pathogenic oomycetes, fungi, bacteria and viruses. Common assay systems such as microplates or standard blotting membranes may be used in arrays which can be created by hand or by using robotics to deposit the samples. Generally arrays may be of two kinds viz., macroarrays and microarrays. Macroarrays contain sample spot sizes of about 300 μm in diameter or larger and they can be easily imaged by existing gel or blot scanners. The membrane-based arrays or filter arrays belong to this category. On the other hand, microarrays have spot sizes less than 200 μm in diameter and they can accommodate thousands of spots. Specialized robotics and imaging equipment are required for microarray system (Shi et al. 2003).

DNA array technology is essentially a reverse dot blot technique useful for characterization of DNA fragments. This is the only currently available technology that enables detection and identification of many microbial pathogens present in a substrate (plants, soil, water or air) in one assay (Lievens et al. 2003; Lievens and Thomma 2005). Two general classes of DNA matrices for hybridization-based array analysis are commonly used. They are membrane-based arrays, where a matrix of specific DNA is bound to a flexible membrane such as nylon and the second one involves the use of higher density chips on a rigid support such as silicon or glass. The detector oligonucleotides are immobilized on a solid support and used for detecting the target pathogen. PCR amplified target DNA is labeled using consensus primers spanning a genomic region harboring specific sequences of the test pathogen. Then the labeled PCR products are hybridized to the array. Theoretically an unlimited number of pathogens may be identified, using a single PCR, provided that sufficient polymorphism(s) exist within the genomic target region. High discriminatory potential associated with immobilized detector oligonucleotides has been found to be crucial for diagnostic applications, since closely related pathogen species may differ in only a single base pair (single nucleotide polymorphism (SNP)) for a target gene. The SNP profiles of fungal pathogens have been used as the basis of detection of large number of pathogens simultaneously.

DNA array technology has been applied for the detection of tomato wilt pathogens *Fusarium oxysporum* and root and foot rot pathogen *Phytophthora infestans* (Lievens et al. 2003, 2004) and *Fusarium* spp. in cereal grains (Nicolaisen et al. 2005). The sequence information for *Synchytrium endobioticum*, causative agent of potato wart disease, is not available. Hence, the 18S rDNA sequences were determined by extracting the DNA from resting spore from infected potato tubers. As the sequences of 18S rDNA of different *Synchytrium* spp. were conserved and showed homology, specific oligonucleotide probes were designed and arrayed onto glass slides for detecting the pathogen. Probes specific for detection of the viruses and viroids infecting potatoes were designed based on the nucleic acid sequences. Total RNA from infected plants was reverse-transcribed, labeled with Cyanine 5 and hybridized with the microarray. An appreciable number of the oligonucleotide probes exhibited high specificity in detecting *S. endobioticum* and viruses. The results revealed the great potential of microarray-based hybridization for detection and identification of several pathogen targets simultaneously. Such a possibility of simultaneous detection of different kinds of pathogens (fungi, bacteria and viruses) will be able to provide the required edge for quarantine and certification agencies to deal with plants and propagative material effectively (Abdullah et al. 2005).

The oomycetes and fungi *Phytophthora nicotianae*, *Pythium ultimum*, *Fusarium oxysporum* f.sp. *lycopersici* and *Verticillium dahliae* were isolated in pure cultures and the perfect match oligonucleotides were selected from either ITS1 or ITS2 sequences from which the detector sequences with a melting temperature of 55°C ± 5°C were obtained by adjusting the length of these oligonucleotides. When only a single nucleotide was substituted, mismatches at the fifth nucleotide were the most selective for *P. ultimum*, allowing SNP discrimination irrespective of amplicon amounts of the nucleotide used in substitution. Similar selectivity of a specific SNP

oligonucleotide depending on its sequence was observed in *P. nicotianae*, *F. oxysporum* f.sp. *lycopersici* and *V. dahliae.* The results showed that high specificity can be obtained with DNA arrays that allow discrimination of single nucleotide sequences and hence, closely related microorganisms can be differentiated. It is suggested that multiple nucleotides harboring the unique polymorphism at different positions may be employed to differentiate SNPs (Lievens et al. 2006).

Various species (>100) of *Pythium* were detected and identified using a DNA array containing 172 oligonucleotides complementary to specific diagnostic regions of the ITS. Positive hybridization reaction with at least one corresponding species-specific oligonucleotide was recorded in all except *P. ostracodes*. Hybridization patterns were distinct for each species and also for strains of each species tested. Further, hybridization patterns were consistent with the identification of the isolates based on morphological characteristics and ITS sequence analyses. DNA array technique detected 13 species of *Pythium*, the identity of which was corroborated by soil dilution method. Identification of soilborne fungal pathogens, using DNA arrays, could be a major step forward for conducting complex epidemiological and ecological investigations (Tambong et al. 2006).

A combination of low-density arrays and a single universal PCR for identification of target sequences was utilized for the development of a DNA microarray protocol. The presence of biotinylated nucleotides in the PCR amplification facilitates detection of amplicons on the capture probes of microarray. Fourteen *Fusarium* spp. capable of producing trichothecene and moniliformin, were detected by the microarray procedure, employing capture probes corresponding to the sequences of the translation elongation factor-1-α (TEF-1-α). A consensus PCR amplification of a part of the TEf-1-α was followed by hybridization to the *Fusarium* chip and the results could be visualized by a colorimetric silverquant detection method. The detection limit of this protocol was 16 copies of genomic DNA, providing the results with 1–2 days, while conventional isolation method required 7–21 days (Kristensen et al. 2007).

Robust SCAR markers were developed based on the RAPD markers. These markers could be employed for specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* (FOC) and *F. oxysporum* f.sp. *radicis-cucumerinum* (FORC) in plant tissues. By implementing these markers in a DNA macroarray, multiple pathogens belonging to the genus *Fusarium* and the species of *F. oxysporum* were detected simultaneously with high level of sensitivity. Three plant samples out of five tested were diagnosed with FORC and two with FOC. Plating on selective medium and examining morphological characteristics confirmed the identity of the pathogens, as determined by DNA macroarray (Lievens et al. 2007). *Fusarium solani* species complex (FSSC) includes morphologically similar, but more than distinct 45 lineages. They were chosen as targets for the development of a macroarray detection system that can be adapted broadly. Oligonucleotides (17–27-mers) were designed from the ITS of the rDNA genes of 17 FSSC isolates that belong to 12 phylogenetically closely related species. Of the 33 oligonucleotides on the array, 21 were able to discriminate all 12 species, some of which had only a single nucleotide difference among them. High specificity of the array system was found to be

due to optimization of the hybridization temperature and oligo probe length which had more substantial effect on the array performance. The array was validated by testing samples from inoculated and field-infected plants (Zhang et al. 2007).

2.1.5.12 Ligation-Based Probe Assay

The multiplex quantitative analyses available earlier, were found to suffer from compromises between the level of multiplexing, throughput and accuracy of quantification. A new ligation-based probe assay employing Plant Research International (PRI)-lock probes has been developed to bridge the gap between high-throughput and multiplex pathogen quantification. Padlock probes developed earlier by Szemes et al. (2005) could not be used for quantification of pathogen DNA. The circularized probes, developed in the later investigation are amplified by using probe-unique primer pairs via real-time PCR, enabling accurate target quantification in a highly multiplex format. These PRI-lock probes are long oligonucleotides with target complementary regions at their 5′ and 3′ends. When the target hybridization is perfectly completed, the PRI-lock probes are circularized via enzymatic ligation, subsequently serving as template for individual, standardized amplification via unique probe-specific probes. High-throughput real-time amplification is accomplished by adaptation to OpenArray™ which can accommodate up to 3,072 reactions/assay. The OpenArray™ has 48 subarrays, allowing parallel testing of up to 48 samples and each subarray contains 64 microscopic through-holes of 33 nl volumes. This assay combines the multiplex capabilities and specificity of ligation reactions with high-throughput real-time PCR, resuting in a flexible, quantitative multiplex diagnostic system.

The PRI-lock probes were designed to detect several economically important plant pathogens at different taxonomic levels – oomycetes, fungi, bacteria and a nematode species. Each of the 13 PRI-lock probes was designed with unique primer building sites allowing quantitative detection. The PRI-lock probes were engineered with a desthibiotin moiety between the primer sites for reversible PRI-lock probe capture, washing and release using streptavidin-coated magnetic beads. This additional purification step effectively removes excess nontarget DNAs and possible enzyme-inhibiting compounds, leading to enhancement of exonuclease activity and consequent reduction in assay background. The OpenArray™ system was applied successfully for the simultaneous detection and quantification of *Phytophthora* spp., *P. infestans*, *Rhizoctonia solani* (AG 2–2, AG 4–1 and AG 4–2), *Fusarium oxysporum*, *Myrothecium roridum*, *Verticillium dahliae*, *V. albo-atrum*, *Erwinia carotovora*, *Agrobacterium tumefaciens* and *Meloidogyne hapla.* The sensitivities of detection of the pathogens were between 10^3 and 10^4 target copies/µl of initial ligation mixture, depending on the PRI-lock probe. Pathogen quantification was equally robust in single target versus mixed target assays. The OpenArray™ system using PRI-lock probes enable very specific, high-throughput, quantitative detection of multiple pathogens over a wide range of target concentrations (van Doorn et al. 2007).

2.1.5.13 Tandem Mass Spectrometry

Mass spectrometry (MS) may be useful to detect a wide variety of characteristic molecules such as lipids, phospholipids, carbohydrates or metabolites. But proteins are well-suited as highly specific biomarkers for detection by MS, since they confer indirect species-specific genetic information in their sequences. Specific proteins and their post-translational products identified by MS or MS/MS have been found to be excellent indicators of the presence of animal and plant viruses and bacterial pathogens. The MS/MS detection of distinct peptides belonging to the coat protein of *Potato virus X* revealed the potential of MS/MS methods to determine the unknown cause of a disease effectively (Cooper et al. 2003). Unlike PCR or ELISA, the liquid chromatography (LC)-tandem mass spectrometry (MS/MS) does not require pathogen specific reagents for the detection of pathogen specific-proteins and peptides. The tandem mass spectrometry (MS/MS) can be used to identify peptides and proteins from complex fungal pathogens. The characteristic proteins were successfully detected from the target pathogens *Ustilago maydis*, *Phytophthora sojae*, *Fusarium graminearum* and *Rhizoctonia solani* (Padliya et al. 2007). This approach may find applications for the detection of plant pathogens whose genomics are available in public.

2.2 Detection of Fungal Pathogens in Seeds and Planting Materials

Seeds and propagative plant materials such as setts, cuttings, tubers and corms, when infected by microbial plant pathogens, become the primary sources of inoculum capable of introducing the disease(s) into a new location or field where the incidence of the disease(s) may be limited or absent. Hence, detection of the fungal pathogens in the true seeds and vegetatively propagated plant materials is considered as an important component of integrated disease management systems (Agarwal and Sinclair1996; Maude 1996). Detection of the pathogens is required because of specific restrictions imposed by respective governments to protect domestic crop production from pathogens likely to be introduced through imported plant materials. Quarantine, indexing and certification programs are in operation in various countries to examine different kinds of plants and planting materials for freedom from diseases and pests. The detection techniques applied for testing the seeds and vegetatively propagated plant materials may be of two types viz., traditional methods and modern molecular methods. Traditional methods comprise of visual inspection for disease symptoms, microscopic examination and culturing the pathogen in culture media. Blotter test, growing-on test and direct plating of seeds on agar media are methods applied for assessing the extent of seed health. Bait tests are useful for the detection of fungal pathogens infecting roots of susceptible crop plants. The modern methods include various immunological assays and nucleic acid-based molecular techniques, the results of which are not affected by the environmental conditions to which the plants/plant materials are exposed prior to testing.

2.2.1 Detection of Fungal Pathogens in Seeds

2.2.1.1 Traditional Methods of Detection of Seedborne Fungal Pathogens

The International Seed Testing Association (ISTA) has published a Handbook on Seed Health Testing containing work sheets (each with a specific ISTA number) with description of methods for detecting seedborne fungi, bacteria and viruses (ISTA 1994). Langerak et al. (1996) have listed the fungal, bacterial and viral pathogens that can be detected in seeds of various crops. The International Seed Health Initiative (ISHI) has been established to coordinate the production and supply of healthy seeds of vegetable crops. Status reports of ISHI to focus the efforts based on the importance of different pathogens transmitted through seeds are made available to the cooperating agencies (Meijerink 1997).

Dry Seed Examination

Dry seeds are examined for the presence of admixtures such as ergot sclerotia, smutted kernels, discolored and shriveled seeds and free insects. A stereoscopic microscope or an illuminated swinging-arm desk magnifier of ×2 magnification can be used for examining samples of generally 400 or more seeds. Fungal fructifications such as pycnidia, acervuli and smut sori can be detected. In the case of some pathogens, incubation of seeds under high humidity conditions may be required to induce the pathogen present in the seeds to sporulate, facilitating detection of seed infection by such pathogens.

Seed Washing Test

The dry examination of seeds may not be useful to detect the presence of certain fungal pathogens, even after incubation. In such cases, a sedimentation or seed washing test has to be adopted for detecting spores of pathogens causing downy mildew, rust and smut diseases. Seed samples (50 seeds) are placed in test tubes containing distilled water (10 ml) and a few drops (10–20) of 95% ethyl alcohol or a detergent. The sample tubes are agitated in a mechanical shaker for 10 min. The aqueous suspension is then centrifuged at 3,000 rpm for 10 min. The supernatant is poured off and the pellet is resuspended in 2 ml of sterile water. Spores or fungal structures present in the suspension can be viewed by examining a few drops of the suspension under the light microscope.

Blotter Test

In the standard blotter test, seeds are sown in petridishes containing three layers of moistened absorbing (blotting) paper. Five to ten seeds (depending on the seed size) are

placed equidistant from one another in each petridish and incubated at 22°C ± 2°C under near ultraviolet (NUV) light with alternate cycles of 12 h of light and darkness for 7 days. The seeds are then examined under a stereomicroscope for the presence of fungal colonies and their characteristics are recorded for identifying the pathogen. The pictorial guide prepared by Ahmed and Ravinder Reddy (1993) is very useful in the identification of seedborne fungi of cereals and legumes.

The seeds have to be surface-sterilized, if the internally seedborne pathogens are to be detected. They may be immersed in a NaOCl solution containing 1% chlorine for 10 min or in a 1.7% NaOCl solution for 1 min followed by immersion in 70% ethanol for 1 min (ISTA 1966). Germination of seeds may be retarded by wetting the blotter paper with 0.1–0.2% solution of the herbicide 2,4-D. This procedure has been employed for the detection of *Leptospheria maculans* (anamorph- *Phoma lingam*) in crucifer seeds (Hewett 1977; Maguire and Gabrielson 1983) and for routine testing of common bean and soybean seeds (Dhingra et al. 1978). Use of 2,4-D is replaced by freezing the seeds for the detection of *Alternaria radicina* in carrot seeds (Pryor et al. 1994). Blotter test results may be inconclusive, if characteristic sporulation does not occur, requiring the use of time-consuming plant inoculation tests for confirmation.

The standard moistened blotter method (SBM) and deep-freezing blotter method (DFB) recommended by ISTA were found to be ineffective for detecting lurked pathogens on fenugreek. Hence, an improved method of seed health testing was developed by soaking blotting paper in solutions of NaOH (0.3 M) and KOH (0.2 M), instead of using tap water for moistening the blotting papers. The alkali treatments resulted in the detection of *Verticillium dahliae* at 6.5–7.5% as against 0.3% in SBM and 1.0% in DFB procedures. In addition, some more pathogens were also detected more efficiently by this new procedure. Stimulation of the growth of *Fusarium semitectum* and *Curvularia* sp. by NaOH was also observed, when this new procedure was followed (Elwakil and Ghoneem 2002). In a further study using the alkaline blotter method (ABM), blotter disks treated with NaOH (0.8%) or KOH (0.4%) were used to detect the seedborne fungi of peanut. *Cephalosporium* sp. and *Verticillium* sp. that were difficult to isolate because of their slow growth rates by SBM and DFB, could be detected. A combination of DFB and ABM procedures increased the efficiency of detection of slow growing fungi associated with peanut seeds. The growth of the slow-growing pathogens was enhanced by alkaline treatment, facilitating their detection even in the presence of saprophytes (Elwakil et al. 2007).

Direct-Plating or Agar Test

Ater surface sterilization, the seeds are plated on appropriate medium or selective medium that specifically encourages the growth of the target pathogen. The colony characteristics of the pathogen growing on the medium are used for identification. For some pathogens such as *Phoma betae*, water agar may suffice (Mangan 1983). The selective media may not prevent in every case the growth of all saprophytic fungi which may overgrow the pathogen, making its identification difficult.

However, use of selective media appears to be more reliable than the blotter test. For detection of pathogens infecting vegetatively propagated plant materials such as corms, the macerated corm tissue is plated in a semiselective medium as in gladiolus infected by *Fusarium oxysporum* and incubated for 1 week. Colonies of *F. oxysporum* could be identified in this medium by this method, though it was not possible to differentiate between pathogenic and nonpathogenic isolates which were morphologically similar (Roebroeck et al. 1990).

Growing-On Test/Seedling Symptom Test

This method involves the planting of specified number of seeds, preferably on sterile soil, determining the number of infected plants and calculating the percentage of infected plants out of total number of seeds planted. These test results are helpful in assessing field performance and estimating the number of infection loci/unit area, if the seed lot under investigation is used for cultivation by farmers. Infection of soybean seeds by *Colletotrichum truncatum* was detected by this procedure (Dhingra et al. 1978). Some pathogens, such as *Sclerotium* sp. could not be detected in soybean seeds on potato dextrose agar (PDA) medium or by blotter test, could be detected by this method (Dhingra and Muchovej 1980). This method is very effective in the case of obligate pathogens causing downy mildew diseases. However, it requires large greenhouse space and also it is time-consuming, making it unsuitable for testing large number of seed lots.

2.2.1.2 Physical Methods

Addition of solutes increases the density of water, resulting in floating of lighter seeds that are ill-filled due to physiological or pathogenic causes. A simple method of removing lighter seeds infected by fungal pathogens, fungal sclerotia from the seed lot, is to immerse the seeds in sodium chloride solution (20%). The infected seeds and fungal structurers will float on the surface of the solution and they can be either mechanically removed or filtered out. The extent of fungal colonization of wheat kernels inoculated with *Fusarium* sp, may be determined by a color image analysis using the MultiScan® 4.01 and calculations were made using MS Excel® This procedure has been employed for rapid evaluation of kernel colonization by fungal pathogens in order to monitor grain quality of food and feed (Wiwart and Korona 1998). Although it may be possible to undertake visual inspection of large wheat seed consignments, harvest delays and missed wheat kernels infected by *Tilletia indica*, causative agent of Karnal bunt disease, cannot be avoided due to inspector fatigue. On the other hand, if a high speed optical sorter is employed, infected seeds may be removed rapidly and efficiently processed at the rate of up to 8,800 kg/h. This technology has the potential for large scale application for removal of bunted grains from wheat meant for food or feed (Dowell et al. 2002).

2.2.1.3 Chemical Methods

The presence of the fungal pathogens in the seed tissues may be observed by staining. Wheat loose smut pathogen *Ustilago nuda* infects embryos of seeds. The embryos are extracted in NaOH (5%) and then stained with trypan blue (Khanzada et al. 1980; Khanzada and Mathur 1988). Infection of wheat and barley respectively by *U. tritici* and *U. nuda* was detected by a modified method. Wheat and barley seeds are incubated in NaOH (10%) containing trypan blue (1 g/l) for 12–16 h. Then the embryos are separated from the endosperm by passing the seeds through different sieves followed by boiling in alkali solution for 15 min. The pathogen mycelium stained by trypan blue may be viewed after washing embryos and boiling again in acetic acid or lactic acid (45–50%) for 1 min (Feodorova 1987). Likewise, by treating the rice seeds with NaOH (0.2%), the infection by *Trichoconiella padwickii* could be inferred by the change of color of the diseased portion of infected seeds to black (Dharam Singh and Maheshwari 2001).

Deterioration of seed quality due to pathogenic fungi is one of the serious obstacle to be overcome by the seed industry. Evolution of characteristic volatiles and odor from infected cereal grains may be an early indication of deterioration of grain quality. The odor of wheat grains infected by common bunt pathogen, *Tilletia caries*, could be detected by an electronic nose which was found to be more efficient than a panel of grain assessors. The electronic nose appears to sense a different characteristic not related to common bunt odor (Börjesson and Johnson 1998). The reliability and efficiency of electronic nose technology was tested for early detection of grain spoilage, based on volatiles as an indicator of fungal activity and differentiation between species affecting wheat, maize and other cereals. The range of volatiles produced by fungi causing grain spoilage in vitro was determined. The key groups of volatile compounds emanating from the cereal grains were determined (Magan and Evans 2000).

2.2.1.4 Immunoassays

Comparitively detection and identification of fungal pathogens based on morphological characteristics may be easier than the bacterial and viral pathogens whose morphological characteristics are limited or insufficient for this purpose. Seed mycoflora includes fast-growing saprophytes which may overgrow the fungal pathogens, making their isolation and examination of morphological characterisitics difficult. Further, the strains, races or varieties of some of the fungal pathogenic species are morphologically indistinguishable. Hence, they have to be detected by more discriminating techniques like immunoassays and nucleic acid-based techniques. This kind of situation exists in the case of rice seedborne pathogens. More than half of 56 fungal pathogens infecting rice have been reported to be seedborne (Mew et al. 1988).

Polyclonal and monoclonal antibodies have been produced against fungal antigens present in culture filtrate, cell fractions, whole cells, cell walls and extracellular

components (Narayanasamy 2001, 2005). *Humicola languinosa* and *Penicillium islandicum* associated with discoloration of stored rice were efficiently detected using specific monoclonal antibodies (MAbs). *P. islandicum* produces a harmful mycotoxin capable of causing liver lesions, cirrhosis and primary liver cancer following consumption of contaminated rice grains. Hence, *P. islandicum* has to be detected rapidly and identified accurately to prevent the use of such contaminated rice grains. *P. islandicum* was detected more effectively and reliably by using specific MAb, compared with direct plating procedure which required long time (Dewey et al. 1989, 1990).

Several seedborne fungi like *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. have been demonstrated to be mycotoxin-producers. Two MAbs capable of reacting with antigens of 12 field and 27 storage fungi were generated. The presence of fungal pathogens in barley seeds was detected using polyclonal antibodies (PAbs) raised against *Penicillium aurantiogriseum* var. *melanoconidium* in indirect ELISA format. A possible linear relationship was noted between absorbance values and the pathogen population increase (Banks et al. 1992). Rice and corn seeds are colonized by the mold fungi *Aspergillus parasiticus*, *Penicillium citrinum* and *Fusarium oxysporum.* These fungi were detected by employing DAS-ELISA test. The amount of mold growth was strongly reflected by the variations in the absorbance values of the reactants. The detection limit of DAS-ELISA used, was 1 μg/ml (Chang and Yu 1997). A later investigation by Wang and Yu (1998) confirmed the effectiveness and reliability of DAS-ELISA test for the detection of nine toxigenic *Aspergillus* spp. in rice and corn seeds/grains. The usefulness of a direct competitive ELISA as a post-column monitoring system after liquid chromatography (LC) for the detection and quantification of fumonisins in maize was demonstrated. The detection limit of this protocol was 0.1 ng of FmB_1 in maize samples and *Alternaria alternata* (AAL) toxin in culture extracts (Yu and Chiu 1998).

Tilletia indica causes Karnal bunt disease in wheat crops which suffer both qualitative and quantitative losses. This pathogen has a protein (64-kDa) with antigenic properties. Antibodies specific to this protein specifically reacted with the pathogen teliospores in a microwell sandwich-ELISA and dipstick immunoassay (Kutilek et al. 2001). *Ustilago nuda* causes the loose smut disease of barley and it is internally seedborne. The pathogen is carried internally in the developing plant at the growing point which is transformed into smutted ear, instead of grain-bearing head at maturity. A DAS-ELISA test with biotinylated detection antibodies was employed to test naturally infected barley seeds. The conventional seed embryo test and DAS-ELISA test produced comparable and corroborative results. However, with artificially inoculated barley seeds, DAS-ELISA test scored higher level of seed infection than the embryo test. It may be possible to assess the efficacy of seed treating chemicals for elimination of the pathogen mycelium from infected seeds, in addition to studying the pathogen biology and characterization of resistance mechanism operating in barley plants by employing this ELISA format (Eibel et al. 2005b).

Phomopsis longicolla seedborne pathogen of soybean seeds was detected by employing ELISA and immunoblotting techniques and their comaparitive efficacy was assessed. By applying seed immunoblot assay (SIBA) for detection of the pathogen,

Table 2.9 Detection of *Macrophomina phaeolina* in cowpea seeds by agar plating and DAS-ELISA methods (Afouda et al. 2009)

	Percentage of infected seeds	
Seed source	Agar plating	DAS-ELISA
Market seeds (Niamey)	7	13
Market seeds (with doubtful symptoms)[a]	92	100
Market seeds (Maradi)	23	47
TN 5–78 (Niger Research Station)	2	0
IT 93K-734 (IITA-Nigeria)	0	0

[a]Discolored seeds with doubtful symptoms of infection

the nonspecific interference observed in ELISA could be overcome. Infected soybean seeds are placed on nitrocellulose paper on which the mycelium of the pathogen grows out. A conspicuous colored blotch formed on the nitrocellulose paper may be recognized, when it is assayed. As the mycelium can be produced only from the viable spores of *P. longicolla*, SIBA test provides the distinct advantage of differentiating the living and dead spores. In contrast, ELISA test results do not offer such a vital information (Gleason et al. 1987). Likewise, SIBA test was applied for the detection of *Tilletia indica* infecting wheat seeds. Colored imprints were formed on nitrocellulose paper on which infected wheat seeds were placed, indicating the presence of live teliospores of the pathogen in the seed lots tested (Anil Kumar et al. 1998). *Macrophomina phaseolina*, causing root rot diseases in a wide range of host plant species, including legumes, is seedborne. The presence of *M. phaseolina* in cowpea seeds was tested by agar plating and DAS-ELISA methods. The pathogen was detected by both agar plating and DAS-ELISA procedures in four of five seedlots obtained from different fields in Niger and Nigeria. However, ELISA format was more sensitive in detecting the pathogen in higher percentages of seeds compared to agar plating procedure which was time consuming and cumbersome (Table 2.9) (Afouda et al. 2009) (Appendix 25).

2.2.1.5 Nucleic Acid-Based Techniques

In general, the nucleic acid-based techniques have been shown to be more specific, sensitive, rapid and reliable than the immunoassays and very small quantities of samples or tissues are sufficient for detection of pathogens in seeds of various crops. In the recent years, nucleic acid-based diagnostic techniques have become the preferred ones for detection, identification and quantification of fungal pathogens constituting the seed microflora.

Peronosclerospora sorghi causing sorghum downy mildew disease was detected in seeds using dot blot hybridization. The target DNA from this pathogen was extracted by grinding up sorghum seeds. Hybridization of probes occurred only with the DNA of *P. sorghi*, but not with the DNA of any other fungi associated with sorghum seeds (Yao et al. 1990). Sensitivity of the assay was significantly enhanced by

using probes generated from the mtDNA rather than from chromosomal DNA. These probes were highly specific and hybridized only with the DNA of *P. sorghi* (Yao et al. 1991). Sunflower downy mildew pathogen *Plasmopara halstedii* was detected by employing primers in PCR assay, especially in the shell fractions (Says-Lesage et al. 2001). A sensitive nested-PCR assay was developed for the detection of *Peronospora arborescens* in the commercial opium poppy seed stocks in Spain. Two primers designed from the sequences of ITS regions of rDNA improved the detection sensitivity of the pathogen significantly (100- to 1,000-fold) compared with the detection limit obtained using single PCR employing the same primers. The frequency of detection of *P. arborescens* in the seeds indicated the possible threat posed by this pathogen for rapid dissemination through the seeds (Montes-Borrego et al. 2009).

Soybean seed decay was reported to be primarily due to *Phomopsis longicolla* and *Diaporthe phaseolorum* and it appears to hasten the seed deterioration when it is present along with the former pathogen. These seedborne pathogens could be detected by employing specific primers PhomI and PhomII derived from the polymorphic regions of pathogen DNAs. The presence of specific bands of PCR amplicons from ten pooled samples as well as from individual seeds could be visualized (Zhang et al. 1997). In a further study, species-specific detection of *D. phaseolorum* and *P. longicolla* was accomplished, using PCR-RFLP analysis and TaqMan chemistry. Fungal DNA was extracted from the soybean seeds, using an ultrasonic processor to break seed coat and cells. Based on the sequences of ITS regions of rDNA, three TaqMan primer/probe sets were designed. Primer/probe set PL-5 amplified a 96-bp fragment of *P. longicolla*, *D. phaseolorum* var. *sojae*. A 86-bp DNA fragment of *P. longicolla* was amplified by the set PL-3, while set DPC-3 amplified a 151-bp DNA fragment of *D. phaseolorum* var. *caulivora.* The detection limit of TaqMan primer/probe sets was as little as 0.15 fg (four copies) of plasmid DNA. When PCR-RFLP analysis was performed for *Diaporthe* and *Phomopsis* detection, the sensitivity was as low as 100 pg of pure DNA. TaqMan detection protocol was the most rapid and efficient for detection of these pathogens in soybean seeds (Zhang et al. 1999) (Appendix 26). The seeds of Tasmanian pyrethrum are infected by *Phoma ligulicola.* The presence of the pathogen was dectected by employing a PCR-based assay. Infection in seed lots down to 0.5% could be detected and the detection limit was 800 fg of *P. ligulicola* DNA. Reliability of amplification of the target fungal DNA was enhanced by addition of bovine serum albumin (BSA) to reduce the activity of inhibitors of PCR present in the pyrethrum seeds. The percent infection of seeds and the viability of *P. ligulicola* depended on the cultivars of pyrethrum (Pethybridge et al. 2006).

The serious threat to the export market for wheat posed by the presence of *Tilletia indica* was perceived, because of either restriction or total prohibition of wheat imports by other countries. In order to concentrate the teliospores of *Tilletia indica* the seed wash of 50-g grain sample was passed through 53 and 20 μm pore size nylon screens. The material retained in the 20 μm screen was suspended in water and then it was examined under the microscope or tested by PCR assay. Two pairs of pathogen-specific primers were employed for identifying the pathogen. Both microscope examination and PCR assay detected the pathogen in the grain

samples artificially infested with *T. indica* at five teliospores/50 g of wheat seeds. Size-selective sieving was found to be faster compared to standard centrifuging method for concentration of the teliospores of *T. indica* (Peterson et al. 2000). A repetitive sequence-based (rep)-PCR has been demonstrated to be effective in differentiating *Tilletia* spp. Each taxon was distinguishable by computer-based analysis of database of combined fingerprints which also indicated the phylogenetic relationship among the isolates of *Tilletia* spp. The results revealed the diagnostic potential of rep-PCR format as an effective diagnostic tool (McDonald et al. 2000). As the existing PCR assays were not capable of differentiating *T. indica* from *T. walkeri* infecting ryegrass, an effective PCR protocol had to be developed. The nucleotide sequences of a 2–3 kb region of the mtDNA, earlier amplified by PCR only from *T. indica*, was determined for three isolates of *T. indica* and three isolates of *T. walkeri.* By using five sets of specific PCR primers, *T. indica* could be consistently detected in wheat samples. Likewise, three *T. walkeri* PCR primer sets produced single bands only with DNA extracted from *T. walkeri* isolates and no detectable PCR amplicon from DNA of *T. indica* could be recognized (Fig. 2.12).

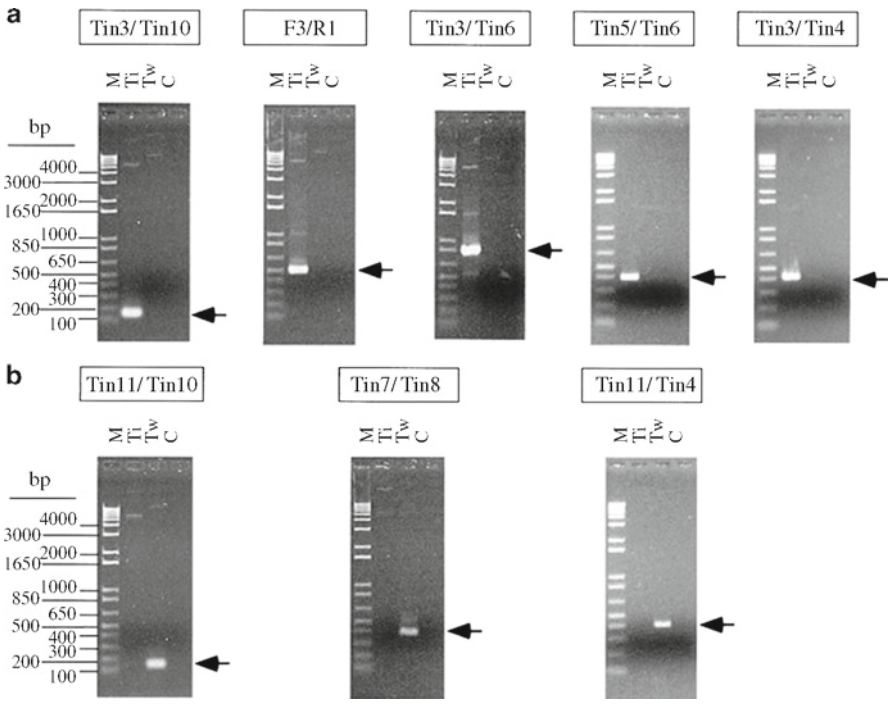

Fig. 2.12 Detection and differentiation of *Tilletia indica* and *T. walkeri* using species-specific primers (**a**) and (**b**) respectively in standard PCR assay. Lane M: molecular weight markers; Lane Ti: *T. indica* isolate Bpop; Lane Tw: *T. walkeri* isolate YRG-001; Lane C: no DNA template (control). *Arrows* indicate pathogen-specific PCR products (Courtesy of Frederick et al. 2000; The American Phytopathological Society, MN, USA)

In addition, a 212-bp amplicon was developed as target sequence in fluorigenic 5 (prime) nuclease for PCR assay by employing the TaqMan® system to detect and differentiate *T. indica* and *T. walkeri* (Frederick et al. 2000).

In a later investigation, a molecular assay with enhanced sensitivity and specificity was developed for rapid detection and differentiation of *Tilletia indica*, causative agent of Karnal bunt which is a quarantineable disease with significant impact on international wheat trade. The technique to multiplex PCR by probe color in real-time PCR analysis was utilized to design a single-tube, five-plex fluorescent assay to detect and identify the different pathogen species simultaneously. Different probes are labeled with dyes that have non-overlapping specific emission spectra for simultaneous detection. The protocol involves the release of DNA from bunt spores, PCR amplification to enrich *Tilletia*-specific templates from released DNA and five-plex real-time PCR to detect, identify and differentiate *T. indica*, *T. walkeri*, *T. ehrhartae*, *T. horrida* and a group comprising *T. caries*, *T. laevis*, *T. contraversa*, *T. bromi* and *T. fusca* in wheat grains. This fluorescent technique could detect even one spore and thus bypass the germination step which is mandatory for testing seeds/grains for the presence of fungal pathogens. This assay utilizes five dual-labeled, species-specific primer pairs in a PCR mix in a single-tube. The amplicons are detected simultaneously by five different fluorescence spectra. This protocol is effective and economically sustainable, because of the high specificity and sensitivity of the assay that demands reduced labor and reagents. Further, this protocol will be also useful for the identification of other contaminant *Tilletia* spp. present in wheat grains (Tan et al. 2009).

Barley scald disease pathogen *Rhynchosporium secalis* overwinters in plant debris and it can infect seeds symptomlessly or induce typical scald symptoms. Lima bean agar medium amended with Bengal rose and streptomycin was used to isolate the pathogen. A species-specific primer set based on sequence analysis of the ITS region of *R. secalis* was effective in detecting the pathogen in symptomless infections. The diagnostic band was observed only in the symptomless seeds of susceptible cultivar, but not in the seed extracts of resistant cultivar (Lee et al. 2001). In another study, a primer set RS8 and RS9 capable of amplifying a 264-bp fragment from DNA of all isolates of *R. secalis* was employed. This primer set did not amplify the DNA from other species tested, indicating the specificity of the primers with a detection limit of 1 pg of pathogen DNA. The test needed only 1 day, while the isolation-plating procedure required 10 days (Lee and Tewari 2001).

The PCR assay is useful for specific pathogen detection, but it cannot be applied for quantification of the pathogen population. Competitive PCR assay involves the use of an internal control that can compete for the same primer set and subsequently amplifies at the same rate as target DNA and the internal control DNA allows for quantification of initiation of concentration of the pathogen DNA. The advantages of competitive PCR are (i) any variable that may affect amplification has the same effect on both target and internal control DNA; (ii) the final ratio of amplified products reflects exactly the initial ratio of targets, rendering the reaction independent of the number of amplification cycles (Celi et al. 1993). The pathogen biomass

present in barley seed was quantified by employing a competitive PCR format, using the primer set RS1 and RS3 derived from ITS region of rDNA genes. The DNA extracted from the seeds with different intensity of infection was subjected to comp-PCR with a heterologous internal control which could compete for the same primer set in the conventional PCR, allowing the quantification of *R. secalis* biomass. The resulting PCR product ratio for each PCR (RS-amplified DNA/internal control template-amplified DNA) registered increases proportionally with increase in levels of infection in seeds. Naturally infected seeds collected for 4 years were utilized to demonstrate the potential of comp-PCR assay as an alternative seed health testing method (Lee et al. 2002) (Appendix 27).

PCR analyses for diagnosing pathogen-specific DNA sequence were performed, using PCR primers derived from ITS regions of rDNA of *Rhynchosporium secalis* and AFLP marker in *Pyrenophora teres* infecting barley seeds. PCR primers RS1–RS2 and RS8–RS9 were able to detect reliably *R. secalis* in barley seeds and leaves. On the other hand, primers PTT-F, PTT-R and PTM-F, PTM-R detected *P. teres* in artificially inoculated barley seeds (Fig. 2.13) (Gubis et al. 2004). *P. teres* is predominantly seedborne, but infected plant debris may also serve as sources of inoculum, whereas *Pyrenophora graminea* is strictly seedborne and hence seed infection levels may increase rapidly, if proper assessment of seed infection is not made. A quantitative PCR assay was developed using Scorpion Amplified Refractory Mutation System (ARMS) technology with real-time PCR detection. In this procedure, a single nucleotide base mismatch in the primer sequence could distinguish *P. teres* from the closely related *P. graminea*. Using the Scorpion primer set, quantification of *P. teres* alone was possible and 2 pg DNA of *P. teres* was readily detected after 35 cycles of amplification (Bates and Taylor 2001).

The presence of *Rhynchosporium secalis* and *Pyrenophora teres* in the barley seeds was detected by employing pathogen-specific primers in a PCR-based assay. The barley seeds were artificially inoculated with *R. secalis* and *P. teres*, the seed infection ranging from 70% to 90% in four samples tested. The total DNA from barley seeds was isolated using the Adgen DNA Extraction System from one gram powder from ground seeds. The primers were derived from the ITS regions of rDNA of *R. secalis* and from AFLP marker in *P. teres.* Electrophoretic analysis of PCR

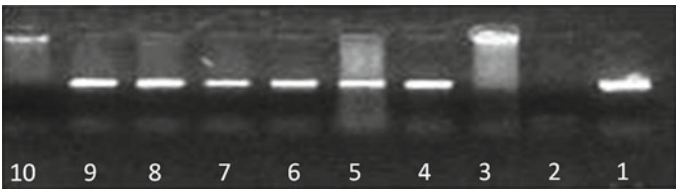

Fig. 2.13 Detection of *Pyrenophora teres* DNA in inoculated barley seeds. Lane 1: positive control (pathogen DNA); Lane 2: negative control (water); Lane 3, 10: DNA extracted from healthy barley cv. Dukos (Negative control); Lane 4–9: DNA extracted from infected barley seed samples; (Courtesy of Gubis et al. 2004; Czech J Genetics and Plant Breeding, Praha, Czech Republic)

amplicons was performed in 1.4% agarose gels. These pathogens were detected in all the samples tested with varying disease intensities (Gubis et al. 2004).

A real-time PCR assay was developed to detect and quantify seedborne infection of *Pyrenophora graminea* in barley. The conventional freezing blotter procedure cannot distinguish *P. graminea* and the closely related *P. teres*. The seed infection threshold for *P. graminea* is lower than that of *P. teres*. PCR primers and a TaqMan probe were designed to target a *P. graminea*-specific DNA sequence. DNA contents of *P. graminea* determined in the barley seeds were positively correlated with seed infection incidence assessed by the freezing blotter method, as well as with the incidience of disease on plants in the greenhouse, the correlation coefficient being $R^2 = 0.59$ ($P < 0.001$). Combining real-time PCR with a fast DNA extraction procedure provided the opportunity for rapid and automated detection and quantification of *P. graminea* DNA in seed samples. The time required for detection of *P. graminea* in barley seeds was reduced from 30 days required for conventional method (greenhouse) to 1 day by adopting the protocol developed in this investigation. The major advantages of the real-time technique are the precision, specificity and speed of providing the results to prevent the rejection of seed lots (Justesen et al. 2008).

Fusarium head blight (FHB) or scab disease of wheat and other cereals is due to different *Fusarium* spp. of which *F. culmorum*, *F. graminearum* (*Gibberella zeae*), *F. sporotrichoides*, *F. sambucinum* and *F. avenaceum* are important, in addition to *Microdochium nivale* (*Monographella nivalis*). These pathogens reduce the grain yield and quality considerably. Further, they produce a group of mycotoxins, trichothecenes among which deoxynivalenol (DON) is potentially more harmful. A competitive PCR-based assay to quantify trichothecene-producing *Fusarium* spp. based on primers derived from the trichodiene synthase gene (*Tri 5*) was developed. The primers specifically amplified a 260-bp product from 25 isolates of the trichothecene-producing *Fusarium* spp. A significant correlation was observed between the amount of trichothecene-producing *Fusarium* spp. and DON concentrations in the grains (Edwards et al. 2001).

Fusarium graminearum causing FHB disease in wheat and barley produces the mycotoxins DON, nivalenol (NIV) and zearalenone (ZEA) capable of inducing mycotoxicoses in humans and animals. A specific primer pair targeting the sequence of the *gaoA* gene coding for the enzyme galactose oxidase for the detection of *F. graminearum* was applied for the detection of this pathogen (Niessen and Vogel 1997). The Light Cycler™ technology involving a combination of rapid in vitro amplification of DNA with real-time detection and quantification of *F. graminearum* was applied. Based on the PCR primers specific to the *tri5* gene, a quantitative group specific assay was developed. This system was rapid, providing the results for 32 samples in 45 min, including quantification and identification of the product. This protocol provided reproducible results (98%) in the range between 0.05 and 6.0 ng of purified *F. graminearum* DNA. The Light Cycler ™ system in combination with SYBR Green I, a fluorescent dye enabling real-time detection of PCR products was applied for the detection and quantification of *F. graminearum* both in pure culture and in contaminated wheat samples for the first time by Schnerr et al. (2001).

A fast, sensitive and easy-to-handle method was developed for the detection of *F. graminearum* contamination in cereal samples. DNA Detection Test Strips™ was first tested, using DNA isolated from the *F. graminearum* cultures and this procedure was compared to agarose gel electrophoresis. A minimum of 0.26 ng of purified target DNA was detectable by the Test Strips™ and the detection results could be obtained in 20 min without the need for the special technical equipment or hazardous fluorescent dyes. A distinct adavantage of using the Test Strips ™ over conventional PCR format was the possibility of both detection and identification of the target pathogen. Even in the case of nonspecific amplification, products which do not hybridize to the specific probe will not be detected by Test Strips™ protocol. The sensitivity of detection by Test Strips™ method was comparable with gel-based DNA detection methods. This method can be applied for routine and screening investigations (Knoll et al. 2002).

Group-specific detection of trichothecene and fumonisin-producing *Fusarium* spp. and specific identification of *F. graminaearum* and *F. verticillioides* in field-collected barley and corn samples were accomplished by employing a fluorogenic (TaqMan®) real-time PCR assay. Primers and probes were designed from genes involved in mycotoxin biosynthesis (*TR16* and *FUM1*). In addition, primers and probes were prepared based on the rDNA sequences of *Fusarium* to provide a genus-specific internal positive control. Barley and corn samples infected by *F. graminearum* and *F. verticillioides* tested positive for the presence of trichothecene and fumonisin (Bluhm et al. 2004). A real-time PCRassay useful for monitoring and quantifying the major *Fusarium* spp. involved in FHB complex was developed. TaqMan® primers and probes showing high specificity to *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Michrodochium nivale* var. *majus* were designed. By maintaining an internal PCR control and using proper dilutions of pure genomic DNAs of the pathogens, the DNA contents of each pathogen in leaves, ears as well as in harvested grains of winter wheat could be determined. TaqMan® technology was shown to be useful to quantify and monitor the dynamics of individual species of the species complex causing FHB disease in cereals (Waalwijk et al. 2004).

The predominance of *Fusarium* species complex may vary depending on the geographical locations and environmental conditions. By using species-specific primers in PCR assays, the components of *Fusarium* species complex could be determined (Nicholson et al. 2004). The ribosomal ITS and a portion of calmodulin gene of *F. proliferatum*, *F. subglutinans* and *F. verticillioides* were sequenced and analyzed to design species-specific primers. Three pairs of primers (PRO1/2, SOB1/2 and VER1/2) produced PCR products of 585, 631 and 578-bp fragments for *F. proliferatum*, *F. subglutinans* and *F. verticillioides* respectively. The toxigenic fungi were detected in maize kernels (Mulé et al. 2004). Fumonisin-producing and fumonisin-nonproducing strains of *F. verticillioides* could be differentiated by employing a PCR assay based on DNA markers unrelated to fumonisin production (González-Jaén et al. 2004). Two pairs of specific primers based on intergenic regions of rDNA units were employed in another investigation, for the detection of *F. verticillioides* strains. The first pair of primers was specific to *F. verticillioides*, whereas the second primer pair could discriminate the major fumonisin-producing

strains that were primarily associated with crops and a minor group of strains, non-fumonisin-producing strains associated with bananas. This protocol using highly specific primer sequence, was found to be simple, rapid and precise, providing sensitive detection, identification and differentiation of *F. verticillioides*, capable of becoming a great risk to animal health (Patiño et al. 2004).

Many genes potentially involved in the virulence of *F. graminearum* (*Fg*), the predominant causal agent of FBH complex, have been identified. It is necessary to detect and quantify the wild-type and mutant strains occurring in the fields to understand the role of these genes in disease development. A SYBR Green-based real-time PCR assay was developed to quantify the total genomic DNA in a plant sample as well as the total genomic DNA of *Fg* that contributed from a strain containing a common selectable marker used to create deletion mutants. This procedure has shown to allow researchers to correlate the amount of disease observed in wheat field trials to the *Fg* mutant strains being investigated (Dyer et al. 2006). In order to identify *Fusarium* spp. present in grains and feed samples, the DNA extraction methods such as DNeasy® Plant Mini Spin Columns (Qiagen), the Bio robot EZ1 (Qiagen) with the DNeasy® Blood and Tissue Kit (Qiagen) and the Fast-DNA® Spin Kit for Soil (Qbiogene) were tested. DNeasy® Plant Mini Spin Columns in combination with sonication gave the best results with respect to *Fusarium* DNA yield. The modified DNeasy® Plant Min Spin protocol was used to analyze 31 wheat samples for the presence of *F. graminearum* and *F. culmorum*. The DNA level of *F. graminearum* could be correlated to the level of DON ($r^2 = 0.9$) and ZEN ($r^2 = 0.6$), whereas there was no correlation between *F. culmorum* and DON/ZEA. The results indicated that *F. graminearum* and not *F. culmorum* was the main producer of DON in Swedish wheat during 2006 (Fredlund et al. 2008).

Quantification of biomass of different *Fusarium* species in wheat and maize is essential to understand the role of individual species in disease development. Quantitative real-time PCR assays were developed based on the primers and probe designed on the sequences of the elongation factor 1a (EF1a) gene for 11 *Fusarium* spp.: *F. graminearum*, *F. culmorum*, *F. poae*, *F. langsethiae*, *F.sporotrichoides*, *F. equiseti*, *F. tricinctum*, *F. avenaceum*, *F. verticillioides*, *F. subglutinans* and *F. proliferatum.* The biomass assessment for different *Fusarium* spp. was performed in 24 wheat and 24 maize samples. The assays were found to be specific and sensitive and the results of quantitative real-time PCR assays were well correlated with the mycotoxin data of the field samples (Nicolaisen et al. 2009).

Detection of *Fusarium* spp. invading seeds of malting barley by plating method is time-consuming and laborious and also it is not possible to gather information about the toxingenic species of *Fusarium* carried by barley grains. Hence, a rapid and simple quantification procedure like real-time PCR is essential to assess the mycotoxin risk in cereals used in cereal-based industry. The TMTR1 and TMFg12, two variants of TaqMan technology were applied to quantify trichothecene-producing *Fusarium* DNA and *F. graminearum* DNA present in barley grain and malt samples. The contents of deoxynivalenol (DON) in barley grains were represented by the *Fusarium* DNA contents determined by TMTR1-trichothecene assay. On the other hand, the TMFg 12 format for *F. graminearum* provided relative DON contents in the North American barley and malt samples (Sarlin et al. 2006). Species-specific

Table 2.10 Overall percent incidence of each pathogen involved in FHB detected in each chaff or grain sample using PCR-based diagnostic (dPCR) and quantification (qPCR) methods respectively (Xu et al. 2008)

	dPCR		qPCR	
Pathogen	Chaff	Grain	Chaff	Grain
Fusarium avenaceum	46	29	19	4
F. culmorum	20	13	2	0
F. graminearum	43	47	18	8
F. poae	54	36	16	4
Microdochium majus	50	28	25	9
M. nivale	56	22	17	1

PCR primers were used to detect *Microdochium nivale*, *M. majus*, *Fusarium avenaceum*, *F. culmorum*, *F. graminearum* and *F. poae* by extracting their DNA from seeds (milled flour) and chaff in diagnostic PCR (dPCR) and competitive PCR formats. The incidence of FHB pathogens with quantifiable amounts of DNA was significantly less ($P < 0.05$) in the grain than in the chaff for all FHB pathogens, except for *F. culmorum* for which there were no significant differences. The amount of quantifiable DNA in the chaff was as much as ten times greater than in the grain (Table 2.10). Accumulation of DON was most strongly associated with the presence of *F. graminearum*, while NIV accumulation was related only with *F. culmorum*. On the other hand, zearalenone (ZON) accumulation was associated with all three *Fusarium* spp. However, the amount of pathogen DNAs and mycotoxins varied greatly at different locations (Xu et al. 2008).

Aspergillus flavus group encloses *A. flavus* and *A. parasiticus* as aflatoxin producers and *A. oryzae* and *A. sojae* as koji molds. A PCR-mediated assay was employed to identify the four aflatoxin-synthesizing genes encoding norsolorinic acid reductase (*nor-1*) versicolorin A dehydrogenase (*ver-1*), sterimatocystin-*o*-methyl-transferase (*omt-1*) and a regulatory protein (*apa-2*) involved in aflatoxin biosynthesis. Fourteen strains of *A. flavus* were found to possess the four target DNA fragments. When peanut kernels were artificially contaminated with *A. parasiticus* and *A. niger* for 7 days, the contaminant DNA was extractable from a piece of cotyledon (ca. 100 mg) and it was subjected to multiplex-PCR assay using four pairs of primers coding for the genes mentioned above. They were successfully detected. The target DNA fragments were detected in kernels infected by *A. parasiticus*, while none was detected in uninoculated kernels or other kernels infected with *A. niger* (RueyShyang et al. 2002).

Verticillium wilt caused by *Verticillium dahliae* is one of the most destructive diseases of olive (*Olea europea*) fruit trees with worldwide distribution. The pathogen has been demonstrated to be seedborne in lettuce (Vallad et al. 2005) and spinach (Toit et al. 2005). Transmission of the pathogen through seeds has been suggested as a possible mode of dissemination to new areas. Seeds and seedlings from symptomatic and asymptomatic mother plants were tested by standard plating and nested-PCR methods. The primers were designed using the sequences of ITS regions of nuclear rRNA genes of *V. dahliae*. The sensitivity of the nested-PCR assay was evaluated by amplifying the crude DNA of conidia. The incidence

Table 2.11 Detection of *Verticillium dahliae* in the seeds of olive by isolation and nested-PCR methods (Karajeh 2006)

Health status	Infected seeds cultivars (%)	
	Shimali[a]	Nebali[a]
Symptomatic trees		
Isolation	15.0 a	11.3 b
Nested-PCR	66.7 a	63.3 a
Asymptomatic trees		
Isolation	7.0 a	7.3 a
Nested-PCR	16.3 a	13.3 a
LSD value	3.9	2.8

LSD Least significant difference
Means of three trees with 25 fruits/tree; values followed by the same letter are not significantly different according to LSD test

of infection by *V. dahliae* in seeds and seedlings was significantly higher with nested-PCR assay than with the plating method, when both symptomatic and asymptomatic trees were tested (Table 2.11). Seed transmission of *V. dahliae* to the progeny of seedlings of two cultivars of olive varied considerably. The results clearly showed that infected olive seeds could be a potential source of inoculum carrying the pathogen to new locations (Karajeh 2006) (Appendix 28).

Various species of *Alternaria* are carried by seeds of different crops. In addition to yield losses caused by them, several toxic or carcinogenic compounds harmful to plants and animals are also produced by them. A PCR-based method for the detection of *Alternaria alternata* and *A. solani* colonizing cereal grains, was developed. Primers based on the sequences of ITS1 and ITS2 regions of the 5.8S rDNA were designed. Amplification of the DNAs of both pathogens occurred, but not of the DNAs of other microorganisms or host plant species tested, indicating the specificity of the assay. The results of both PCR assay and conventional isolation method were comparable, as both methods gave positive results for eight of ten samples tested. However, the PCR assay required only 8 h for obtaining results, while the isolation method needed several days. As the time required for obtaining the results is the most crucial factor for accepting or rejecting shipment of grains, PCR assay offering the results in a short time, will be preferable (Zur et al. 2002).

Of the three species of *Allternaria* forming the primary sources of inoculum carried on carrot seeds, *A. alternata* and *A. radicina* have a high toxigenic potential, while *A. dauci* can infect the plants, as the other two pathogenic species. A PCR-based assay was developed for the detection of these pathogens, since the conventional deep-freezer-blotter and plating on selective medium were laborious and time-consuming. Primers were designed based on the sequences of ITS regions of the rDNA repeat for the detection of *Alternaria* spp. in the seeds and roots of the carrot plants. These primers were highly specific, sensitive and capable of differentiating these pathogens. As the results of PCR assay and conventional

methods were similar, PCR assay offers the advantage of providing early and reliable results, making it the method of choice, when results are needed rapidly (Konstantinova et al. 2002).

Alternaria brassicae causes an important seedborne black spot disease of cruciferous crops. Detection and identification of *A. brassicae* in seeds forms the crucial step for producing disease-free seeds which constitutes the disease management system. A PCR-based detection protocol was formulated using specific primers designed from the sequences of ITS regions of nuclear rDNA of *A. brassicae*, *A. brassicola* and *A. japonica* involved in the black spot disease. These pathogens could be detected in the seed macerates (Iacomi-Vasilescu et al. 2002). In a later study, the sequences of two clusterd genes potentially involved in the pathogenicity were used to design two different sets of primers that were employed in conventional and real-time PCR formats. *A. brassicae* was specifically detected in DNA extracted from seed. Real-time PCR assay provided more sensitive and specific detection and differentiation of *A. brassicola* and *A. japonica* in radish, *A. alternata* in radish and cabbage, *Stemphylium botryosum*, *Penicillium* sp. and *Aspergillus* in cabbage and *Verticillium* sp. in tomato seeds. Real-time PCR assay could be used for quantification of natural infection levels in cabbage and radish seeds (Guillemete et al. 2004).

Colletotrichum gossypii causing anthracnose and *C. gossypii* var. *cephalosporioides* causing ramulose in cotton (*Gossypium hirsutum*) are seedborne and morphologically indistinguishable. The conventional blotter testing method cannot differentiate these two pathogens. Amplified fragment length polymorphism (AFLP) markers were evaluated for detection and differentiation of these pathogens. The AFLP analysis showed a total of 318 polymorphic and 16 monomorphic bands revealing clear distinction between these two pathogenic forms of the ten *C. gossypii* isolates tested. A distinct advantage of applying AFLP analysis is the possibility of determining genetic diversity within pathogen populations from small amounts of pathogen DNA over the laborious and time-consuming isolation method (Silva-Mann et al. 2005).

A rapid, specific and sensitive PCR-based assay was developed for the detection of the anthracnose pathogen *Colletotrichum lindemuthianum* in bean seeds. Five forward primers were designed based on the sequences of rDNA region consisting of 5.8S gene and ITS1 and 2 of 4 *C. lindemuthianum* races and 17 *Colletotrichum* spp. downloaded from GenBank. One forward primer, based on the specificity of detection, was selected and used in combination with ITS4 to specifically detect *C. lindemuthianum.* A PCR product of 461-bp was generated from the DNA of 16 representative isolates of *C. lindemuthianum*, but not from other *Colletotrichum* spp. A nested PCR format was used for enhancing the sensitivity of detection, the limit of detection being 10 fg of pathogen DNA and 1% of infected seed powder. The protocol could be completed within 24 h as against a 2-week period required for culturing the pathogen (Fig. 2.14) (Chen et al. 2007).

Neck rot disease of onions is caused by three species of *Botrytis* viz., *B. aclada*, *B. allii* and *B. byssoidea* carried on the onion seeds. A magnetic capture hybridization and polymerase chain reaction (MCH-PCR) assay was developed for the

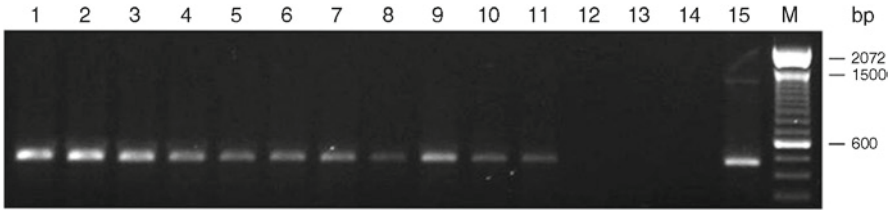

Fig. 2.14 Detection of *Colletotrichum lindemuthianum*-specific DNA fragment in bean seed powder using nested PCR assay. Lane M: 100 bp DNA ladder; seed powder tested consisted of mixture of infected and healthy seeds in different proportions. Lane 1: 100%; Lane 2: 80%; Lane 3: 60%; Lane 4: 40%; Lane 5: 20%; Lane 6: 10%; Lane 7: 8%; Lane 8: 6%; Lane 9: 4%; Lane 10: 2%; Lane 11: 1%; Lane 12: 0%; Lane 13: anthracnose resistant bean genotype G2333; Lane 14: negative control (water); Lane 15: positive control (pathogen DNA) (Courtesy of Chen et al. 2007; The American Phytopathological Society, MN, USA)

detection of *B. aclada* in onion seeds. This procedure reduced the time required to test the onion seeds from 10 to 14 days by isolation method to less than 24 h. MCH-PCR format detected the pathogen DNA from aqueous solutions containing 100 fg DNA/ml. MCH-PCR assay was more sensitive and efficient than conventional PCR format, in detecting the pathogen in seedlots with 4.8% and 9.9% infection in artificially inoculated seeds. The pathogen was also detected in naturally infected seedlots (Walcott et al. 2004). The ribosomal intergenic spacer (IGS) regions of the target pathogen species and nontarget *Botrytis* spp. were sequenced, aligned for designing a primer pair specific to *B. aclada*, *B. allii* and *B. byssoidea*. These primers were employed in a real-time fluorescent PCR assay using SYBR chemistry for detection and quantification of these seedborne pathogens. The primers reliably detected 10 fg of genomic DNA/PCR reaction extracted from pathogen cultures. The pathogens present in the seeds of 23 commercial seed lots were quantified by applying real-time PCR format which was shown to be more sensitive than the conventional plating method. Five of the 23 seed lots tested negative to the agar plating method. However, the presence of the pathogens in these five seed lots was confirmed by the PCR-based protocol developed in this investigation. But the incidence of neck rot disease had no bearing on the extent of seed infection determined by using the PCR-based assay (Chilvers et al. 2007).

2.2.2 Detection of Fungal Pathogens in Propagative Planting Materials

Vegetatively propagated plant materials such as tubers, corms, bulbs and setts are infected by fungal pathogens through inoculum present in the aerial plant parts of the same plant or soil or irrigation water or air. The infected propagative materials carry the inoculum to short or long distances and also to subsequent generations, if

no attempt is made to assess the health status of these plant materials. When the infected materials are stored, the pathogens multiply and cause deterioration in the quality, resulting in significant reduction in the market values. The yield of crops raised by vegetative propagation such as potatoes, onions, yams, banana, strawberry, sugarcane and cassava depend largely on the use of disease-free planting materials. Hence, it is advisable and mandatory in certain crops, to use certified planting materials to ensure the freedom from designated diseases that can otherwise, cause serious losses to the growers.

2.2.2.1 Conventional Methods of Detection

Visible external symptoms of infection by fungal pathogens in potato tubers, carrot roots, onion bulbs, sugarcane setts and strawberry cuttings may be recognized. The infected materials can be removed during visual examination. The symptoms on the planting materials may be useful in the idenfication of some fungal pathogens, as in the case of potato powdery scab disease caused by *Spongospora subterranea* and black scurf disease caused by *Rhizoctonia solani*. However, identification of the fungal pathogens may not be so straight forward and easy in most cases. Fungal pathogens causing latent/quiescent infections or indistinct symptoms have to be detected by isolation of the pathogen in appropriate culture media followed by examination of morphological characteristics under light microscope. Immunoassays or nucleic acid-based techniques have been very extensively employed for the sensitive and reliable detection and identification of fungal pathogens present in the propagative materials which may or may not exhibit recognizable external symptoms, thus facilitating the elimination of infected plant materials.

2.2.2.2 Immunoassays

Immunoassays have been applied successfully for the detection of soilborne fungal pathogens that have invaded the propagative plant materials. The polyclonal antibodies (PAbs) generated against purified mycelial proteins from *Verticillium dahliae* were used for the detection of this pathogen in potato tubers (Sundaram et al. 1991). The presence of *Spongospora subterranea* was detected in potato tuber extract by using the PAbs raised against the homogenates of spore balls (cystosori) in ELISA test which had a detection limit of 0.8 spore ball equivalent/ml (Harrison et al. 1993). A specific protein of pathogen origin can be employed for the detection of the pathogen causing the disease under investigation. A polyclonal antiserum was produced against a 101-kDa polypeptide present in *Colletotrichum falcatum*, causative agent of sugarcane red rot disease. The PAbs generated against this protein could be employed to detect *C. falcatum* in the root eyes, buds, leafscar and pith region in the stalks from which the setts are prepared for planting in the next season (Viswanathan et al. 1998).

2.2.2.3 Nucleic Acid-Based Techniques

High levels of sensitivity and specificity of nucleic acid-based diagnostic tests have been demonstrated for the detection and identification of fungal pathogens present in the vegetatively propagated plant materials. Different species of *Phytophthora*, including *P. infestans* infecting potatoes and tomato (causing late blight disease) were detected by a PCR-based assay. Primers designed from the sequences of the ITS regions specific to *P. infestans* were employed to detect this pathogen in infected field samples of potato and tomato (Trout et al. 1997). Six taxonomic groups of *Phytophthora* spp. including *P. infestans* could be identified by adopting a PCR-based assay depending on the amplification of 5.8S rDNA gene and ITS4 and ITS5 primers developed by Liew et al. (1998). Potato tubers were inoculated with *P. infestans* which was detected by PCR in light sprouts and stems in different growth stages. The biomass of the pathogen was below the detection limit during the course of plant growth, probably due to dilution effects. However, the symptoms induced later indicated the ability of the pathogen to cause symptoms even at very low concentration (Appel et al. 2001). A rapid method of detection of *P. infestans* in potato tubers was developed, using three oligonucleotide primers designed from the ITS sequences of *P. infestans*. The primer pair PiS-1/Pi2A-2 was employed to detect the pathogen in the potato tubers. The sample tissues were collected from tuber lesions, margins around the lesion or symptomless areas at various distances from tuber lesions in inoculated tubers. The primer pair generated and amplified products of expected size (550 bp) with samples collected from tubers. This primer set amplified this unique fragment only from *P. infestans* DNA and potato tubers infected by *P. infestans*, indicating the specificity of the assay (Jyan et al. 2002).

Phytophthora infestans and *P. erythroseptica* (causative agent of pink rot of potato tubers) could be detected in potato tubers as early as 72 h after inoculation, by employing specific primers designed based on the sequences of the DNAs of these pathogens. The infection by these pathogens could be recognized well in advance of development of disease symptoms on the tubers (Tooley et al. 1998). In a later study, primer pair specific to *P. infestans* based on sequences of ITS region amplified a 613-bp product was employed. In a single round PCR assay, 0.5 pg pure DNA of *P. infestans* was detected. The pathogen present in the seed potato tubers could be detected in addition to extracts of leaves and stem tissues. The nested-PCR format used in this investigation has a practical application for testing seed tubers. In most samples that were PCR-positive, had no visible symptoms of the disease, indicating the usefulness of this protocol for reliable detection of latent infection of seed tubers (Hussain et al. 2005).

Potato tubers infected by skin spot disease caused by *Polyscytalum pustulans* has reduced market value, necessitating the development of an efficient and rapid detection method, since the existing methods are time consuming and laborious. A real-time PCR assay was developed for the detection and quantification of *P. pustulans* in potato tubers. A single PCR product of identical size of 1065-bp and sequence of ITS1/ITS2 and 5.8S rDNA were amplified with universal primers ITS4/ITS5 from DNA of both *P. pustulans* isolates Pp3 and Pp14 tested. A set of

real-time PCR primers PpustF1/PpustR2 and a TaqMan probe PpustPr1 were subsequently designed to unique sequences within the ITS1 regions of Pp3 and Pp14 isolates. DNA extracted from all isolates of *P. pustulans* from pure cultures produced a signal in real-time assay. Likewise, DNA extracted from dried tuber peelings infected by *P. pustulans* also produced the expected amplification. DNA extracts of nontarget pathogens infecting potato tubers did not produce the signal, indicating the specificity of the assay. The real-time PCR assay could detect the pathogen in symptomless tubers at attogram (ag) levels (Lees et al. 2009).

Spongospora subterranea is a soilborne biotrophic pathogen belonging to Plasmodiophorales. Masses of resting spores aggregating to form spore balls (cystosori) are present on the potato tuber surface, seriously reducing the tuber quality and marketability. The only effective way to control the disease is to plant pathogen-free tubers in non-infested soils. Hence, detection of *S. subterranea* in the potato tubers rapidly and precisely is essential. Primer pairs Spo 8/9 and Sps 1/2 were designed from the ITS sequences of the pathogen and both primer pairs were specific for the identification of *S. subterranea* (Bulman and Marshall 1988; Bell et al. 1999). S. *subterranea* could be detected in potato peel and tuber washings by employing specific primers (Sps 1 and Sps 2) based on the sequences of the ITS regions 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 the PCR assay. The detection threshold of the assay was determined to be DNA equivalent to 2.5×10^5 cystosori or one zoospore/PCR. Disease risk assessment of the potato seed tuber stocks may be performed using this PCR format (Bell et al. 1999). A real-time PCR was developed for the detection and quantification of *S. subterranea* in plant tissues in addition to soil and water (van de Graaf et al. 2003).

A method of directly detecting *Spongospora subterranea* in potato tubers, combines a fast two-step automated approach to DNA extraction with a sensitive TaqMan® PCR assay for rapidly processing the samples. Real-time primers and probe were designed using the sequences of ITS region of the pathogen. The assay was pathogen-specific and this protocol was effective for detecting *S. subterranea* directly on a range of potato cultivars from different locations in United Kingdom. Further, the assay was found to be useful for the detection of the pathogen in the tubers at more stages in its life cycle and not at the cystosori stage only as in the case of ELISA test. The sensitivity of TaqMan® PCR format was at least 100-fold greater than that of ELISA test or conventional PCR assay (Ward et al. 2004).

In order to detect the pathogen in naturally infected symptomatic and asymptomatic potato tubers and also to detect the pathogen in other infected symptomless host plants, a PCR assay using primers SsF and SsR designed from the ITS regions of *S. subterranea* f.sp. *subterranea* was developed. These primers amplified a 434-bp product from the DNA of spore balls, but not from DNA of healthy potato, common scab tuber and taxonomically related plamodiophorids. The presence of the pathogen in naturally infected symptomatic and asymptomatic tubers and other host plants such as tomato and tobacco was detected at 14 days after inoculation. The PCR protocol developed in this investigation has the potential for routine

detection of this pathogen in plant tissues with or without symptoms of infection (Qu et al. 2006). A sensitive and semiquantitative real-time PCR assay specific for *S. subterranea* developed by van de Graaf et al. (2005) was employed for the detection of this pathogen in potato tubers. The percentage of latent infection varied from 27 to 80, indicating the danger of using tubers from infected plants as seed tubers (Lees et al. 2008).

Potato dry rot disease with worldwide distribution seems to be due to different species of *Fusarium*, of which *F. sulphureum* is the most frequently encountered pathogen in North America and some European countries. A micro-plate (MP)-PCR-ELISA technique and a real-time PCR procedure were applied for the detection of pathogenic *Fusarium* spp. associated with the dry rot disease. The ITS1 and/ITS2 regions of the rDNA genes of the isolates of *F. coeruleum*, *F. sulphureum*, *F. avenaceum* and *F. culmorum* were amplified with the universal primers ITS5 and ITS4. Specific detection of *F. coeruleum* and *F. sulphureum* was achieved by designing primers based on regions of dissimilarity. Additional sets of species-specific primers and probes were designed in the ITS regions of all *Fusarium* spp. tested to meet the optimal requirements of the fluorescent technology based on amplicons between 50- and 150-bp in length. The detection limits for seed potato peel extracts (0.5 ml) for PCR-ELISA assay were 12.5–25.0 macroconidia, depending on *Fusarium* spp. or 50–100 fg (femtograms: 10^{-15} g) of genomic DNA of the pathogen species concerned. TaqMan technique could reliably detect attogram (ag – 10^{-18} g) levels of genomic DNA of different isolates. Real-time PCR technique detected all the four *Fusarium* spp. either singly or in combination in potato seed stocks sampled from commercial stores. Pathogen DNA contents increased with increase in disease intensity on seed tubers observed. Both PCR-ELISA and real-time PCR procedures had high levels of sensitivity and specificity, producing similar results. However, real-time PCR needs more expensive equipments, although reagent costs are less than that of PCR-ELISA technique (Cullen et al. 2005).

Species-specific nested primers (CcINFI/CcNRI) were employed for the detection of *Colletotrichum coccodes* causing black dot disease of potato. A rapid procedure for the direct extraction of DNA from potato tubers and soil was developed, the limit of detection being 3.0 spores/g or equivalent of 0.06 microsclerotia/g of soil. *C. coccodes* present in the extracts of potato peel could be detected by this PCR assay (Cullen et al. 2002). Potato silver scurf disease pathogen *Helminthosporium solani* was detected by a PCR-based assay, using specific primers Hs1F1/Hs2R1. A 447-bp product was amplified by these primers from the DNA extracted from 20 infected potato tissue samples and 54 single spore isolates of *H. solani.* The pathogen detection by PCR was rapid, providing the results in a day, which were similar to those obtained from conventional plating method which required about 4 weeks (Errampalli et al. 2001).

Potato wart disease pathogen *Synchytrium endobioticum* has a worldwide distribution and exists as at least 30 biotypes. The resting spores carried by the tubers may be introduced into new areas or into a new field in the same area where it can survive in the soil for 30–70 years (Hampson 1993). The 18S rDNA

of the pathogen was sequenced by extracting the DNA from the resting spores from infected tubers. DNA array technology was applied for the detection of *S. endobioticum*. Species-specific probes were designed and arrayed onto glass slides. Probes specific for viruses infecting potatoes were also designed based on nucleotide sequences of the viruses to be detected. A significant number of probes showed high specificity in detecting the fungal and viral pathogens in microarray-based hybridization for identification of multiple pathogen targets (Abdullah et al. 2005).

In a later investigation, a real-time PCR assay was developed for quantitative detection of *Synchytrium endobioticum* in potato tuber plant tissues and also in the soil samples. The DNA isolated from the sporangia in fresh wart tissues using $CsCl_2$ centrifugation, warts and different plant parts of potato were tested by using specific primers and a TaqMan probe designed from the ITS region of the multicopy rDNA gene. Coamplification of target DNA along with an internal competitor DNA fragment (cytochrome oxidase gene of potato plant) guarded against possible false-negative results. The detection limit of this protocol was 1 fg of genomic DNA of *S. endobioticum*. Amplification of a smaller product of 84-bp made this procedure more efficient, resulting in lower detection limits compared to the methods available earlier. The pathogen could also be detected in stolons from plants with warts and also from symptomless plants. The sensivity of the procedure developed in this investigation improved the detection of *S. endobioticum* by 100-fold and proved to be reliable for precise diagnosis of this important disease of potato (van Gent-Pelzer et al. 2010).

Incidence of cavity spot disease accounts for appreciable losses in carrot crops. *Pythium violae*, *P. sulcatum*, *P. sylvaticum*, *P. intermedium* and a new species tentatively named *P. 'vipa'* are considered to be responsible for the cavity spot disease in Norway (Hermansen et al. 2007). PCR primers based on the ITS sequences, were designed for the identification of the five *Pythium* spp. associated with cavity spot disease. The PCR primers amplified the fragments of expected size in all target species with no cross-reaction to other species or fungal isolates from carrots tested. The detection limits differed depending on the primers used, the two of the most sensitive ones allowing detection of as little as 5 fg DNA of the pathogen species. All the five species were detected in the lesions from diseased carrots. The signals were weak in carrot samples without symptoms (Fig. 2.15). Among the *Pythium* spp., most sensitive detection was obtained for *P. intermedium* and *P.* '*vipa*' with a detection limit of 5 fg of DNA of these two species, whereas PCR assay was the least sensitive for *P. violae*, although this pathogen could be detected in carrot tissues and also in field soils (Klemsdal et al. 2008) (Appendix 29).

Detection and quantification of *Botrytis aclada*, causative agent of onion necrotic disease in onion bulbs, was achieved by employing a real-time PCR assay which is based on TaqMan probe-based chemistry. The pathogen fragment of L45–550 region and a DNA sequence from onion serine acetyl transferase gene (*SAT1*) as an internal control was used. The detection limits of the real-time PCR assay was 10 pg/µl of pathogen genomic DNA. The presence of onion tissues in the

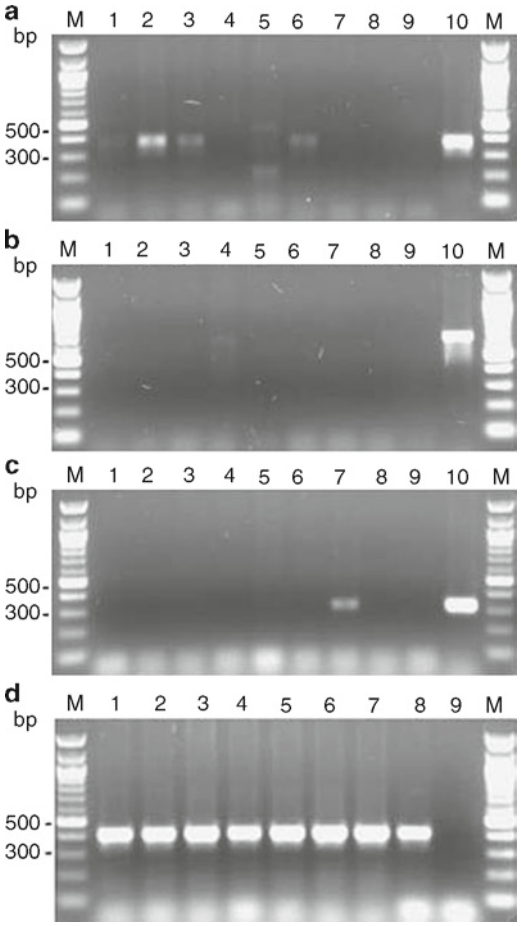

Fig. 2.15 Detection of cavity spot pathogens in symptomless carrot samples using PCR assay. (**a**) *Pythium intermedium*, (**b**) *P. sulcatum*, (**c**) P. '*vipa*', (**d**) PCR reaction with primer pair IST3 and IST4 used as a control reaction. Lanes 1–8: detection of three *Pythium* spp. in eight random symptomless carrot samples; Lane 9: negative control (water) in place of DNA; Lane 10: pure DNA from respective *Pythium* spp. as positive control (Courtesy of Klemsdal et al. 2008; British Society for Plant Pathology, Hertfordshire, England)

sample did not affect the assay results. Different quantities of *B. aclada* mycelium growing on onion disks inoculated with different pathogen populations could be precisely determined by the real-time PCR assay. The results of the assay were correlated to the visual observations made duing the incubation period. The results showed that the protocol might be useful for determining the pathogen mycelium in bulbs during growth, harvest and storage (Coolong et al. 2008).

The importance of indexing the mother plants from which the cuttings are taken for vegetative propagation for freedom from disease(s) has been well realized especially in the case of virus diseases. Petri disease (black goo decline) caused by

Phaeomoniella chlamydospora is considered to be responsible for serious problems in newly planted vineyards. Infection can be spread through cuttings taken from infected mother vines and contaminated cuttings appeared to occur during nursery procedures (Fourie and Halleen 2000). Detection of *P. chlamydospora* contamination at different stages during the propagation process was attempted using nested-PCR assay (Whiteman et al. 2004). Later a quantitative real-time PCR assay was developed using SYBR® Green technology that enabled detection of *P. chlamydospora* in the buds of infected grapevines (Overton et al. 2004). Quantitative real-time PCR procedure was applied to detect and quantify *P. chalmydospora* at different stages during grapevine propagation in the nursery (Wiechel et al. 2005). Various methods, using PCR, nested-PCR and quantitative PCR (SYBR® Green and TaqMan®) were tested for their efficacy to detect *P. chlamydospora* during propagation. The nested-PCR assay was the most sensitive for detection followed by the quantitative PCR methods and the single PCR assay was the least sensitive in detecting this pathogen in the plant tissues (Edwards et al. 2007).

2.2.3 Detection of Fungal Pathogens in Postharvest Produce

Fungal pathogens can infect not only the standing crops at different stages of growth, but they also infect the produce prior to or after harvest during transit and storage. Infection of floral parts externally and internally may lead to development of symptoms either in the field or later during storage. The symptoms of infection may be visible at different periods after havest depending on the host–pathogen combination, existing environmental conditions and resistance/susceptibility levels to the disease(s) concerned. Pathogens may remain dormant in immature fruits and vegetables and become active when suitable physiological conditions become available, as the fruits ripen leading to the development of characteristic symptoms based on which some of the fungal pathogens can be identified.

2.2.3.1 Plating-Isolation Methods

The fungal pathogens infecting fruits and vegetables can be isolated using appropriate artificial media and they may be identified up to species level based on the descriptions of morphological characteristics, such as spore morphology, sporulation patterns, production and characteristics of sporulating structures producing asexual and sexual spore forms. Temperature optima for the growth of the fungus may be an useful characteristic helping in the pathogen identification. *Sphaeropsis pyriputrescens* causing fruit rot disease of d'Anjou pears requires for its growth a temperature range of 0°C to 25°C with an optimum between 15°C and 20°C. This pathogen cannot develop at temperatures above 30°C. This cultural characteristic is useful as a practical criterion for its tentative identification (Xiao and Rogers 2004).

2.2.3.2 Physical Methods

Significant changes in the structure and physiological functions of fruits and vegetables following infection by fungal pathogens have been recorded. Dried fig (*Ficus carica*) fruits infected by *Aspergillus* spp. emit characteristic bright greenish yellow fluorescence (BGYF) under long-wave light. *A. flavus* and *A. parasiticus* capable of producing the mycotoxin aflatoxin and *A. tamari* and *A. albiacens* were associated with BGYF observed in naturally infected figs. By observing the presence of BGYF under some specific conditions prevailing in California, it may be possible to eliminate the diseased figs (Doster and Michailides 1998). Infection of tomatoes by *Fusarium oxysporum* and *Rhizopus stolonifer* was observed to alter the visible and near infrared (NIR) spectra. Spectral signatures in the frequency domain were analysed using discriminate analysis and models capable of detecting spore-free (control) and inoculated tomatoes. The tomatoes with conidia of the fungal pathogens on their surface could be detected and differentiated accurately (Hahn 2002). On inoculation with *Botrytis cinerea*, causing the gray mold disease, the ripening tomatoes stored at 20°C, produced acetaldehyde, ethanol, ethylene and carbondioxide and their contents were determined. Of these compounds, ethylene was detected at more than 24 h before the first decay symptom became visible. Production of other compounds was recorded much later. Production of ethylene was suggested as an early marker of infection of tomato by *B. cinerea* that could possibly be used for the early recognition of fungal infections (Polevaya et al. 2002).

A solid-phase extraction (SPME) fiber was used to trap volatiles from potatoes cv. Russet Burbank inoculated with *Pythium ultimum*, *P. infestans* or *Fusarium sambucinum* and gas chromatography with flame ionization detector (GC-FID) to fingerprint trapped volatiles. Depending on the disease intensity, the amount of volatiles produced showed variation. In addition, pathogen-specific volatiles were differentiated. The chromatographs of *F. sambucinum* showed two unique peaks at retention time (RT), whereas *P. infestans* produced few peaks and the profile was quite similar to uninoculated control tubers. In contrast, *P. ultimum* produced many peaks and a distinct peak at RT = 1.71 min (Kushalappa and Lui 2002).

2.2.3.3 Chemical Methods

Chemicals have been applied on the harvested fruits either to hasten the ripening or to remove natural pigments that may mask the presence of the pathogens. Treatment of grapevine berries with paraquat may reveal the latent infection of *Botrytis cinerea* (Gindrat and Pezet 1994). Likewise, latent infection of plums by *Monilinia fructicola* could be detected by treatment of fruits with paraquat, helping the growers for taking timely preventive/curative measures (Northover and Cerkauskas 1994). Latent infection of banana by the anthracnose pathogen *Colletotrichum musae* could be detected by treatment with paraquat (500 ppm a.i.) (Rajeswari et al. 1997). The relative efficacy of paraquat and freezing in detecting infection of

strawberry by *Colletotrichum acutatum* and *C. gloeosporioides* in field grown plants and plants inoculated in the greenhouse was assessed. The petioles were killed by both treatments. After incubation for 5–7 days, the acervuli (fruiting bodies of the pathogen) appeared more frequently in the treated petioles than on control petioles. Detection by freezing may be preferable, as it is a nonhazardous procedure (Mertely and Legard 2004).

2.2.3.4 Immunoassays

Immunoassays have been successfully employed for detection and quantification of postharvest fungal pathogens infecting fruits and vegetables. *Botrytis cinerea*, causing gray mold disease in many fruits, was detected and quantified in pear stems after 6 and 8 months of cold storage by employing ELISA test and also by isolation on selective medium. ELISA test was more sensitive than the isolation procedure. Quantitative ELISA format showed that over 200 μg of *B. cinerea* biomass/g of stem tissue was present in visibly rotted tissues, whereas the stems from fruits without visible symptom had about 35 μg/g of tissue. The MAb BC-12-CA4, with high specificity could be employed to trace the infection path of *B. cinerea* and to detect latent infections (Meyer and Spotts 2000). The presence of the powdery mildew pathogen *Uncinula necator* in the grape berries could be detected by using the antiserum generated against the antigens present on the conidia and hyphae. The antibody reacted with three antigens with MW of 21, 29 and >250 kDa present on the conidia in an immunofluorescence assay and also in the ELISA test (Markovic et al. 2002). The activity of invertase in grape berries was stimulated following infection by *Botrytis cinerea*. A new invertase similar to *Botryits* invertase (BIT) was also stimulated in infected berries. The presence of BIT could be detected using anti-BIT IgY antibodies produced in chicken, revealing the possibility of detecting *B. cinerea* infection of grape berries and elimination of infected bunches in order to prevent further spread of the disease during transit and storage (Ruiz and Ruffner 2002).

Fungal pathogens infecting vegetables have been detected by employing both PAbs and MAbs. *Pythium violae* causing cavity spot disease was detected in field-grown carrot roots by employing PAbs in ELISA test (Lyons and White 1992). The MAbs specific to certain epitopes on the cell walls of *Botrytis cinerea* were employed to detect the pathogens in gray mold diseased tomato and other vegetables (Dewey 1998). By using an amperometric immunoenzyme sensor, the possibility of detecting *Phoma betae* antigen at a wide range of concentrations in carrots and beet and also in the seeds was indicated by Khaldeeva et al. (2001).

2.2.3.5 Nucleic Acid-Based Techniques

The nucleic acid-based diagnostic techniques have been shown to be more rapid, reliable, sensitive and specific compared to immunoassays and conventional

detection methods, as in the case of seeds and clonal propagative plant materials. The isolates (39) of *Colletotrichum gloeosporioides*, causing anthracnose diseases of various fruits were detected and differentiated using the probes designed from the ITS regions of rDNA of this pathogen (Mills et al. 1992). The strawberry pathogens *C. 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 absence of amplification of DNA sequences from non-strawberry isolates of *Colletotrichum* (Martinez-Culebras et al. 2003).

The PCR primers specific to the 3′ regions of the intron present only in *Monilinia fructicola* infecting plums together with the small subunit (SSU) rDNA primer NS5, were designed. These primers amplified specifically a 444-bp fragment of the DNA of *M. fructicola* and differentiated related species of *M. fructigena* and *M. laxa.* These primers amplified this PCR product also from plum tissue infected by *M. fructicola*, but not from the other related fungal pathogens, indicating the specificity and reliability of the PCR protocol (Fulton and Brown 1997). *M. fructicola* causing brown rot disease of sweet cherry could be detected at the early stages of infection by employing two primer sets that were designed from DNA sequences of either rDNA (MF 5/ITS4/ITS3) or an RAPD fragment (X-09 int F3/X-09R) that specifically amplified DNA from isolates of *M. fructicola* and *Monilinia* spp. respectively. The specificity of these primers was revealed by the absence of the amplicons from the DNA of *Botrytis cinerea* (gray mold pathogen) and other fungi commonly associated with sweet cherry. Brown rot infection in artificially inoculated sweet cherry fruits could be detected before the appearance of any visible symptoms. Visible and quiescent infections in field-collected fruits were also inferred by the protocol developed in this investigation (Förster and Adaskaveg 2000).

The sequences of microsatellite regions of the genome of *M. fructicola* were used for designing primers. The microsatellite primer M13 amplified the species-specific sequences of the DNA fragment. By using the external and internal primer pairs (EmIfG + EMfR and IMfF + IMfR) amplified 571-bp and 468-bp fragments from *M. fructicola*, but not from other fungi associated with stone fruits (Ma et al. 2003). A multiplex-PCR was developed for the detection and identification of *Monilinia* spp. and *Monilia polystroma* infecting stone fruits. The multiplex-PCR assay consistently produced a 402-bp amplicon from *M. fructigena*, a 535-bp product from *M. fructicola* and a 351-bp product from *M. laxa*. On the other hand, a 425-bp PCR product was obtained for *M. polystroma.* The pathogens present on the naturally or artificially inoculated stored apples were detected and identified precisely (Côté et al. 2004). By applying a multiplex-PCR format using species-specific primers, a newly isolated pathogen *Neofabrae alba*, causing bull's eye rot disease of pear was detected and identified. Likewise, another species *N. perennans* occurring in Washington was also identified. The involvement of these two species in the bull's eye rot disease in nine European pear cultivars, Asian pear and quince was confirmed by the multiplex-PCR protocol developed in this investigation (Henriquez et al. 2004).

Identification and differentiation of *Alternaria infectori*, *A. arborescens* and *A. tenuissima* considered to be involved in core rot of red apple cultivars in South Africa, was possible by performing sequence analysis of the ITS1 and ITS2 regions of the nuclear rDNA gene. The major pathogens associated with core rot disease were indicated to belong to *A. tenuissima* species group (Serdani et al. 1998, 2002). *Alternaria alternata* is known to produce host-specific AM-toxin. The gene *AMT* that plays an important role in the biosynthesis of AM-toxin was cloned and characterized. The sequences of the *AMT* gene were used for designing the primer and employed successfully for the detection of AM-toxin-producing isolates of *A. alternata* apple pathotype (Johnson et al. 2000). A new species of *Alternaria* in YaLi pear fruits was intercepted at US ports. A PCR assay using specific primers for *A. gaisen* showed that none of the isolates from the Ya Li pear fruit from China was like *A. gaisen*. The AMT gene-based assay proved that Ya Li isolates were not similar to *A. mali*. However, all isolates from Ya Li pear produced a 350-bp product with ß-tubulin primers. Hence, this pathogen isolated from Ya Li pear was considered to be a new species and named as *A. yaliinficiens* sp. nov. (Roberts 2005).

Penicillium spp. causing blue mold disease in apple and pear were recovered from fruits as well as from water and floatation tanks in commercial apple juice facilities. The isolates of *Penicillium* spp. were characterized by employing RFLP of the region including the ITS1 and ITS2 and the 5.8S rRNA gene of ribosomal DNA region and RAPD primers. RAPD analysis was found to be a rapid and reliable tool to identify and group the isolates into *P. expansum* and *P. solitum*. The involvement of *P. solitum* in blue mold disease was brought out for the first time by Pianzzola et al. (2004). Isolates of *Geotrichum candidum* causing sour rot disease of citus fruits are morphologically indistinguishable from noncitrus (nonpathogenic) isolates of the fungus. PCR-RFLP analysis of rDNA ITS and PCR using specific primers to pathogenicity (PG) genes from each type could identify the pathogenic isolates and also differentiate them from noncitrus isolates (Fig. 2.16) (Nakamura et al. 2008).

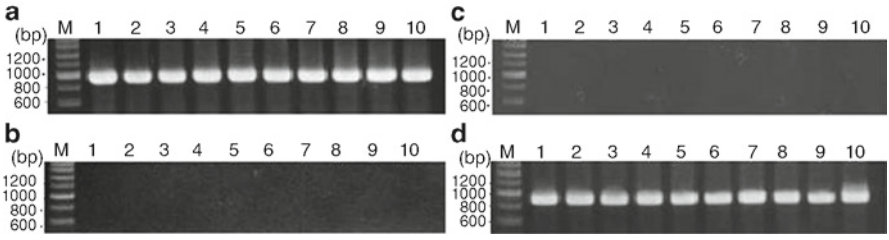

Fig. 2.16 Detection of citrus and noncitrus isolates of *Geotrichum candidum* in citrus fruit tissues using PCR-RFLP analysis by amplifying PG gene of the pathogen followed by digestion with restriction enzyme. (**a**) and (**b**) citrus type isolates. Lane M: 200 bp ladder marker; Lane 1: S31; Lane 2: S7; Lane 3: 148; Lane 4: S181; Lane 5: M13; Lane 6: Te2; Lane 7: Pt3; Lane 8: R2; Lane 9: D2; Lane 10: Kk10. (**c**) and (**d**) Noncitrus type isolates. Lane 1: 63; Lane 2: R9; Lane 3: R10; Lane 4: 21; Lane 5: R22; Lane 6: Tm5; Lane 7: Ig1; Lane 8: W4; Lane 9: W6; Lane 10: Mm2. (**a**) and (**c**) PCR specific to *S31pg1* gene; **b** and **d**: PCR specific to *S63pg1* gene (Courtesy of Nakamura et al. 2008; The Mycological Society of Japan, Kyoto and Springer Science and Business Media, Heidelberg, Germany)

Guignardia citricarpa (anamorph – *Phyllosticta citricarpa*) causes citrus black spot disease which is of quarantine importance. The harmless morphologically similar *G. mangiferae*, an endophyte, is often incorrectly identified as *G. citricarpa* resulting in the withholding of mango consignments. Identification by isolation and morphological characteristics requires 5–14 days with an efficiency of less than 50%. As citrus consignments have to be cleared pathogen-free at the harbor, the value of the consignment decreases rapidly with each additional day spent in holding. Development of a same-day test to unequivocally identify *G. citricarpa* and distinguish it from *G. mangiferae* is essential. Application of the species-specific primer set CITIC1 and CAMEL2 in conjunction with the ITS4 primer yielded PCR amplification ~580-bp and 430-bp fragments for *G. citricarpa* and *G. mangiferae* respectively. Repeated tests confirmed the accuracy and sensitivity of the protocol. Furthermore, no positive PCR amplification occurred with *Colletotrichum gloeosporioides* which is a common contaminant in black spot lesions. This method was further improved to directly isolate DNA from fruit lesions by means of the DNeasy Plant Mini Kit (Qiagen) protocol. This procedure eliminates the need for culturing this slow-growing pathogen, consequently saving substantially the time required to just 1 day to test and verify the presence or absence of *G. citircarpa* in export consignments (Meyer et al. 2006). In another investigation, the available primers were found to be inefficient in differentiating *G. citricarpa* from *G. mangiferae*, when the DNA was extracted directly from single characteristic black spot lesions on citrus fruit. Hence, new primer pairs were designed for both species from the ITS regions that were highly sensitive and specific for detection of *G. citricarpa* using DNA extracted from single fruit lesions by a rapid extraction procedure developed in this investigation (Peres et al. 2007).

Elsinoe fawcetti (*Ef*) and *E. australis* (*Ea*) causative agents of citrus scab and sweet orange scab diseases respectively, were detected, identified and differentiated by employing 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. *Ef* present in the lesions on fruits and leaves could be detected by Efaw-1 and Efaw-2 primer sets, whereas *Ea* was detected in lesions on sweet orange using primer pairs Eaut-1, Eaut-2, Eaut-3 and Eaut-4 (Hyun et al. 2007).

Botrytis cinerea, the causative agent of grapevine Botrytis bunch rot disease remains in the quiescent phase in infected berries in the early stages and becomes activated as the berries mature. For effective management of the bunch rot disease, it is essential to detect and quantify the pathogen population in the quiescent phase. The standard tissue freezing and incubation procedure and two real-time quantitative PCR (qPCR) formats were evaluated for their efficacy in detecting *B. cinerea* in berries at different stages of maturity. The bioassay involving berry surface sterilization, killing of host tissues by freezing and subsequent fungal colonization of the dead berry provided a qualitative detection of *B. cinerea*. Although freezing bioassay was more effective for detecting the infection at early stages than qPCR assay, it was unable to quantify fungal colonization. Of the two qPCR assays, the SYBR

Green format was able to detect and quantify *B. cinerea* with a linear response to a dilution series. But this format was not specific enough to prevent signal amplification in the grape DNA-only negative control. On the other hand, qPCR based on TaqMan chemistry overcame this limitation and allowed detection down to 3.2 pg of *B. cinerea* DNA, with a detection limit of 100 fg. The freezing assay detetected 64.3% incidence of infected pea-sized berries, but qPCR formats failed to detecet any *B. cinerea* DNA in such berries. It was suggested that the combined ability of both assays may be utilized for reliable detection of quiescent infection of berries by *B. cinerea* (Cadle-Davidson 2008).

Oomycete pathogens infecting potato tubers have been detected by employing different nucleic acid-based techniques. Primers were designed based on the sequences of the ITS1 region of rDNAs of *Pythium ultimum* and *P. aphanidermatum* associated with leak syndrome in potato tubers. These primers reliably detected the two pathogens and also differentiated them. This was required because both produce symptoms similar to pink rot disease caused by *Phytophthora erythroseptica* (Triki et al. 2001). Sensitive detection of *Phytophthora infestans* causing the late blight disease in potato tubers was accomplished by a PCR-based assay, using the PINF and ITS5 primers. The pathogen DNA from a single sporangium or oospore could be amplified by PCR after CTAB or NaOH lysis extraction methods. The PINF and ITS5 primers were shown to be a powerful tool for rapid and sensitive detection of zoospores, sporangia and oospores of *P. infestans*, providing a firm basis for elimination of infected tubers, resulting in the restriction of the spread of the pathogen from the infected to healthy tubers during transit and storage (Wangsomboondee and Ristaino 2002).

Tomatoes are infected by several fungal pathogens both in the field and storage conditions. The primer set PB80-1F/PB80-1R specifically amplified a 831-bp fragment in the PCR assay from *Botrytis cinerea* and *B. elliptica.* Both pathogens could be detected and differentiated by the PCR format developed (Chen et al. 1998). *B. cinerea* infection in greenhouse-grown tomatoes was detected by a dot blot hybridization technique. DNA from fungal cultures and infected tomato tissues were amplified by using specific primers in PCR. Probes labeled with digoxigenin were employed for hybridization of amplified sequences. The probe Bot1 hybridized positively with DNA of all isolates of *B. cinerea* and also with fresh or frozen plant tissues infected by *B. cinerea* received from research centers and commercial greenhouses, providing rapid and reliable results (Mathur and Ukhede 2002). The isolates AN1 and AN2 were obtained from immature (green) bell pepper exhibiting anthracnose disease symptoms and these two isolates were identified as *Colletotrichum acutatum* based on morphological and cultural characteristics and a PCR-based assay. The *C. acutatum* species-specific CaInt-2 in conjunction with the ITS4 primer, amplified a 480-bp fragment from the genomic DNA from AN1 and AN2 and reference *C. acutatum* isolates, but not from *C. gloeosporioides*. In contrast, a 450-bp fragment was amplified from the genomic DNA of two *C. gloeosporioides* reference isolates. Thus, the identity of the causative agent of pepper anthracnose was confirmed as *C. acutatum* by the PCR assay (Lewis Ivey et al. 2004).

Appendix 1: General and Selective Media for Isolation of Fungal Pathogens

A. General media

Czapek Dox agar	Solution A
Sodium nitrate	40 g
Potassium chloride	10 g
Magnesium sulphate (hydrous)	10 g
Ferrous sulfate (hydrous)	0.2 g
Distilled water	1 liter
	Solution B
Dipotassium hydrogen phosphate	20 g
Distilled water	1 l
(store the solutions A and B separately in a refrigerator)	
Prepare the mixture of A and B	
Stock solution A	50 ml
Stock solution B	50 ml
Distilled water	900 ml
Sucrose (analar)	30 g
Oxiod agar No.3	20 g
Just before autoclaving add for 1 l	
Zinc sulfate (1.0 g/100 ml water)	1.0 ml
Cupric sulfate (0.5 g/100 ml water)	1.0 ml
Malt extract agar	
White bread malt extract	20 g
Oxoid agar No.3	20 g
Tap water	1 l
Oat agar	
Oat meal ground	30 g
Oxoid agar No.3	20 g
Tap water	1 l
Potato carrot agar	
Grated potato	20 g
Grated carrot	20 g
Oxoid agar No.3	20 g
Tap water	1 l
Potato dextrose agar	
Potatoes	200 g
Oxoid agar No.3	20 g
Dextrose	15 g
Tap water	1 l
V8 agar	
V8 vegetable juice	200 ml
Oxoid agar No.3	20 g
Distilled water	800 ml

(continued)

Appendix 1 (continued)

B. Selective media	
CW medium for *Alternaria brassicola* (Wu and Chen 1999)	
Galactose	30 g
Calcium nitrate	3 g
Dipotassium hydrogen phosphate	1 g
Magnesium sulfate	1 g
Benomyl	100 ppm
Chloramphenicol	100 ppm
Agar	20 g
Distilled water	1 l
Media for *Botrytis cinerea* (Edwards and Seddon 2001)	
(i) Botrytis selective medium	
Glucose	2 g
$NaNO_3$	0.1 g
K_2HPO_4	0.1 g
$MgSO_4.7H_2O$	0.2 g
KCl	0.1 g
Chloramphenicol	0.02 g
Maneb 80 (80% manganese ethyl bisdithiocarbamate)	0.02 g
Rose Bengal	0.05 g
Tannic acid	5.0 g
Oxoid agar No.3	20 g
Water	1 l
Adjust the pH to 4.5 using 1M NaOH prior to addition of agar	
(ii) Botrytis spore trap medium (BSTM)	
Rubigan (12% fenarimol) is used intead of Rose Bengal usedin BSM above	
(iii) Diluted supplemented Malt extract agar (dsMEA)	
Malt extract broth (Oxoid)	4.0 g
Chloramphenicol	0.2 g
Rose Bengal	0.05 g
Oxoid agar No.3	15 g
Water	1 l
Semi-selective medium for *Colletotrichum gloeosporioides* (Ekefan et al. 2000)	
Basal medium – PDA amended with	
Pencycuron	50 mg/l
Tolclofos-methyl	10 mg/l
Streptomycin sulfate	100 mg/l
Chloramphenicol	100 mg/l
Chlortetracycline	100 mg/l
Adjust the pH to 5.0	
Komada's Selective medium for *Fusarium* spp. (Arie et al. 1995)	
K_2HPO_4	1 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
Fe-EDTA	10 mg
L-asparagine monohydrate	2.0 mg
D-(+) galactose	20 g
Pentachloronitrobenzene (PCNB)	0.75 g (a.i.)

(continued)

Appendix 1 (continued)

Sodium chlorate	0.5 g
Sodium tetraborate decahydrate	1 g
Chloramphenicol	0.25 g
Agar	15 g
Distilled water	1 l
Fusarium* selective (FS) medium for *Fusarium circinatum (Schweigkofler et al. 2004)	
Bacto peptone	15 g
Agar	20 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Streptomycin sulfate	0.3 g
Ampicillin	0.1 g
Pentachloronitrobenzene	0.2 g
PDA	39 g
Water	1 l
R-PDA medium for *Gaeumannomyces graminis* var. *tritici* (Duffy and Weller 1994)	
Peeled boiled potatoes	40 g
Dextrose	4 g
Agar	18 g
Deionized water	1 l
Add after autoclaving and adjusting the pH to 6.0–6.5	
Rifampicin	100 µg/ml
Tolclofos-methyl	10 µg/ml

Appendix 2: Assessment of Vegetative Compatibility Relationships Among *Verticillium dahliae* Strains (Joaquim and Rowe 1990; Daayf et al. 1995)

A. Generation of *nit* mutants

(i) Prepare monoconidial subcultures of isolates of the test pathogen in small culture vials containing sterilized soil, perlite and peat moss (1:1:1 v/v/v) and store at 5°C.

(ii) Cut agar discs (5 mm diameter) using a cork borer from the edge of wild-type colonies of the pathogen growing on potato dextrose agar (PDA); transfer to chlorate minimal medium (CMM) amended with 30 g/l of potassium chlorate kept in petridishes (9 cm diameter) and incubate for 10 days at 25°C.

(iii) Cut a small segment (1 cm diameter) from the margin of the chlorate-resistant colony; transfer into a tube containing sterile water (8 ml); agitate well to disperse the conidia from the medium and adjust the conidial concentration to 1×10^4/ml using a hemacytometer.

(iv) Dispense aliquots of 50 µl of conidial suspension into petridishes containing CMM; incubate for about 40 h and transfer individual germinated conidia

onto minimal medium (MM) placed in the petridishes to select distinct chlorate-resistant monoconidial strains.

(v) Observe the colony morphology; thin and expansive mycelium on MM medium indicates the inability to metabolize nitrate; these colonies are considered as *nit* mutatnts.

B. Characterization of *nit* mutants

(i) Transfer a segment of the mycelium of each chlorate-resistant mutant onto basal medium (MM without nitrogen source) supplemented with one of the following nitrogen sources: (a) sodium nitrate (0.2 g/l), (b) sodium nitrite (0.4 g/l), (c) hypoxanthine (0.5 g/l), or (d) ammonium tartarate (0.8 g/l) buffered with calcium carbonate and examine the growth response of each mutant in each nitrogen source.

(ii) Assign *nit* mutants the phenotype identity as follows:
   (a) *Nit1* mutants – unable to utilize nitrate, but capable of using nitrite, ammonium, hypoxanthine and uric acid.
   (b) *Nit* mutants – unable to use nitrate and hypoxanthine, but can use the other three nitrogen sources.

C. Assignment of strains to vegetative compatibility groups (VCGs)

(i) Prepare complementary tester strains (Nit1 and NitM) for the strains to be assigned to VCG.

(ii) Pair the tester strains with *nit* mutants by placing a *Nit1* or *NitM* mutant derived from one strain at the center of each plate containing MM; place Nit1 and NitM (derived from a tester strain representing a specific VCG) each 1.0–1.5 cm apart on either side.

(iii) Perform pairing twice for each mutant.

(iv) Observe the prototrophic growth developing at the mycelial interface between the *nit* mutant positioned at the center; score the density of growth after 18–24 days of inoculation.

Appendix 3: Media for Generation and Selection of Vegetative Compatibility Groups (VCGs) of *Fusarium oxysporum* (Correll et al. 1987)

A. Basal medium: Sucrose – 30 g; KH_2PO_4 – 1 g; $MgSO_4.7H_2O$ – 0.5 g; KCl – 0.5 g; $FeSO_4.7H_2O$ – 10 mg; agar – 20 g; trace element solution 0.2 ml; water 1,000 ml (Trace element solution: $ZnSO_4.7H_2O$ – 5.0 g; $Fe(NH_4)_2(SO_4)_2.6H_2O$ – 1.0 g; $CuSO_4.5H_2O$ – 0.25 g; $MnSO_4.H_2O$ – 50 mg; H_3BO_4 – 50 mg; NaMoO4.$2H_2O$ – 50 mg; water – 95 ml)

B. Complete medium: Basal medium – 1 l; NaNO – 2.0 g; N-Z amine (Sheffield) – 2.5 g; yeast extract (Difco) – 1 g; vitamin solution – 10 ml (Vitamin solution: Thiamine HCl – 100 mg; riboflavin – 30 mg; pyridoxine HCl – 75 mg; D-pantothenate-Ca – 200 mg; p-aminobenzoic acid – 5 mg; nicotinamide – 75 mg;

cholineCl – 200 mg; folic acid – 5 mg; D-biotin – 5 mg; myoinositol – 4 mg; ethanol (50%) – 100 ml)

C. Minimal medium: Basal medium 1 l; $NaNO_3$ – 2 g

D. Minimal agar medium with chlorate (MMC): Minimal medium 1 l; L-asparagine 1.6 g; $NaNO_3$ – 2 g; $KClO_3$ – 15 g

E. Potato dextrose agar medium with chlorate (PDC): Potato dextrose broth (Difco) – 24 g; agar – 20 g; $KClO_3$ – 15 g; water – 1,000 ml

Appendix 4: Generation of Antibodies Against Fungi (Banks et al. 1992)

A. Preparation of antigen

(i) Prepare spore suspensions using 0.01% Tween 80; wash thrice by centrifugation; inoculate 1 ml of spore suspension (10^6 spores/ml) into 100 ml of liquid medium supplemented with NaCl (100 g/l) and incubate at 25°C for 7 days in the dark by placing the flask with contents on a rotary shaker.

(ii) Transfer the mycelium by filtering into a sintered glass filter; wash with sterile water and then with sterile phosphate-buffered saline (PBS) containing 2.9 g $Na_2HPO_4.12H_2O$, 0.2 g KH_2PO_4, 8.0 g NaCl and 0.2 g KCl and 1,000 ml distilled water; freeze overnight at −20°C; thaw and transfer to centrifuge tubes and dry in vacuum dryer.

(iii) Collect the mycelium and add 50 ml of liquid nitrogen; mince the mycelium in a blender for 1 min and grind in a mortar with a pestle to a fine powder.

(iv) Suspend the mycelial powder in PBS (200 mg in 100 ml); centrifuge at 4,500 rpm (3,000 g) for 10 min at 4°C and divide the supernatant containing soluble nitrogen into 0.5 ml aliquots and store at −20°C.

(v) Estimate the total protein content of the antigen preparation.

B. Production of polyclonal antiserum

(i) Mix soluble antigen preparation with equal volumes of Freund's complete adjuvant (Difco) to produce a final protein concentration of the mixture at 1 mg/ml.

(ii) Inject rabbits intramuscularly with 1 ml of the mixture at predetermined intervals.

(iii) Bleed the animal at 4 weeks after the first injection and subsequently at 14, 16 and 18 weeks.

(iv) Separate the serum after completion of clotting of blood cells followed by centrifugation.

C. Production of monoclonal antiserum

(i) Mix soluble antigen preparation with an equal amount of Freund's complete adjuvant to yield a final protein concentration of 1 mg/ml.

(ii) Inject a BALB/c mouse, after anaesthetization with 0.1 ml of the immunogen intraperitoneally and subsequently at 2, 4, 6 and 8 weeks after the first injection with PBS and remove the spleen after sacrificing the animal by cervical dislocation.

(iii) Carry out fusion of splenocytes with selected myeloma cell line (P3-NS-1-Ag4) at a ratio of 1×10^8: 5×10^7 by gentle addition of 2 ml of 30% polyethylene glycol (PEG) (w/v) over 60 s.
(iv) Add 10 ml of warm serum-free RPMI 1640 medium (Gibco) over next 60 s with gentle stirring; add another 20 ml of RPMI and centrifuge for 3 min at 400 g at room temperature.
(v) Suspend the pellet of cells in 50 ml of growth medium (RPMI 1640) with 20% Myclone fetal calf serum (FCS) (v/v); dispense cell suspension into five 96-well microplates at 100 µl/well.
(vi) Add 110 µl of hypoxanthine aminopterin–thymidine (HAT) medium diluted to 1:50 in growth medium to each well in the fusion plates.
(vii) Add growth medium + HAT on 2, 4, 7 and 10 days by removing 100 µl of the medium and replacing with 100 µl of fresh medium.
(viii) Screen the hybridoma cells for efficiency of antibody production by indirect ELISA procedure.
(ix) Clone healthy growing hybridoma twice by limiting dilution in a non-selective medium; preserve by freezing slowly in 7.5% dimethyl sulfoxide (DMSO) and store in liquid nitrogen.

Appendix 5: Detection of *Botrytis cinerea* by Enzyme-linked Immunosorbent Assay (ELISA) Test (Bossi and Dewey 1992)

A. Preparation of antigen
(i) Prepare surface washings of the pathogen (*B. cinerea*) grown on PDA for 17–20 days at 21°C, using 5 ml/petridish of phosphate-buffered saline (PBS) containing 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.25 g KH_2PO_4, and water 1,000 ml at pH 7.2 and remove the wash suspension by suction.
(ii) Centrifuge the wash fluid for 3 min at 13,000 g to remove the fungal debris and dilute the supernatant with PBS to have tenfold dilutions.
(iii) Remove the high MW carbohydrates and glycoproteins by passing the cell-free wash fluid through a Centricon 30-kDa filter (Amicon No. 4208) to prevent induction of nonspecific antibodies; freeze-dry the filtrate and redissolve the contents in 1 ml of distilled water and use it as the antigen.

B. Enzyme-linked immunosorbent assay (ELISA)
(i) Coat the wells (in triplicate) in the 96-well microtiter plates with PBS surface washing fluid (50 µl/well) overnight and wash the wells four times allowing two min for each washing followed by a brief washing with distilled water.
(ii) Air-dry the plates in a laminar flow hood and seal them in a polythene bag and store at 4°C.
(iii) Incubate the plates successfully with hybridoma supernatants for 1 h, then with a 1/200 dilution of a commercial goat antimouse polyvalent (IgG + IgM)

peroxidase conjugate and finally with PBS with 0.05% Tween-20 (PBST) for 1 h more.

(iv) Add the substrate solution containing tetramethyl benzidine (100 µg/ml) for 30 min.

(v) Maintain the controls incubated tissue culture medium containing 5% fetal bovine serum (FBS) in place of hybridoma supernatant.

(vi) Stop the reaction by adding 3 M H_2SO_4 (50 µl/well); determine the intensity of color developed in each well using ELISA reader at 450 nm.

(vii) Absorbance levels more than three times greater than those of controls indicate positive reaction and presence of antigen protein.

Appendix 6: Quantitative Detection of *Mycosphaerella fijiensis* by Triple Antibody Sandwich (TAS)-ELISA (Otero et al. 2007)

A. Preparation of antigens

(i) Cultivate the fungal pathogen in appropriate medium under optimal growth conditions required; transfer the mycelial disks from the nutrient medium to 250 ml tissue culture flasks containing 50 ml of sterile potato dextrose broth (Difco) and incubate for 3–7 days at room temperature.

(ii) Transfer the mycelial suspensions aseptically to 250 ml flasks; incubate with shaking (80 rpm) at 26°C and harvest the mycelia using cellulose filters in a Buchner funnel under vacuum.

(iii) Dry the harvested mycelia (10 g) of 18 days old; mince in liquid nitrogen and resuspend the homogenate in protein extraction buffer containing 50 mM Tris HCl, pH 8.0, 1 mM phenylmethane sulphonyl fluoride (PMSF), 2 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM 1-4-dithio-DL-threitol under agitation at room temperature.

(iv) Precipitate the protein at 80% ammonium sulfate followed by destalting (Sephadex G-25) into phosphate-buffered saline (PBS) and determine the protein contents of the samples by the Coomassie method (Bradford 1976).

(v) Prepare the secreted protein antigen from the metabolized pathogen culture suspension media by concentration, precipitation at 80% ammonium sulfate and dialysis against PBS and determine protein content of the sample as done earlier (step iv).

(vi) Prepare the antigens from the leaves by washing with distilled water; cut them into fragments; powder the fragments (10 g) using liquid nitrogen followed by mixing (1:3 w/v) with alkaline extraction buffer containing Tris (50 mM), EDTA (10 mM), ascorbic acid (0.2%), sodium chloride (150 mM), 2-mercaptoethanol (20 mM), PMSF (0.57 mM), Tritox X-100 (1.5%, pH 7.5); keep the mixture at 4°C for 1 h; centrifuge at 2,000 × g for 10 min; precipitate the protein at 80% ammonium sulfate; dialyze against PBS and estimate the protein content as done earlier (step iv).

B. Preparation of polyclonal antiserum

(i) Emulsify mycelial antigen preparation (100 µg) in 2 ml of complete Freund's adjuvant (Sigma) and inject into female New Zealand adult rabbits subcutaneously and inject the same dose of antigen emulsified in incomplete Freund's adjuvant (Sigma) at 2-week interval until the titer rises to 1:32 by Ouchterlony double immunodiffusion method.

(ii) Bleed the animal; precipitate at 50% ammonium sulfate, desalt and fractionate on a matrix of diethyl aminoethyl Sepharose (Amersham-Bioscience) to have suitable IgG fraction.

(iii) Determine the protein content following bicinchroninic acid method (Smith et al. 1985).

C. Preparation of monoclonal antiserum

(i) Immunize 6–8 weeks old female BALB/c mice by intraperitoneal route with 50 µg of mycelial antigen in 1:1 (v/v) emulsion of complete Freund's adjuvant (Sigma) and PBS and administer subsequent doses of mycelial antigen (50 µg) emulsified with incomplete Freund's adjuvant (1:1 v/v) and PBS by subcutaneous route at 2 and 4 weeks after initial immunization.

(ii) Obtain serum before and at 2 weeks after each immunization and analyze for antibodies reacting to pathogen mycelial antigen and assess the antibody response using an indirect ELISA.

(iii) Give booster injection by intravenous route to the mouse with the highest antibody titer, with 25 µg of antigen in PBS; sacrifice the mouse by CO_2 asphyxiation and remove the spleen.

(iv) Follow the polyethylene glycol-based procedure for fusion of spleen cells with murine plasmacytoma cells Sp. 2/0-Ag 14 (ATCC No. CRL-1581) and identify positive clones by evaluating the supernatant using indirect ELISA method, using hybridoma culture medium.

(v) Clone the hybridoma-producing antibody reactive to both mycelial and secreted antigens of the fungal pathogen using limiting dilution to recover homogeneous hybridoma cell line.

(vi) Inoculate the selected hybridoma (2H6H8) into BALB/c mice by intraperitoneal route; collect the ascetic fluid and purify the antibody using affinity chromatography in a Protein G Sepharose Fast Flow (Amersham-Biosciences) column as per the manufacturer's recommendations.

(vii) Dialyze the antibody against PBS and determine the protein concentration as done earlier (Step B. iii); sterilize the antibody solution by filtration through a 0.22 µm nitrocellulose membrane.

D. Double antibody sandwich (DAS)-ELISA test

(i) Coat the microtiter plates with 100 µl of a solution containing 10 µg/ml of the target pathogen in carbonate–bicarbonate buffer pH 9.6 and incubated at 4°C for 16 h.

(ii) Wash the plates thrice with PBS-T after completion of the incubation period; block the wells using 3% BSA in PBS for 1 h to prevent nonspecific binding;

dispense PAb solution (50–200 µg/ml) diluted in PBS-T solution to the antigen coated wells and incubate at 37°C for 1 h.

(iii) Wash the wells as done before; transfer a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma) to the wells and incubate for 1 h.

(iv) Add *p*-nitrophyenyl phosphate disodium hydrate in diethanolamine buffer, pH 9.8 and determine absorbance at 405 nm, after stopping the reaction by adding concentrated sulfuric acid.

E. Triple antibody system (TAS)-ELISA test

(i) Coat the microplates with 100 µl of a solution containing 10 µg/ml of MAb recognizing the mycelial antigen in carbonate-bicarbonate buffer, pH 9.6; incubate for 16 h at 4°C; wash the plates thrice with PBS-T after completion of incubation period.

(ii) Block the wells as done before (step D iii); dilute the antigen (1.25–40 µg/ml) in the blocking solution and transfer to the wells and incubate for 2 h at 37°C.

(iii) Dispense 20 µg/ml of the second antibody PAb (anti-pathogen) and follow other steps as in DAS-ELISA test.

Appendix 7: Detection of Resting Spores of *Plasmodiophora brassicae* in Plant Tissues by ELISA (Orihara and Yamamoto 1998)

A. Preparation of immunogen

(i) Homogenize club root-infected roots and hypocotyls (850 g) in distilled water for 5 min using a blender; filter the homogenate through eight layers of gauze; centrifuge the filtrate at 3,000 rpm for 20 min; resuspend the pellet in distilled water and repeat centrifugation cycle five times.

(ii) Prepare a sucrose density column (with 20% and 40% sucrose solutions) in a transparent centrifuge tube; overlayer the final suspension containing resting spores and plant cell debris on sucrose gradient column and centrifuge at 3,000 rpm for 20 min.

(iii) Collect the layer containing resting spores; wash with distilled water five times and store at −20°C.

B. Preparation of antiserum

(i) Inject the rabbit intramuscularly with 0.5 ml of immunogen preparation (6×10^7) purified resting spores/ml of 0.85% NaCl solution); inject again intramuscularly with a mixture of 1 ml of immunogen and 1 ml of Freund's complete adjuvant after an interval of 2 weeks; administer additional dose of immunogen (0.5 ml containing 5.4×10^7 resting spores/ml) intravenously.

(ii) Collect the blood serum after a rest period of 2 weeks; purify the antibodies by ammonium sulfate preparation and DEAE-cellulose column chromatography.

C. Indirect ELISA

(i) Collect the club root-infected roots and hypocotyls and similar healthy tissues and store at −20°C; homogenize the samples separately in distilled water for 5 min; filter as done earlier (step A i above) and adjust the spore concentration to 1×10^6 spores/ml and dilute healthy samples to the same volume.

(ii) Suspend the sample extracts in coating buffer (carbonate) and dilute to required level and transfer 200 µl of each sample to two wells of microtiter plates and incubate overnight at 4°C.

(iii) Wash the wells thrice with PBS-containing polyvinyl pyrrolidone (2%) and BSA (2%) and incubate for 1 h at room temperature.

(iv) Dispense to each well PBS containing 2 µg/ml of anti-resting spore IgG; incubate at 37°C for 4 h and wash the wells as done earlier.

(v) Add PBS containing goat anti-rabbit IgG-alkaline phosphate conjugate at a dilution of 1/2,000; incubate for 4 h at 37°C and wash the wells as done earlier.

(vi) Add 1 ml of diethanolamine (10%), pH 9.8 containing *p*-nitrophenyl phosphate (enzyme substrate) to each well; incubate for 5 min at 37°C in the dark and record the absorbance values at 405 nm using an ELISA reader.

D. Dot immunobinding assay (DIBA)

(i) Spot the samples of 2 µl onto a 40 cm^2 nitrocellulose membrane sheet (Trans-Blot, BIO-RAD, USA); air dry and block nonspecific binding sites by immersing the membrane in a buffer solution consisting of 20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20, pH 7.5 (TTBS), 2% polyvinyl pyrrolidone (PVP) and 2% BSA.

(ii) Treat the membrane with 0.1–0.2 µg/ml of anti-resting spore IgG in TTBS containing 2% PVP and 0.2% BSA (TTBSPB) for 1 h at room temperature.

(iii) Treat the membrane with alkaline phosphatase conjugated goat anti-rabbit IgG in TTBSPB for 1 h and then with buffer consisting of 0.1 M Tris-HCl, 0.1 M NaCl and $MgCl_2$, pH 9.5 containing 0.33 mg/ml of nitroblue tetrazolium substrate and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt prediluted with *N,N*-dimethylformamide.

Appendix 8: Detection of *Fusarium* spp. by Direct Tissue Blot Immunoassay (DTBIA) (Arie et al. 1995; Arie et al. 1998)

(i) Prepare cross sections of stems of infected plants (3 mm thick); place them on nitrocellulose membrane (0.45 µm, pore size) (Trans-Blot Transfer Medium, BIO-RAD USA) saturated with Tris-buffered saline (TBS), pH 7.0 for 10–30 min for direct tissue blotting.

(ii) Immerse the membrane in a blocking solution containing fetal calf serum (FCS) (10% v/v) and BSA 1.0% w/v) in TBS (FB-TBS) for 1 h at room temperature.

(iii) Incubate with specific MAb (AP 19–2) diluted in FB-TBS for 1 h at room temperature and wash the membrane thrice in TBS containing Tween-20 (0.1%) (TBST).

(iv) Incubate the membrane with a mixture of biotinylated anti-mouse IgM-goat IGg, diluted to 1:500 in FB-TBS and horseradish peroxidase-avidin D conjugates (diluted 500 times) for 1 h at room temperature.
(v) Wash the membrane with TBST for 5 min and repeat washing twice; immerse the membrane in substrate solution containing 4-chloro-1-naphthol and 0.02% hydrogen peroxide (v/v).
(vi) Observe for the development of blue color indicating positive reaction.

Appendix 9: Detection of *Fusarium* spp. in Tomato by Immunofluorescence Assay (Arie et al. 1995)

(i) Cut transverse sections (3 cm diameter, 0.3 mm thick) from fresh stem, crown or root of test plants using a sharp razor blade.
(ii) Immerse the sections in blocking solution containing 1% gelatin and 10% BSA in 0.1 M phosphate buffered saline (PBS, pH 7.0) for 2 h at room temperature.
(iii) Soak the sections in the MAb (AP19-2) solution diluted in PBS containing 0.1% Tween-20 (PBST) for 2 h at room temperature and then wash the sections thrice in PBST.
(iv) Incubate the sections with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM diluted 500 times with PBST for 2 h at room temperature.
(v) Observe the sections under a reflecting fluorescence microscope (BHS-RF-A, Olympus, Japan) by ß-excitation.

Appendix 10: Detection of *Polymyxa graminis* by Fluorescent Antibody Technique (FAT) (Delfosse et al. 2000)

(i) Prepare root fragments (~5 mm long) from healthy and infected plants; fix them in 3% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2 prepared under vacuum); wash in phosphate buffer; dehydrate in graded series of ethanol and embed in epoxy resin (Ladd Research Industries, USA).
(ii) Cut transverse sections (~8 µm thick) of embedded fragments; transfer to glass multispot slides and heat briefly on a hot plate at 50°C for the sections to adhere to the glass.
(iii) Conjugate GAR-IgG (Sigma R-3128, Sigma Chemical Co., USA) with fluorescein 5-isothiocyanate (FITC) (Sigma F-7250) by the procedure detailed below.
(iv) Dissolve 15 mg FITC in 1 ml of dimethyl sulfoxide (Sigma D-2650) and mix with 14 ml of 0.1 M sodium carbonate buffer, pH 9.6; dissolve one mg of GAR-IgG in 1 ml of carbonate buffer; dialyze against 15 ml of FITC solution overnight in cold room and remove excess FITC by dialysis against PBS.

(v) Cross-absorb crude antiserum against the pathogen (*P. graminis*) with an equal volume of healthy sorghum root extract (dried roots at 0.4% w/v) prepared in conjugate buffer; remove the immuoprecipitate by centrifugation and repeat the cross-absorption process three times.
(vi) Extract IgG for the pathogen from the supernatant with neutral ammonium sulfate and use at a concentration (100 µg/ml).
(vii) Stain the thin sections in glass multispot slides using 20 µl of reagent/window in each step; soak sections in PBS-Tween containing 10% low fat milk (blocking buffer) for 1 h at 37°C and wash the sections under a gentle stream of distilled water.
(viii) Soak the slides in pathogen IgG for 3 h at 37°C or overnight at 5°C prepared in blocking buffer and wash the sections in distilled water.
(ix) Add FITC-labeled GAR-IgG at a dilution of 1:20 prepared in blocking buffer; incubate for 3 h at 37°C and wash the sections in distilled water.
(x) Mount the sections in 90% glycerol in 0.1 M PBS; examine under the microscope with a provision for epifluorescence and photograph the desired tissues of healthy and infected roots for inference, using a Kodak 400 ASA color reversal film at a magnification of × 80 or × 100.

Appendix 11: Detection of *Botrytis cinerea* by Protein A-Gold Labeling Technique (Svircev et al. 1986)

A. Immunogen preparation
(i) Separate the mat of mycelia and spores; treat with 0.5% formalin; centrifuge the suspension of fungal cells at 1,700 g for 10 min; resuspend the pellet in distilled water; repeat washing and centrifugation cycle three times and resuspend the mycelial mass in 2 ml of Freund' complete adjuvant to have a concentration of 10^6 cells/ml.
(ii) Adminster the fungal preparation intramuscularly followed by the booster dose (second injection) after an interval of 2 weeks.
(iii) Collect the blood, separate the antiserum after centrifugation and store at –20°C.

B. Preparation of proteina-gold label
(i) Prepare the colloidal gold particles (15 mm diameter) by adding 4 ml of aqueous sodium citrate (1%) to 100 ml of a boiling solution of chloroauric acid (0.01); cool the mixture for 5 min until a wine red color develops and store at 4°C in the dark.
(ii) Prepare the protein A-gold complex by adjusting the pH of colloidal gold suspension (10 ml) to pH 6.9 using potassium carbonate and add 0.3 ng protein A (Sigma, USA) in 0.2 ml of distilled water.
(iii) Centrifuge at 48,000 g at 4°C to remove the excess unbound protein A.
(iv) Resuspend the dark red protein A-gold pellet in 10 ml of 0.01 M PBS, pH 7.4 and store at 4°C.

Protein A-gold label may be stable for 6–8 weeks.

C. Protein A-gold labeling

(i) Float thin sections of plant tissue to be tested onto a saturated sodium periodate solution to remove osmium tetroxide used as a fixative for 2–3 min.
(ii) Wash the sections with distilled water three times and treat with 1% ovalbumin for 5 min to block non-specific binding sites.
(iii) Float the sections on drops of specific antiserum placed on coated electron microscope grids and wash the sections thoroughly by passing the grids through a series of water drops.
(iv) Treat the sections with protein A-gold solution for 30 min; wash with drops of water as done earlier and stain with 3% uranyl acetate for 20 min.
(v) Examine the grids under the electron microscope.

Appendix 12: Detection of Fungal Pathogens with DNA Probes (Tisserat et al. 1991)

(i) Transfer 200–400 mg of infected plant tissues in a 1.5 ml microfuge tube; freeze by adding liquid nitrogen and grind with smooth lipped steel rod.
(ii) Suspend the macerate in 600 µl 2 × CTAB buffer (2 × CTAB = 1.4 M NaCl, 2% hexadecyl triethyl ammonium bromide, 1% 2-mercaptoethanol, 10 mM Tris-HCl, pH 8.0 and extract with chloroform.
(iii) Precipitate by adding 0.8 volume of isopropanol and resuspend the pelleted DNA in 40 µl TE buffer (TE = 10 mM Tris, pH 7.6; 1 mM EDTA).
(iv) Denature DNA at 95°C for 4 min; transfer 20 µl to a nylon membrane in a slot-blot apparatus and bake at 80°C for 2 h.
(v) Hybridize with as for Southern hybridizations.

Appendix 13: Identification of Fungal Pathogens by Repetitive DNA Polymorphism (Panabieres et al. 1989)

A. Fungal DNA preparation

(i) Cultivate the target fungus in an appropriate medium; harvest the cultures by filtration on filter paper under vacuum; rinse the mycelia in 250 ml of distilled water and store by freezing.
(ii) Grind the frozen mycelium (250 mg) in liquid nitrogen; suspend the powdered mycelium in 0.5 ml of NIB buffer containing 100 mM NaCl, 30 mM Tris-HCl, pH 8.0; 10 mM ß-mercaptoethanol; 0.5% NP-40 9v/v) and centrifuge for 1 min at 12,000 g.
(iii) Resuspend the pellet in NIB buffer; repeat the procedure in (ii) above; resuspend the pellet in 0.8 ml of homogenization buffer consisting of 0.1 M NaCl, 0.2 M sucrose and 10 mM EDTA; add 0.2 ml of lysis buffer containing 0.25 M EDTA; 0.5 M Tris, pH 9.2 and 2.5% sodium dodecylsulfate and incubate at 55°C for 30 min.

(iv) Extract twice with one volume of phenol-chloroform isoamyalcohol (50:48:2) and then with one volume of ether.
(v) Add one volume of ethanol; centrifuge for one min in a microcentrifuge at room temperature and collect the DNA as pellet.
(vi) Wash the pellet with 70% ethanol; centrifuge again; resuspend in 50 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA) and store at −20°C.

B. Digestion of DNA and electrophoresis analysis
(i) Digest 5 µg of total DNA overnight with 20 units of restriction enzyme as per the manufacturer's instruction.
(ii) Separate DNA fragments on 1% agarose gels at 5 V/cm in 90 mM Tris borate buffer, pH 8.3.
(iii) Stain the gels with ethidium bromide; view under UV light.

Appendix 14: Rapid Extraction of DNA from *Fusarium oxysporum* (Plyler et al. 1999)

(i) Remove 1-cm^2 block of fungal growth from the colony edge of single-spore cultures; place the mycelium in a 1.5-ml Eppendorf tube containing 150 µl Tris-EDTA (TE) buffer and grind the mycelium using sterile wooden sticks.
(ii) Place the tubes in liquid nitrogen for 3–4 min; thaw the tubes in a water bath at 22°C for 5 min; return the tube to liquid nitrogen followed by thawing at 22°C and repeat the cycle three or four times.
(iii) Place the tubes at 65°C in a water bath for 15 min; centrifuge at 11,500 g for 10 min; transfer the supernatant to new Eppendorf tube; add an equal volume of chloroform-octanol (24:1) to the supernatant and mix the contents by vigorous shaking.
(iv) Centrifuge at 12,000 g for 10 min and dilute the contents by tenfold for use in PCR assays. If necessary adopt the following additional steps for further purification.
(v) Mix 24 µl of the supernatant with 16 µl of isopropanol in a separate tube to precipitate DNA; centrifuge for 5 min and drain isopropanol from the tubes.
(vi) Add 16 µl of 70% ethanol to wash the pellet; centrifuge for 5 min; dry the pellet under a laminar flow hood; resupend the pellet in 2 µl TE buffer and use it for PCR amplification.

Appendix 15: Extraction of Genomic DNA from *Claviceps* sp. by Magnetic Separation (Scott Jr et al. 2002)

(i) Cultivate the fungus (*Claviceps africana*) in YM broth (Difco) at 22°C in darkness and lyophilize the mycelium.
(ii) Grind 5–10 mg of lyophilized mycelium to a fine powder in liquid nitrogen; resuspend the ground material in 250 µl of DNA X –Tract™ solution 1

(D2 BioTechnologies Inc., USA); mix the suspension with an equal volume of DNA X-Tract™ solution 2 (high salt buffer) in a 1.5 ml microfuge tube and add 500 µl of chloroform/isoamyl alcohol (24:1).

(iii) Vortex the mixture vigorously; centrifuge at 10,000 g for 5 min; transfer the aqueous phase to a new tube; mix the suspension with 250 µl of DNA X Tract™ precipitation solution and 250 µl of DNA X-Tract™ solution 3 and incubate for 30 min on ice.

(iv) Precipitate the DNA by centrifuging at 10,000 g for 15 min in a microcentrifuge and save the pellet.

(v) Resuspend the maganetic particles (Dynabeads, DNA Direct System Dynal Inc., USA) by gentle swirling to get a homogenous dispersion of magnetic microparticles in solution and equilibrate to room temperature as per manufacturer's instructions.

(vi) Transfer 200 µl of magnetic particle solution to a sterile 1.5 ml microcentrifuge tube and place the tube in a magnetic stand (Dynal MPC) to allow the magnetic particles to complex to the sides of the tube and transfer the supernatant, after 2 min, to the tube containing the pellet of DNA (step iv above).

(vii) Resuspend the pellet by flicking and breaking up with a pipette tip; transfer back the contents to the tube containing the magnetic microparticles (step vi above) and incubate the magnetic microparticles-DNA mixture for 10 min at room temperature.

(viii) Place the tube again in the magnetic stand to allow the DNA-magnetic microparticle complex to aggregate to the sides of the tube and carefully pipette out the supernatant solution.

(ix) Resuspend the complex in 200 µl of washing buffer; place the tube in the magnetic stand; allow it stand till the supernatant becomes clear and repeat washing once again.

(x) Resuspend the complex in 30 µl of resuspension buffer and use the suspension either directly or after dilution (1:10) in PCR reactions.

(xi) Alternatively, elute the DNA by incubation at 65°C for 5 min and place the tube in the magnetic stand to allow the magnetic microparticles to complex to the sides of the tube.

(xii) Transfer the supernatant containing the DNA to new tube for use in PCR.

Appendix 16: Extraction of DNA from Fungal Cultures (Griffin et al. 2002)

(i) Grow the test fungal pathogen in suitable medium; transfer 1 ml of culture suspension to a sterile cryogenic storage tube containing 200 µl of sterile glycerol and store at −70°C.

(ii) Streak out the fungus onto plates containing R2A agar (Fisher Scientific, USA) and incubate for 2 days at room temperature.

(iii) Transfer the fungal tissue (~2.5 mg) from each isolate/species in a sterile 1.5 ml microcentrifuge tube and add to each tube 400 µl of AP1 buffer (DNeasy Plant Mini Kit, Qiagen) and 4 µl RNase (supplied with the kit).
(iv) Apply freeze/thaw cycle to lyse fungal cells using crushed ice/ethanol and a boiling water bath; repeat the cycle seven times and boil for 30 min in a water bath, after the last cycle of freeze/thaw cycle.
(v) Use a sterile 1 ml micropipette tip to grind any visible tissue in the tubes briefly (5 s) between the tip and conical bottom of the microcentrifuge tube.
(vi) Follow DNeasy Plant Mini Kit 'Protocol for isolation of DNA from plant tissue procedure starting with step 4 (add 130 µl of buffer AP2…).
(vii) Elute the DNA in 50 µl buffer AE and use 5 µl of diluted DNA for PCR amplification.

B. Bead-beating extraction of fungal DNA
(i) Streak the isolates from storage (−70°C) onto plates containing R2A agar and incubate for 2 days at room temperature.
(ii) Transfer the fungal tissue (2.5 mg) of each isolate/species to sterile 2 ml cryogenic/microcentirfuge tubes fitted with an O-ring; add to each sample 400 µl AP1 buffer (DNeasy Plant Mini Kit) and 4 µl of RNase A (from the kit); transfer sterile glass bead (~100 µl, 0.1 mm diameter) (BioSpec Products Inc., USA) and load the tubes in a Mini-BeadBeater-8 (BioSpec Products).
(iii) Allow the beater to work for 2 min at maximum speed and repeat the bead-beating/cooling cycle twice.
(iv) Centrifuge the samples for 10 min at 14,000 rpm in microcentrifuge.
(v) Transfer the supernatant fluid from each tube separately to sterile 1.5 ml microcentrifuge tubes.
(vi) Perform DNeasy Plant Mini Kit-Protocol for isolation of DNA from plant tissues procedure starting from step 4 (add 130 µl of buffer AP2).
(vii) Elute the DNA in 50 µl buffer AE and use 5 µl of eluted DNA in PCR assay.

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

A. Growing and disruption of pathogen mycelium
(i) Use appropriate medium kept in petridishes for multiplication of the target pathogen and after the required incubation period gently scrap the mycelium from the top surface of the medium.
(ii) Dispense 1 ml of PARP-V6 broth amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin and 25 ppm pentachloronitrobenzene (PCNB) into each of the 24-deepwell (DW) Uniplate microtitter plates (Whatman Inc., USA) containing 10-ml wells; transfer wefts of mycelium scrapped from culture plates; cover the plates with ryan breathable tape and incubate the plates for 6 days at room temperature.

(iii) Dispense the glass balls using a Millipore dry dispensing plate; transfer the pathogen colonies developing into a 96-well 2-ml DW plate containing three 3 mm glass balls/well; cover the plates with Aeraseal ryon breathable tape (PGC Scientifics, USA) and freeze the contents at −80°C for at lease 1 h.
(iv) Lyophilize the samples for a period of 48 h; use the Labconco stoppering tray drying systems (STDS) (Labconco Corp., USA) with incubation chamber at 0°C for 24 h, followed by 24 h with incubation at 23°C.
(v) Remove the samples from the chamber and apply a capmat immediately to deepwell plates with a capmat applicator (CMA) (Fisher Scientific).
(vi) Disrupt the samples with MM 300 for a total of 2 min on the highest setting of 30 rpm; rotate the 96-well deepwell plate 180°, after bashing 1 min and bash again for an additional minute.

B. Extraction of DNA (Adatation of Qiagen DNeasy 96 Plant Kit)
(i) Centrifuge the plates containing pulverized dried mycelium at 4,600 g for 5 min and remove the capmat carefully.
(ii) Transfer 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 (Loveland Industries, Colorado, USA) 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 five to ten times and incubate them in a 65°C chamber for 20 min.
(iv) Centrifuge the plates at 4,600 g 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 five to ten times; incubate at −20°C for 30 min to overnight and centrifuge the plates at 4,600 g 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 6.33% ethanol solution using the Apricot (Handle hazardous guanidine chloride carefully and use mask for eye protection) and apply a new capmat.
(vii) Agitate the plates as done earlier to mix the solution; transfer 1 ml of the mixture to a Nunc spin column plate (Nalge Nunc Inc., NY, USA) sitting on a 2 ml DW plate and centrifuge at 4,600 g 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 4,600 g for 5 min.
(ix) Wash the membrane again by adding 500 µl of 95% ethanol; centrifuge at 4,600 g for 5 min and incubate the spin column plate at 65°C for 5 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 4,600 g for 2 min and assess the quality of DNA by separation on a 1% agar gel.

Appendix 18: Rapid PCR-Based Method for the Detection of Fungal Pathogen (Harmon et al. 2003)

A. Extraction of DNA of fungal pathogen

(i) Grow the fungal pathogen (*Magnaporthe oryzae*) in appropriate medium (V 8 juice agar, Campbell Soup Co. USA) and maintain the culture conditions that favor optimal growth.

(ii) Grind the mycelium in liquid nitrogen; suspend in 0.4 ml of phenol and 0.8 ml of fungal genomic DNA extraction buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris, pH 8.0, 0.5% SDS and 0.1% ß-mercaptoethanol) in tubes and incubate for 5 min at 60°C.

(iii) Agitate the tubes gently; allow them to cool; add 0.4 ml of chloroform/isoamyl alcohol (24:1 v/v) and centrifuge at 16,000 g for 10 min.

(iv) Extract 0.7 ml of the upper phase with an equal volume of chloroform/isoamyl alcohol (24:1, v/v); separate the upper phase and precipitate DNA with 1.0 ml of cold ethanol containing 150 mM sodium acetate.

(v) Allow the pellet to dry for 5 min and dissolve in 0.5 ml of TE buffer (1 mM EDTA,10 mM Tris, pH 8.0).

(vi) Treat with RNase (50 µg); extract with phenol/chloroform and precipitate with ethanol; dissolve the pellet in 0.1 ml of TE buffer, pH 8.0; determine the DNA contents spectrophotometrically and adjust the final concentration to 50 ng/µl.

B. Extraction of DNA from plant tissues

(i) Place the pieces of infected leaf blades in 1.5 ml Eppendorf tubes; add sufficient extract solution (100 µl) (from the Extract-N-Amp Kit, Sigma Chemical Co. USA) to cover the leaf tissues and incubate for 10 min at 95°C.

(ii) Add equal volume of dilution solution (from the kit); homogenize the sample in the tube using a polypropylene pestle; place in ice and dilute 5 µl aliquot tenfold in sterile distilled water.

C. PCR amplification and detection of diagnostic amplicon

(i) Use primers pfh2a and pfh2b capable of amplifying the 687-bp region of the Pot2 transposon.

(ii) Perform PCR in a 50 µl rection mixture with DNA Taq polymerase (Promega, Madison, USA) and purify genomic DNA from pathogen isolates.

(iii) Perform PCR for plant samples in a 20 µl reaction mixture from the kit in a DNA thermal cycler (Perkin Elmer Cetus, CT, USA).

(iv) PCR program consists of initial denaturation of 2 min at 94°C; 30 cycles of 45 s denaturation at 94°C; 45 s of annealing at 55°C; 45 s of extension at 72°C and final extension at 72°C for 10 min.

(v) Resolve the amplicon after electrophoresis in a 1% agarose gel; stain for 10 min in an ethidium bromide solution (10 µg/ml) and visualize the bands with UV light.

(vi) Use photoimaging system (Stratagene, CA, USA) for getting gel images.

Appendix 19: Detection of Powdery Mildew Pathogens by PCR-mediated Method (Chen et al. 2008)

A. Extraction of DNA from obligate fungal pathogens

(i) Scrape fungal mycelium from diseased leaf tissues; transfer into 2-ml microfuge tube; freeze the mycelium in liquid nitrogen and grind it into a powder using a plastic pestle.
(ii) Add 700 µl of lysis buffer containing 50 mM Tris-HCl, pH 7.2, 50 mM EDTA, pH 7.2, 3% SDS, 1% mercaptoethanol; vortex the contents and heat in a water bath at 65°C for 1 h.
(iii) Extract DNA solution; mix well with 700 µl of phenol/chloroform; centrifuge at 12,000 × g for 10 min; separate the upper phase; mix with 500 µl of chloroform and centrifuge at 12, 000 × g for 4 min.
(iv) Transfer the aqueous phase into a new 1.5 ml Eppendorf tube; add 50 µl of 3 M sodium acetate and 500 µl of isopropanol and centrifuge at 12, 000 × g for 20 min.
(v) Was the DNA pellet with 500 µl of 70% ethanol and centrifuge at 12, 000 × g for 20 min.
(vi) Air-dry the DNA pellet; dissolve in 0.5 ml of TE buffer containing 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 to have a concentration of ~200–500 µg /ml.

B. Primers and PCR amplification

(i) Use ITS universal primer pair PN23/PN34.
(ii) Use PCR reaction mixture containing 0.15 mM dNTPs, 0.4 µM primers, 1 U *Taq* polymerase (BioBasic), 1 × PCR buffer with 1.5 mM $MgCl_2$ and 10 µg of template DNA.
(iii) Add sterile distilled water to have a final volume of 25 µl.
(iv) Perform PCR amplification using a thermal cycler under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles consisting of denaturation at 94°C for 40 s; annealing at 62°C for 1 min; DNA synthesis at 72°C for 1.5 min; final extension at 72°C for 5 min.
(v) Separate PCR product (5 µl) by gel electrophoresis on a horizontal 2% agarose gel and stain the bands with ethidium bromide (0.5 µg/ml).
(vi) Visualize the bands under UV light and photograph.

Appendix 20: Detection of Rust Pathogen by PCR-Based Method (Wang et al. 2008)

A. Extraction of DNA from rust pathogen

(i) Freeze pure samples of urediospores (from artificially inoculated wheat seedlings) in liquid nitrogen and store at −70°C till needed.

(ii) Transfer 25 mg of urediospores to 2 ml tube; add 500 µl extraction buffer (50 mM Trsi-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA) and homogenize with a plastic pestle.
(iii) Add 5 µl proteinase K (1 mg/ml); make up the volume to 1.0 ml with extraction buffer and incubate for 30 min at 65°C.
(iv) Divide the mixture into two equal parts in two microfuge tubes; extract with phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) and chloroform respectively; transfer the top aqueous phase to a clean tube; add an equal volume of cold isopropanol and incubate for 1 h at −20°C.
(v) Centrifuge the contents at 12,000 rpm for 20 min at 4°C to precipitate the nucleic acid; rinse the pellet twice with cold 70% ethanol; dry and dissolve in 0.1 ml TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).
(vi) Add 1 µl of ribonuclease (10 mg/ml, final concentration 20 µg/ml) and incubate at 4°C overnight to digest the RNA completely.
(vii) Reprecipitate the DNA; rinse it with cold 70% ethanol; dissolve in 50 µl of TE buffer and quantify the DNA spectrophotometrically.

B. PCR amplification
(i) Use primers specific for the pathogen (*Puccinia striiformis*) Pst1 and Pst2.
(ii) Perform amplification in aliquots of 25 µl containing 20 ng DNA template, 2.5 µl 10 × reaction buffer (750 mM Tris-HCl, 200 mM $(NH_4)2SO_4$, 0.1 Tween-20) 25 mM $MgCl_2$, 2.5 mM each of dATP, dCTP, dGTP, dTTP, 0.2 µM primer and 1 U Taq polymerase and make up the volume to 25 µl with sterile distilled water.
(iii) Provide optimal conditions using a thermal cycler: initial denaturation at 94°C for 3 min; 34 cycles of amplification each consisting of denaturation at 94°C for 50 s, primer annealing at 50–60°C for 90 s and primer extension at 72°C for 2 min; final extension step at 72°C for 10 min.
(iv) Resolve the amplicons in 1.5% agarose gels, electrophoresed at 10 V/cm for 60–90 min along with a molecular size marker set.

Appendix 21: Detection of *Colletotrichum acutatum* by Arbitrarily Primed (AP)-PCR (Yoshida et al. 2007)

(i) Grow the fungal pathogen in potato dextrose agar (PDA) for 4–7 days at ~20°C.
(ii) Extract the genomic DNA from each isolate using the Wizard Genomic DNA Purification Kit (Promega) as per the manufacturer's recommendations.
(iii) Use a total volume of 25 µl of ~100 ng of genomic DNA, Read-to-Go-RAPD Analysis Beads (GE Healthcare) and uu$(CAG)_5$ (5′-CAGCAGCAGCAGCAG-3′) or $(GACAC)_3$ (5′-GACACGACACG ACAC-3′) primer.

(iv) Carry out the reactions using the GeneampPCR System 9600 (Applied Biosystems) starting with a 2-min denaturation at 95°C, followed by 45 cycles consisting of 1 min at 95°C, 1 min at either 60°C (for $(CAG)_5$) or 48°C (for $(GACAC)_3$) and 2 min at 72°C.

(v) Resolve the amplicons using 2% agarose gels in TBE buffer and view the bands under UV light after staining with ethidium bromide.

Appendix 22: Detection of *Puccinia coronata* by Real-Time PCR Assay (Jackson et al. 2006)

A. Pathogen DNA extraction and amplification

(i) Place standard weights (10^2–10^5 μg) of uredinospores or sections of infected leaf tissues in 1.5 ml tubes (Qiagen); lyophilize the pathogen tissues and add ~30 mg 0.1 mm diameter zirconia/silica beads (Biospec) and 100 mg of 0.5 mm diameter zirconia/silica beads to each tube.

(ii) For leaf samples add additional 3.2 mm stainless steel beads (Biospec) and 2.3 mm stainless beads and grind the tissues on a vibration mill (Retsch MM 300 USA) for 30 min at 30 Hz (3 O oscillations/s).

(iii) Centrifuge the tube-contents at ~6,000 × g for 10 min; add 500 μl of CTAB extraction buffer and mix the contents well.

(iv) Grind the samples for 15 min on the vibration mill; place in water bath at 65°C for 25 min.

(v) Add chloroform-isoamyl alcohol (24:1); centrifuge at 6000 × g for 15 min and separate the supernatant.

(vi) Precipitate DNA with isopropanol; wash the pellet with 70% ethanol and dissolve the pellet in 200 μl of TE buffer containing 10 μl/ml RNase.

(vii) Determine the purity and quantity of the DNA spectrophotometrically at A_{280} nm and A_{260} nm and store the DNA preparations at 4°C.

B. Conventional and real-time PCR assays

(i) Perform amplifications at 50°C for 60 s and 95°C for 10 min; then 40 cycles at 95°C for 15 s and 60°C for 60 s in a total volume of 50 μl containing 28.6 μl sterile double distilled (dd) water, 5.0 μl 10 × buffer, 3.0 μl 25 mM Mg^{2+}, 0.4 μl 5 U/ml Ampli TaqDNA polymerase (Applied Biosystems), 1.0 μl 10 mM dNTPs, 1.0 μl of each of forward and reverse primer (300 nM) and 10 μl DNA template (20 ng/μl).

(ii) Visualize the PCR amplicons on 2% agarose (SIGMA) gels stained with ethidium bromide after 2 h at 90 V (approximate distance of 5 cm from the wells) using a Fluorochem 8800 Image System (Alpha Innotech Corp. CA, USA).

(iii) Perform real-time PCR amplifications using a 96-well optical reaction plate in an ABI Prism 7000 Sequence detection system; follow thermal cycling conditions as in conventional PCR amplification (step B (i) above).

(iv) Use reaction volumes of 50 µl containing 13.4 µl sterile dd water, 25 µl TaqMan Universal master mix (Applied Biosystems), 0.3 µl of each forward and reverse primer (300 nM), 10 µl TaqMan probe (200 nM) and 10 µl DNA template.

Appendix 23: Detection of *Colletotrichum* spp. in Strawberry Plants by Real-Time PCR Assays (Garrido et al. 2009)

A. Extraction of DNA from strawberry plant tissues

(i) Place the weighed samples (0.25–1.0 g) in extraction bags (Bioreba) with 8–10 volumes of CTAB lysis buffer containing 12% sodium phosphate buffer, pH 8.0, 2% CTAB, 1.5 M NaCl, supplemented with 2% antifoam B emulsion (Sigma Aldrich); homogenize the samples to a paste-like consistency using a Homex grinder (Bioreba); transfer the homogenate to clean 2-ml centrifuge tubes and centrifuge for 5 min at 10,000 g to pellet the cell debris.

(ii) Dispense 600 µl of lysate (supernatant) to fresh 2-ml tubes containing 200 µl chloroform; mix by vortexing and centrifuge for 5 min at 13,000 g.

(iii) Transfer 500 µl of aqueous layer to clean 2-ml tubes containing 500 µl isopropanol and 50 µl MagneSil® Paramagnetic Particles (Promega); and incubate for 10 min at room temperature.

(iv) Extract the DNA using a robotic magnetic particle processor (Kingfisher ML, ThermoScientific); load the Kingfiser 5-ml tube strips as detailed below:

(a) tube 1: 1 ml sample containing the MagneSil® beads; (b) tube 2: 1 ml GITC lysis buffer containing 5.25 M guanidiniumthiocyanate, 50 mM Tris HCl pH 6.4, 20 mM EDTA and 13 g/l Triton X-100; (c) tubes 3 and 4: 1 ml 70% ethanol; (d) tube 5: 200 µl sterile distilled water.

(v) Use a total genomic DNA program (Kingfisher ML, ThermoScientific) to purify the DNA in each sample; transfer DNA collected in tube 5 to fresh 1.5-ml microcentrifuge tubes and store all DNA samples at −20°C, until needed for use in real-time PCR.

B. Real-time PCR formats

(i) Set up all real-time PCR assays in 96- or 384-well reaction plates.

(ii) Set up all SYBR® green assays, use with Uni 58SSybr F1/Uni 58SSybr R2 primers with an Absolute™ QPCR SYBR® Green ROX (500 nM) Mix Kit (AB gene) as follows.

12.5 µl SYBR® Green Mix, 0.375 µl ROX, passive reference (diluted 1:50), 300 nM primers and 10 µl diluted DNA extract made up to 25 µl using molecular grade water; carry out SYBR® green assays in duplicate with generic cycling conditions: 95°C for 10 min and 40 cylcles of 60°C for 1 min and 95°C for 15 s, followed by a dissociation step consisting of

a single transfer from 60°C to 95°C at a ramp rate of 2% within an ABI Prism 7900 HT Sequence Detector Systen (PE Biosystems).

(iii) Analyze the melting curves, after each run, to check for the presence of non-specific amplification products.

(iv) Set up all TaqMan® assays using PCR Core Reagent Kits (PE Biosystems) consisting of 1 × buffer A, 0.025-U μl^{-1} AmpliTaq Gold, 0.2 mM dNTPs and 5.5 mM $MgCl_2$.

(v) Use all sets of primers at 300 nM and probes at 100 nM; add 1 µl DNA extract, giving a final volume of 25 µl/reaction.

(vi) Maintain negative controls containing nuclease-free water instead of DNA for each run.

(vii) Carry out TaqManR PCR reaction in duplicate at 50°C for 2 min and 45 cycles of 95°C for 15 s and 60°C for 1 min.

(viii) Assess the C_T values for each reaction using SEQUENCE DETECTION SOFTWARE v2.2.2 (PE Biosystems).

Appendix 24: Detection of *Phytophthora cactorum* by RAPD-PCR Technique (Causin et al. 2005)

A. Extraction of DNA of fungal pathogen

(i) Crush the fungal mycelium (~200 mg wet weight) in liquid nitrogen using pestle and mortar; transfer immediately the macerate into a microcentrifuge tube; add 1 ml of lysis buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 mM NaCl, 2% cetyltrimethylammonium bromide (CTAB), 1% polyvinylpyrrolidone (PVP), 1% mercaptoethanol) and incubate at 65°C for 60 min.

(ii) Add 1 ml chloroform/isoamyl alcohol (24:1 v/v), shake the contents for 1 h in ice for completion of protein denaturation and centrifuge at 17,300 g for 10 min to separate the phases.

(iii) Recover the aqueous phase carefully; precipitate the DNA by adding 2/3 volume of isopropanol and 1/10 volume 3 M sodium acetate, pH 5.2 and allow the sample to remain at −20°C for 20 min.

(iv) Centrifuge at 17,300 g for 10 min; wash the pellet with 70% ethanol (v/v); repeat the cycle of pelleting and washing processes and dry the pellet at room temperature.

(v) Resuspend the pellet in 100 µl of TE buffer consisting of 10 mM Tris-HCL and 1 mM EDTA) and store at −20°C till required.

B. Screening for RAPD markers

(i) Use the DNA extracted from the test fungus for testing the 10-mer RAPD primers of the OPA series (OPA-1 – OPA-11) (Operon Technologies Inc., CA, USA).

(ii) Perform the reactions in 25 µl volumes with 10–15 ng of template DNA, 100 µM of each dNTP, 2.5 µl of 10 × buffer (200 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton® × 100), 2 mM Mg Cl_2, 0.2 µM of RAPD primer

and 1 Unit of *Taq* DNA polymerase (Promega Corp. USA); overlay a drop of sterile mineral oil on the reaction mix and maintain negative controls without the template DNA to check for DNA contamination of the reagents.

(iii) Provide the following conditions using the Thermo Cycler (Cycler TM, Bio-Rad, Italy): initial denaturation at 94°C for 2 min and 30 s; 45 cycles of amplification consisting of 94°C for 30 s, annealing at 38°C for 1 min, extension at 72°C for 2 min; final extension of 5 min at 72°C after cycling.

(iv) Separate the amplicons using 1.5% TBE buffer (45 mM Tris-borate, 1 mM EDTA) in agarose gels for 2 h followed by staining with ethidium bromide and photographing under UV illuminator (302 nm).

(v) Elute the RAPD band specific for the target pathogen directly from the agarose gel using the Agarose gel DNA Extraction Kit (Boehringer Mannheim Corp. USA).

(vi) Ligate the purified DNA into a plasmid pGEM-T Vector System (Promega) as per the manufacturer's instructions.

(vii) Transform *Escherichia coli* strain JM 109 competent cells using the plasmids and identify recombinant column by the blue-white color selection after 12 h of growth at 37°C on LB agar medium (1.8% trypan-NaCl, 0.5% yeast extract, 1.6% agar agar) containing ampicillin, IPTG (isopropyl ß-D-1-thigalactopyranoside) and X-Gal (5-Bromo-4-chloro-3-indolyl ß-D-galactopyranoside) as per manufacturer's recommendation.

(viii) Purify the plasmids from LB/ampicilling liquid cultures of selected colonies using a High Pure Plasmid Isolation Kit (Boehringer Mannheim Corp.) following the manufacture's instructions.

Appendix 25: Detection of *Macrophomina phaseolina* in Cowpea Seeds by DAS-ELISA Technique (Afouda et al. 2009)

A. Preparation of antigen

(i) Grow the pathogen in polysulfon membrane filters (HT-200 Tuffryn, Gelman, Germany) supported by inert fibre for 4 days in petridishes containing 10 ml of potato dextrose broth (PDB); harvest the mycelium from the filter; homogenize in phosphate buffered saline (PBS, pH 7.4) and centrifuge at 30,000 × g for 10 min and at 130,000 × g for 30 min.

(ii) Estimate the protein concentration by the method of Bradford (1976); adjust the protein concentration to 1 mg/ml and store aliquots of 1 ml in 2-ml Eppendorf centrifuge tubes at −20°C, until required.

(iii) Dialyze the culture filtrate, after harvesting the mycelium against PBS overnight at 7°C; concentrate by ultracentrifugation; determine the protein concentration; adjust the protein concentration to 0.1 mg/ml and store at −20°C, until required.

B. Preparation of antiserum

(i) Immunize the rabbits by injecting 1 ml of antigen emulsified with Fruend's adjuvant (Difco); space the first three injections at 2-week intervals and fourth after 4 months, as booster injection.

(ii) Bleed the rabbits at 1 week after each injection; store the antiserum supplemented with 0.05% sodium azide at 4°C and label the antisera generated against the mycelium and culture filtrate separately before storing.

(iii) Purify the immunoglobulins (IgG) in the antiserum by precipitation with 50% ammonium sulfate, followed by suspension in half-strength PBS and passage through a DEAE-Fractogel columns (Merck, Germany).

(iv) Collect 1 ml aliquots from the column; adjust their final OD to 1.45 at 280 nm corresponding ~1 mg IgG/ml and store at −20°C.

(v) For biotinulation of IgG, dialyze 1 ml of purified IgG overnight at 7°C against coupling buffer (containing 10 g NaCl, 10 g $NaHCO_3$ and 1,000 ml water, pH 7.5) with three changes of buffer solution; add 50 µl of biotinylation reagent (1 mg X-NHS-Biotin, Sigma, Germany) to the IgG; incubate for 30 min at room temperature; stop the reaction by adding 50 µl 1 M Tris-HCL, pH 7.4; dialyze the product overnight in saline (0.85% NaCl); add 50% glycerol and 1% bovine serum albumin (BSA) and store the mixture at −20°C.

C. Preparation of seed extract

(i) Soak the seeds singly in the wells of microtiter plates containing 1 ml PBS-T (PBS with 0.05% Tween 20) and 2% polyvinyl pyrrolidone (PVP); incubate the plates for 24 h at 4°C ;crush the seed into the buffer solution and incubate for a further period of 24 h at 4°C.

(ii) Centrifuge the plates and use the supernatant for detection of the target pathogen.

D. Double-Antibody Sandwich (DAS)-ELISA technique

(i) Coat the wells of microplates with IgG-diluted to 1: 1,000 (v/v) in 0.05 M carbonate buffer, pH 9.6; incubate at 4°C overnight and wash the plates thrice with half-strength PBS-T and subsequently between each step mentioned below.

(ii) Block unspecific reactive surfaces of each well with 200 µl of 0.2% BSA dissolved in coating buffer (0.05 M sodium carbonate buffer, pH 9.6); incubate the plates at room temperature for 2 h.

(iii) Dilute the antigen preparation suitably with PBS-T with 2% PVP; add 200 µl antigen solution to each well; incubate at 4°C overnight.

(iv) Dilute the biotinylated IgG (1:1,000, v/v) in PBS-T buffer with 0.2% BSA; incubate at 4°C overnight.

(v) Add streptavidin-alkaline phosphatase (Sigma) conjugate diluted (1:1,000, v/v) in conjugate buffer (half-strength PBS-T with 0.2% BSA and incubate at 37°C for 30 min.

(vi) Add 100 µl/well the substrate (1 mg.ml of p-nitrophenyl phosphate) in 10% diethanolamine, pH 9.8 and record the absorbance values at 405 nm using

the ELISA reader after allowing the reaction for 1–2 h at 37°C; absorbance values that are more than twice that of healthy control are considered to be positive reactions.

Appendix 26: Detection of Fungal Pathogens in Soybean Seeds by PCR-RFLP Technique (Zhang et al. 1999)

A. Extraction of DNA from soybean seeds

(i) Treat the soybean seeds with 95% ethanol for 30 s, 0.5% NaOCl for 1 min, 2.5% paraquat (Gramoxone, Zeneca Corp. USA) for 2 min and rinse the seeds three times in double-distilled (dd) water.

(ii) Squeeze the disinfected seeds to release the seed coats that are individually placed into a 1.5 ml microfuge tube with 250 µl of extraction buffer containing 50 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, 100 mM NaCl, 1.0% sodium dodecyl sulfate and 10 mM ß-mercaptoethanol.

(iii) Break the seed coats using an ultrasonic processor (Biospec Products) and a tapered microtip (5 mm diameter) for 10 s; mix with 150 µl of potassium acetate (5 M, pH 5.2) and incubate on ice for 20 min.

(iv) Centrifuge at 12,000 × g for 10 min; determine the DNA concentrations of the supernatant (extracted from the seed coat) by measuring OD at 260 nm in an UV spectrophotometer (Perkin-Elmer Applied Biosystems).

B. Extraction of DNA from the mycelium growing out of seeds on potato dextrose agar (PDA)

(i) Plate 100 surface-disinfested seeds from each seed lot on PDA and incubate at 27°C for 24–36 h.

(ii) Cut out 5 × 10 × 2 mm PDA plugs with mycelial growth originating from individual seeds; place into a microfuge tube (1.5 ml) containing 250 µl of extraction buffer and break the cells using the ultrasonic processor.

(iii) Centrifuge and save the supernatants as DNA extracts.

C. PCR-RFLP Assay

(i) Plate 100 seeds from each seed lots on PDA medium and extract the DNA from the pathogens as per steps B (i)–(iii).

(ii) Perform PCRs with a DNA thermal cycler (Perkin-Elmer Applied Biosystems) using the reaction mixture containing 50 mM KCl, 2.5 mM MgCl2, 10 mM Tris-HCl, pH 8.3, 0.2 mM each of dTTP, dATP, dGTP and dCTP, 50 pmol of the primers, 2.5 units of *Taq* polymerase and 25 ng of genomic DNA in a final volume of 50 µl.

(iii) Incubate the reactants at 96°C for 3 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min.

(iv) Check the amplification efficiency by subjecting to agarose gel electrophoresis using 5 µl of PCR amplicons.
(v) Digest the PCR amplicons (7 µl) with suitable restriction enzymes (5–10 units of *Alu* I, *Mse* I, *Hha* I, *Rsa* I and *Scr*F I) as per the manufacturer's instructions using 1.5 µl of buffer (10×) and 6.5 µl of double distilled water at 37°C for 2–4 h.
(vi) Size fractionate the enzyme-digested PCR amplicons on a mixed agarose gel of 1% ultra pure agarose (Amresco, USA) at 3.5 V/cm and stain with ethidium bromide.
(vii) Visualize on a UV-transilluminator and photograph.

Appendix 27: Detection of *Rhynchosporium secalis* in Barley Seeds by Competitive PCR (Lee et al. 2002)

A. Extraction of DNA from seeds
(i) Surface sterilize the seeds (100/sample) with ethanol for 30 s; wash them with several times with distilled water; dry at 22°C and grind to a fine powder using a mixer mill grinder.
(ii) Extract total DNA from the seed powder (0.1 g) and adopt the cetyltrimethyl ammonium bromide (CTAB) procedure.
(iii) Use the DNA equivalent of 0.1 mg dry seed weight in 1 µl for PCR.

B. PCR amplification
(i) Use a reaction mixture (25 µl) containing 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 250 nM of each forward and reverse primer and 0.6 units of *Taq*DNA polymerase.
(ii) Use primer sets designed from ITS regions of target pathogen DNA.
(iii) Perform PCR amplification with the following conditions: initial denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min with final extension at 72°C for 10 min.
(iv) Use 8 µl of PCR amplicons for separating them on a 1.5 agarose gel in Tris-borate-EDTA buffer at 100 V for 1 h; stain the gel with ethidium bromide for 15 min and visualize the PCR products on a UV transilluminator.

C. Competitive PCR
(i) Prepare a heterologous internal control using a competitive DNA Construction Kit (Takara Shuzo Co. Ltd., Japan) which has 5′-and 3′-termini identical to the fungal target primary sites (RS1 and RS3), but no internal sequence homology to the target sequence.
(ii) Generate the competitor fragment (445-bp) as per the manufacturer's recommendation.
(iii) Use 0.1 g milled seed powder (from 100-seed sample) for DNA extraction and use the extracts in the presence of constant amount of the internal control template DNA.

(iv) Calculate the mean PCR product ratios obtained from three replicates of each level of infection (disease intensity); plot the ratios against percentage of seed infection and generate a standard curve by reference to which the quantification of fungal DNA in field-infected barley seed can be made.
(v) Calculate the mean levels of PCR product ratios of samples and subsamples of naturally infected seeds and convert to ng of fungal DNA/mg seed material using the standard calibration curve.

Appendix 28: Detection of *Verticillium dahliae* in Olive Seeds by Nested PCR (Karajeh 2006)

A. Extraction of pathogen DNA from infected seeds
(i) Grind the seed samples (10 seeds/sample) in liquid nitrogen using a Phillips screwdriver; homogenize the seed powder in 1.5 ml preheated 65°C) extraction buffer consisting of 50 mM Tris-HCl, pH 8.0, 700 mM NaCl, 10 mM EDTA-Na_2; 2% CTAB, 0.5% 2-mercaptoethanol (v/v) and 1.0% polyvinyl pyrrolidone (PVP).
(ii) Dispense the homogenate into two 1.5 ml microtubes; incubate at 65°C for 15 min with occasional mixing and extract with 0.6 ml chloroform-isoamyl alcohol (24:1) and centrifuge at 14,000 g for 5 min.
(iii) Transfer the top aqueous phase to a new tube containing 2 µl RNase A (10 mg/ml water); incubate for 15 min at room temperature and precipitate protein using 0.5 volume of 3 M sodium acetate, pH 5.2.
(iv) Add 0.3 ml isopropanol to each microtube; incubate at −20°C overnight and centrifuge at 14,000 g for 5 min.
(v) Wash the DNA pellet with 1 ml 70% ethanol, air-dry and dissolve in 100 µl TE buffer consisting of 10 mM Tris, 1 mM EDTA, pH 8.0.
(vi) Estimate DNA concentration using agarose gel electrophoresis and with a spectrophotometer at 260 nm.

B. DNA amplification using nested PCR assay
(i) Carry out the first round amplification with the primer pair NESF 18S and NESR 28S from the highly conserved DNA sequences of 18S amd 28S genes that flank ITS region of the pathogen DNA and identify the product about 480-bp in size.
(ii) Transfer one microliter of the product of first amplification; perform second amplification with pathogen-specific ITS primers FVD and RVD and identify the product 330-bp in size.
(iii) Perform the PCR in a total volume of 25 µl with each reaction containing the following: 0.2 mM dNTPs (an equal molar mixture of dATP, dGTP. dCTP and dTTP), 0.5 U of *Taq* DNA polymerase, 0.25 mM of each forward and reverse primer, 1 × PCR buffer (10×: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0 at 25°C) and 1 Triton X-100, 1 µl of the final DNA extract (about 50 ng).

(iv) Maintain a negative control (water) for each round to detect contamination with template DNA and olive DNA extract.
(v) Provide the following conditions using Eppendorf Master cycler: initial DNA denaturation for 2 min at 94°C, followed by 35 cycles each consisting of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s and the final extension for 3 min at 72°C.
(vi) Analyze the PCR products by agarose gel electrophoresis with 0.5 × TBE buffer consisting of 10 × buffer of 0.9 M Tris, 0.9 M boric acid and 20 mM EDTA; stain with ethidium bromide and visualize on a UV transilluminator.

Appendix 29: Detection of *Pythium* spp. in Carrot Tissue by PCR Assay (Klemsdal et al. 2008)

A. Extraction of DNA from the oomycete and carrot tissue
(i) Cultivate the pathogen in potato dextrose agar (PDA) covered with cellophane at 20°C for 4–6 days; harvest the mycelium; grind it to a fine powder in liquid nitrogen and extract the DNA using DNeasy Plant Mini Kit (Qiagen Inc.) as per manufacturer's recommendations.
(ii) Collect carrots with and without symptoms of infection at harvest from the fields; wash them with water; take the peels carefully from the top to the tip in each carrot; freeze dry the peels overnight and grind them to a fine powder using pestle and mortar.
(iii) Transfer carrot tissue powder (50 mg) to a microcentrifuge tube; extract the DNA using the GenElute Plant Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's recommendations, except that elution of DNA from the binding column once with 100 µl TE buffer pH 7.5 and pre-warm the extract to 65°C.
(iv) Purify the DNA further using Micro BioSpin Chromatography columns (BioRad Laboratories Ltd.) filled with insoluble polyvinyl polypyrrolidone (PVPP).
(v) Load each column with 400 µl sterile water placed in a microcentrifuge tube and centrifuge for 5 min at 1,500 g.
(vi) Transfer the column to a new centrifuge tube and load the DNA onto the PVPP surface.
(vii) Collect the purified DNA as pellet after centrifuging at 1,500 g for 5 min.

B. Polymerase Chain Reaction Assay
(i) Perform the assay in a total volume of 25 µl with final concentration of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.2 mM dNTPs, 0.1 mg/ml bovine serum albumin (BSA) and 0.1 mM $MgCl_2$.
(ii) Use 25 pmol of each primer and 0.6 U Ampli *Taq* polymerase (Applied Biosystems).

(iii) Use 1 µl of the DNA extracted from carrot tissue as template; use universal primers ITS3 and ITS4 as positive control for DNA extracted from carrot tissues.
(iv) Perform amplifications in a GeneAmp® PCR System 9700 thermal cycler (Applied Systems) programmed for initial denaturation at 94°C for 5 min followed by 45 cycles of 20 s at 94°C, and 30 s annealing at 72°C.
(v) Use the following annealing temperatures for *Pythium sylvaticum*: 56°C; *P.* '*vipa*': 57°C; *P. sulcatum* and *P. intermedium*: 60°C and *P. violae*: 61°C.
(vi) Separate the PCR amplicons by electrophoresis through 1.2% agarose gels; stain with ethidium bromide and photograph in UV light on a GelDoc 1000 (BioRad Laboratories Ltd. USA).

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Chapter 3
Detection of Fungal Pathogens in the Environment

Abstract The presence of fungal pathogens in the environment has to be detected and also quantified rapidly and precisely, because the inoculum for infection of crop plants comes from the pathogen propagules present in the soil, water, air and alternative host plant species. Soilborne pathogens may have different degrees of saprophytic ability, utilizing the organic matter present in the soil for their survival in the absence of crop plants. They produce different structures such as chlamydospores and sexual spores that are long-lived and are capable of surviving in the soil for several years. Fungal structures may be carried by irrigation water or rain water from one part of the field to other parts or to different fields. It has been possible to detect and identify the fungal pathogens in the irrigation water, recycled water used for growing hydroponic plants and also in wash water in the storage facilities for fruits and vegetables. The pathogens infecting aerial plant parts/organs are generally disseminated by wind to different locations. Traditionally spore traps have been used for assessing the spore load of air. Various biological, immunological and nucleic acid-based techniques have been employed for the detection, identification and quantification of pathogen propagules in the environment. Significant improvements have been made in the sensitivity and specificity of detection of fungal pathogens by applying immunoassys and nucleic acid-based methods that are capable of providing reproducible results rapidly and reliably. The relative usefulness and limitations of the detection techniques applied for the detection of fungal pathogens in the environment are discussed.

Studies on the ecology of plant hosts provide information on the influence of environment on the growth and development of plants. Likewise, the influence of the environment on the microbial plant pathogens is studied to understand the extent of population build up resulting in the incidence of diseases in different agroecological conditions. Crop husbandry techniques aim to increase the crop yield to the maximum levels. But some of these techniques like monoculture and excessive application of nutrients may provide favorable conditions for pathogen development. Epidemiology deals with effects of biotic and abiotic environments on disease development in plant populations. Microbial pathogens multiply at different rates in a given set of environmental conditions and exhibit wide variations in their pathogenic potential (aggressiveness). The crop plants also have varying growth

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patterns and respond differently based on their levels of susceptibility/ resistance to environmental factors and microbial pathogens.

The modes of dispersal/dissemination of fungal pathogens have significant influence on the incidence and subsequent spread of the diseases. Inoculum present in the soil, water and air has a vital role in infection and subsequent development of diseases in crop plants. Soilborne fungal pathogens may have different degrees of saprophytic ability. They may be able to survive in the soil for long time through resting spores that are resistant to adverse conditions. The foliar pathogens are dispersed by wind that carries the spores to locations that may be separated by mountains or seas. Rain water and irrigation water may spread the soilborne pathogens from field to field, while river waters may help the pathogens reach distant locations. Spores present on aerial plant parts may be dispersed by rain water in the case of some fungal pathogens. Detection of fungal pathogens in the plant environment is essential to assess the pathogen presence and its population levels have to be quantified to plan effective disease management systems.

3.1 Detection of Fungal Pathogens in Soil

Soil microflora consists of microorganisms that are either beneficial or harmful to plants. Fungus-like and fungal pathogens cause many destructive crop diseases that are difficult to be contained. Damping-off, root rots, collar rots, stem rot and wilt diseases are induced by the fungal pathogens present in the soil. The soilborne pathogens may be grouped into three categories: (i) soil inhabiting pathogens that can survive as a saprophyte independently in the absence of a susceptible host plant species; (ii) soil invading or root inhabiting pathogens that have a saprophytic or parasitic phase depending on the presence of a susceptible host plant species; (iii) obligate parasites that require the presence of a susceptible plant species during their entire parasitic life. The soil invading fungi have a greater infection potential and parasitic specialization compared with soil inhabiting pathogens. The soilborne fungal pathogens have to be detected by applying suitable technique(s), since the symptoms of infection can be recognized in most cases only after the pathogen has established well inside the infected plants and at that stage, it may be difficult to save the infected plants. Nevertheless, it may be possible to restrict further spread of the pathogens to neighboring plants.

3.1.1 Bioassays

Bioassays are the frequently employed method for the detection of soilborne fungal pathogens. In the case of nonculturable obligate parasites like *Plasmodiophora brassicae*, *Olpidium brassicae* and *Polymyxa betae*, bioassys were the only practical

method for the detection, prior to the development of immunoassays and nucleic acid-based techniques. Sensitive indicator plant species is grown in the test soil samples presumed to be infested by the target pathogen. Development of characteristic symptoms of the disease concerned in the indicator plants after required period of incubation is considered to indicate the presence of the target pathogen(s) in the test soil samples. Damping-off disease due to *Pythium* spp. and *Rhizoctonia* spp., wilting/vascular discoloration due to *Fusarium* spp., presence of galls due to *Plasmodiophora* sp., and rotting due to *Phytophthora* spp. and *Pyrenochaeta* sp. may be recognized by examining the symptoms of infection. *Phytophthora fragariae* var. *fragariae* was detected by incorporating the infected root tissues in the soil-less planting mix followed by planting a highly susceptible *Fragaria semperflorens* var. *alpina* (Baron Solemacher) plants. The bait plants are examined after a period of 3–6 weeks. The infected plants collapse due to infection and the presence of oospores and red coloration of stele can be seen in the infected roots. A similar test was employed for the detection of *P. fragariae* var. *rubi* in infected raspberry plants (Duncan 1980, 1990). However, under certain conditions the symptoms of infection by *Rhizoctonia* were not expressed, while serological tests revealed the presence of the pathogen (Christensen et al. 2001).

Detection of oomycetes *Pythium* spp. and *Phytophthora* spp. may be accomplished by baiting and plating procedure. Sterilized seeds of susceptible plants are used as baits for the pathogen in the water extract of soil. After an incubation period of about 12 h, the seeds are placed on a suitable agar medium that favors the growth of the pathogen. The mycelial growth is then examined under the microscope. The baiting test result was confirmed by the ELISA test (Yuen et al. 1998). Root rot diseases of several crops grown in hydroponic culture and ebb-and-flow irrigation systems are caused by *Pythium helicoides*, *P. aphanidermatum* and *P. myriotylum.* These oomycete pathogens can develop at temperatures higher than 40°C. A baiting technique was developed to detect these high-temperature pathogens present in nutrient solutions. Seeds of cucumber, tomato, radish, hemp, perilla and millet and leaves of bent grass and rose were tested as baits in hydroponic cultures. Hemp, perilla and radish seeds and bent grass and rose leaves were the most efficient in recovering the zoospores of these pathogens. In the sensitivity tests, bent grass leaf traps (BLTs) could detect three *Pythium* spp. after only 1 day exposure to suspensions of 40 zoospores/l of water and the frequency of detection increased with zoospore density and with baiting period. It was possible to detect the pathogen in the nutrient solution 23 days before disease spread to noninoculated roses. The use of baits has been shown to be an effective approach for monitoring high-temperature *Pythium* spp. in recirculating hydorponic culture systems (Watanabe et al. 2008).

The pepper leaf disk assay was developed for the detection of *Phytophthora capsici* in soil samples. Five pepper leaf disks (0.5 cm diameter) are floated in saturation water held on soil samples placed in plastic cups. After 24 h, the leaf disks are removed, surface-disinfested with 0.5% sodium hypochlorite solution for 1 min, rinsed in sterile water and plated on Masago's Phytophthora selective medium (Masago et al. 1977). After incubation for 72 h, colonies of *P. capsisci* developing

on the leaf disks are counted and the percentages of leaf disks colonized are calculated (Larkin et al. 1995). A quantitative baiting assay system was developed for the detection of *P. cinnamomi* in the soil involving the use of blue lupin (*Lupinus angustifolius*) radicles. This system using racks of tubes could be employed for efficient handling of large number of soil samples to find out the presence or absence of this pathogen. The factors influencing baiting efficiency were studied. The optimum temperature was found to be ~25°C for baiting and 20–25°C for bait incubation. The peak of zoospore release from naturally infested soil occurred on the first day. Infection on radicles decreased as the distance between the radicle tip and location of inoculum increased (Eden et al. 2000). These factors have to be taken into account, while formulating experiments for the detection of this soilborne pathogen by baiting method.

The efficacy of recovery of *Phytophthora ramorum*, causative agent of sudden death of oak, from the soil was assessed by using rhododendron leaves and pears as baits. Natural inoculum of *P. ramorum* associated with bay laurel (*Umbellularia californica*) tanoak (*Lithocarpus densiflorus*) and redwood (*Sequoia sempervirens*) were determined by monthly baiting. Rhododendron leaf baits were superior to pear baits for sporangia detection. But neither of these baits could detect chlamydospores of the pathogen from the soil. Most inoculum was associated with bay laurel and recovery was higher in soil than in litter. As the pathogen survives and produces chlamydospores in forest soils oversummer, it is important that this form of inoculum has to be assessed by more effective detection method(s) (Fichtner et al. 2007).

Recycling irrigation in nursery and crop production systems potentially increases the risk of spreading a wide range of microbial plant pathogens. Contaminated irrigation water has been recognized as an important source of inoculum for *Phytophthora* species that cause several economically important crop diseases. The efficiency of bait species viz., *Camellia japonica*, *Ilex crenata* and *Rhododendron catawbiense*, bait type (whole leaf or leaf disc), bait depth and duration and growth media for the detection of *Phytophthora* spp. in irrigation runoff containment basin was assessed. Irrespective of culture medium, baiting with rhododendron leaves for 7 days was the most efficient in recovering the greatest diversity and populations of *Phytophthora* spp. with minimum interference from *Pythium* spp. The flexible (two-rope) bait deployment system was superior to the fixed (one-rope) system, minimizing the risk of bait loss to remain at designated depths from the surface under inclement weather. Further, the flexible bait-deployment system was useful to study the horizontal and vertical distribution of *Phytophthora* spp. in containment basins (Ghimire et al. 2009).

3.1.1.1 Direct-Plating Method

The natural soilborne populations of fungal pathogens have to be assessed both qualitatively and quantitatively to determine the influence of epidemiological factors on disease incidence and spread. Soil-dilution technique has been applied for the

isolation of *Verticillium* spp. from naturally infested fresh soils (Jordan 1971). Estimation of *V. dahliae* propagules persisting in air-dried soils was carried out using the modified Anderson Sampler technique. Propagule counts for soils which had been cropped to cotton increased substantially between two sampling dates. The modified method was more accurate and sensitive detecting 2.8 times more microsclerotia per gram of soil (Butterfield and DeVay 1977).

Soil dilution plating assay cannot be used to count for the oospores which do not readily germinate on any agar medium. Hence, assay methods that stimulate oospore germination may improve the detection efficiency of the pathogens in the soil. Baiting assays can detect lower levels of inoculum than dilution plating, but tend to be less quantitative (Goodwin et al. 1990). The efficacy of standard soil dilution plating on selective medium, saturation water, pepper leaf disk assay after 'sample saturation and resaturation of soil with water' procedure was compared for the detection and quantification of specific propagule types of *Phytophthora capsici* in soil. Sporangial inoculum was detected at one ppg (propagules per gram) of soil with all assays when soil water matric potential was controlled during incubation. No single procedure was able to detect and quantify accurately all propagule types (zoospores, oospores or sporangia and mycelial fragments) of *P. capsici*. The leaf disk assay proved to be the best in detecting all propagule types, but not sufficiently quantitative to estimate the inoculum densities (Larkin et al. 1995).

Rhizoctonia solani and *R. oryzae* are the serious root pathogens of wheat and barley. *R. oryzae* is a fast-growing pathogen, while *R. solani* AG-8 is difficult to isolate even by using a semiselective medium. A quantitative assay of active hyphae was developed using wooden toothpicks as baits inserted into soil samples. The toothpicks are removed after 2 days in soil and placed on a selective medium (water agar amended with benomyl at 1 μg/ml and chloramphenicol at 100 μg/ml). Developing colonies are counted after 24 or 48 h under a dissecting microscope. *R. solani* and *R. oryzae* could be distinguished by hyphal colony characteristics. This method was tested in natural soils spiked with known inoculum densities of *R. solani* AG-8 and *R. oryzae*. This simple and inexpensive procedure has the potential for application for detection and diagnosis in growers' fields and for studies on the ecology and edpidemiology of *Rhizoctonia* spp. (Paulitz and Schroeder 2005).

3.1.2 Immunoassays

As the results of soil dilution plating method showed wide variations in the number of propagules (microsclerotia) from the samples, both between methods used and within a method between laboratories, the need for developing a method that can provide reliable and reproducible results was realized. Immunoassays as an alternative to soil dilution procedure were applied to improve the sensitivity and reliability of detection of soilborne fungal pathogens. The immunoassays based on interaction between antigen(s) on or produced by the pathogen and polyclonal or monoclonal antibodies (PAbs or MAbs) were developed. Different formats of enzyme-linked

immunosorbent assay (ELISA) have been tested for their usefulness in detecting the soilborne pathogens. MAb-based techniques allow discrimination of biomass components, if they are raised against constitutively expressed antigens that secreted during hyphal development. The accuracy of immunoassays depends on the specificity of the antibody used in the test. Immunoassays cannot distinguish between viable and dead cells and hence they can be used as a secondary confirmation step for baiting and plating methods.

The PAbs and MAbs were raised against secreted proteins from an anastomosis group (AG)-8 isolate of *R. solani.* PAbs raised against total secreted proteins cross-reacted in immunoblotting experiments with all *R. solani* isolates. But MAbs to secreted proteins showed greater degree of specificity for AG-8 isolates. An IgM MAb recognized a 40-kDa protein specific to AG-8 isolates, while the IgG MAb was more specific by reacting with 38-, 40- and 55-kDa proteins from AG-8 isolates and cross reacting with few isolates of other AGs (Matthew and Brooker 1991). In a later study, MAbs capable of specifically recognizing antigen from *R. solani* were employed for the detection of the pathogen present in the soil (Thornton et al. 1993). In a further study, the usefulness of combining baiting and double MAb-ELISA test was demonstrated. This protocol permits recovery of *R. solani* isolates from colonized baits for determination of their anastomosis group affiliation and pathogenicity. The isolates pathogenic to lettuce were identified as AG-4 group (Thornton et al. 1999).

A fungal capture sandwich ELISA was developed by using the polyclonal antiserum produced against soluble protein extracts of chlamydospores and mycelium of *Thielaviopsis basicola*, causing black rot of cotton and the IgG was labeled with biotin. This ELISA format detected both brown and gray cultural types of *T. basicola*. There was negligible cross-reaction with other soilborne fungi present in cotton field soil. This assay had a detection limit between 1 and 20 ng of pathogen protein (Holz et al. 1994). A polyclonal antibody generated against the cell walls of *Pythium ultimum* was employed as the capture antibody, while a MAb specific for recognition was used in indirect DAS-ELISA test for the detection of the pathogen in the roots of beans, cabbage and sugar beet seedlings grown in infested soils, with one percent root infection (Yuen et al. 1998). The MAb specific to the glycoprotein present in the cell walls of *Pythium sulcatum* were applied in indirect competitive ELISA format for detecting the pathogen efficiently in the soil samples from fields where infected carrots were grown (Kageyama et al. 2002).

Commercial immunoassay kits have been developed for the detection of fungal pathogens in the soil as well as in plants. A dipstick immunoassay was developed based on detection of cysts of *Phytophthora cinnamomi* labeled with MAb attached to nylon membrane. The pathogen could be detected in a wide range of soil samples collected from fields planted to several crop plant species (Cahill and Hardham 1994). The Albert Phytophthora "flow through" immunoassay and multiwell ELISA kits (Agri-Screen) were found to be effective for the detection of *P. capsici* and *P. cactorum* in the soil samples. MAbs provided more specific detection and characterization of *P. capsici* isolates (Miller et al. 1994). Commercial ELISA kits

have been used to detect *Phytophthora citrophthora* in soils as well as in root samples (Timmer et al. 1993).

The resting spores of *Plasmodiophora brassicae*, causative agent of clubroot disease of crucifers, were quantified in soil using the seedling bait assays for gall development. This assay is time-consuming and laborious, in addition to lack of reproducibility of results. The indirect ELISA test was developed for specific detection of *P. brassicae* in artificially inoculated soil samples. The antiserum prepared against the resting spores of *P. brassicae* was capable of detecting *P. brassicae* in the soil and plant tissues. The detection limit of the assay was 1×10^4/ml of the soil suspension. The resting spore density in soil that can cause economical loss was estimated to be 1×10^3 to 1×10^4 spores/ g of dry soil. In addition, dot immunobinding assay (DIBA) was also evaluated for the detection of *P. brassicae* in soils. DIBA test also detected the pathogen as efficiently as ELISA and detection limit of the assay was 1×10^4 spores/ml in artificially infested soil samples (Orihara and Yamamoto 1998) (Appendix 1).

3.1.3 Nucleic Acid-Based Techniques

Nucleic acid-based techniques have been applied for qualitative detection and quantification of fungal pathogens in the soils. Microbial DNA, RNA or both types of nucleic acids have been extracted from the soil for ecological studies. DNA analysis has been used most frequently, because DNA is more stable and less expensive to be extracted from the soil. The key problem with DNA analysis is that it does not reflect the abundance of or level of activity of the microorganisms in the soil samples. DNA contained in the dead cells will also be extracted, giving an incorrect estimate of pathogen populations in different samples analyzed. In contrast, the advantage of analyzing RNA is that it is generally present in high amounts only in actively metabolizing cells. Hence, analysis of RNA is more reflective of the portion of the soil microbial community that is active at the time of sampling. The soil is a very complex environment that creates numerous barriers to the isolation, identification and quantification of soilborne fungal pathogens. The advent of molecular techniques has provided revolutionary new insights into the detection and enumeration of soilborne fungal pathogens, in addition to the availability of information for identifying unknown fungal species from their DNA sequences.

The simplicity of polymerase chain reaction (PCR) assay, together with its potential to detect small numbers of target organisms without the need for culturing of cells, easily makes it a preferable method for detection and monitoring pathogen populations in soils. Over the last few decades methods for extraction of DNA from soil samples have been significantly improved. Species-specific DNA probes generated from cloned random DNA fragments derived from genomic DNA that has been earlier digested with restriction endonuclease, offer many advantages over classical detection methods. There is no need to obtain a pure culture of the target pathogen, due to the high specificity of the probes. Further, DNA contents are the

same at different stages in the life cycle of the fungal pathogens. Hence, the probes can detect the target pathogen at any stage of pathogen development, in contrast to immunoassays. DNA probes exhibit speed, sensitivity and specificity in detection of the pathogens, since repetitive sequences of the genomic DNA are used preferably for probe preparation.

Rhizoctonia solani AG-8, inducing root rot and damping-off diseases in several crops, was detected in soil samples by using a specific DNA probe pRAG12. The specificity and high copy number of AG-8 probe provide the means of a sensitive diagnostic assay for *R. solani* in infested soil (Whisson et al. 1995). A plasmid DNA fragment designated PE-42 hybridized to DNA of all 22 isolates of *R. solani* AG-2-2-IV causing large patch disease of Zoysia grass, but not to the DNA of other pathogens infecting Zoysia grass, revealing the specificity of Southern hybridization assay with PE042 plasmid DNA (Takamatsu et al. 1998). *R. solani* AG-1-1A causing rice sheath blight disease was detected and identified rapidly using primers designed from unique regions within ITS regions of rDNA. The pathogen could be precisely identified in paddy field soils and rice plant tissues by this PCR protocol (Matsumoto and Matsuyama 1998). A quantitative real-time PCR format was developed for the detection, identification and quantification of *R. solani* AG-3 from soil samples (Lees et al. 2002).

A slot-blot hybridization procedure was developed for the detection of *Gaeumannomyces graminis* var. *tritici* (Ggt) in the soil samples. A specific plasmid DNA probe pG158 hybridized strongly to pathogenic isolates of Ggt, moderately to *G. graminis* var. *avenae*, but not to any of the nonpathogenic isolates of Ggt which were morphologically similar. It is necessary to differentiate pathogenic and nonpathogenic isolates of Ggt to relate the soil population to the incidence of wheat take-all disease caused by Ggt. The probe pG158 could detect the pathogenic isolates both in the soil and wheat roots (Harvey and Ophel-Keller 1996). A reverse dot-blot hybridization protocol for identification of oomycetes is based on assays of oligonucleotides labeled with DIG. This procedure showed far fewer cross-hybridization than the one based on entire amplified internal transcribed spacer (ITS) fragments. By just recording the positive hybridization reaction between the DNA labeled directly from the sample and the specific oligonucleotides immobilized on nylon membrane, the pathogens such as *Pythium aphanidermatum*, *P. ultimum*, *P. acanthium* and *Phytophthora cinnamomi* could be detected and identified (Lévesque et al. 1998).

A sensitive and specific PCR-based method was developed for detecting *Pythium myriotylum* causing ginger soft rot disease, in soil samples. Oospores of *P. myriotylum* were separated from large soil particles by floatation in sucrose solution. The thick-walled oospores were disrupted by vortexing with sea sand. Pathogen DNA was extracted by CTAB method. PCR amplification of a 150-bp target sequence of *P. myriotylum* indicated the presence of the pathogen in the soil samples (Fig. 3.1). This procedure was applied for the detection of *P. myriotylum* in the field soils prior to planting ginger crops. The detection limit of the PCR format was ten oospores per gram of soil (Wang and Chang 2003) (Appendix 2). Five species-specific primers were designed from the sequence of ITS regions of

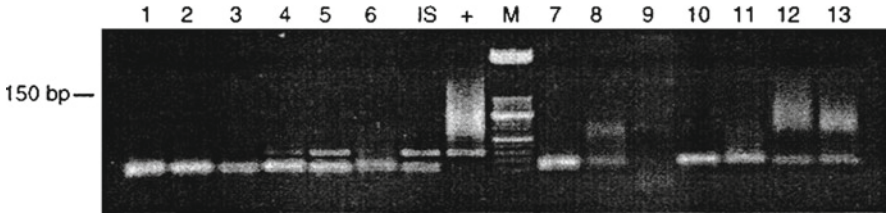

Fig. 3.1 Detection of *Pythium myriotylum* in the field soil samples using PCR assay. Lane M: 50 bp DNA ladder; Lane +: *Pythium myriotylum* DNA; Lane IS: artificially inoculated soil containing 10^3 oospores of *P. myriotylum*; Lanes 1–10: soil samples; Lane 11: autoclaved soil; Lanes 12 and 13: negative control. Note the presence of pathogen-specific band (150-bp) in the lanes 4 to 6 and IS (Courtesy of Wang and Chang 2003; The Society for Applied Microbiology/Wiley-Blackwell, Oxford, UK)

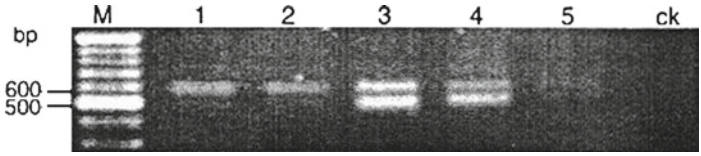

Fig. 3.2 Detection of *Pythium dimorphum* DNA extracted from soil sample. Lane 1: *P. dimorphum* total DNA; Lane 2: *P. dimorphum* in autoclaved soil; Lanes 3–5: *P. dimorphum* extracted from different soils (Courtesy of Wang et al. 2003; The Society for Applied Microbiology/Wiley-Blackwell, Oxford, UK)

rDNA of *Pythium* spp. Specific detection of *P. aphanidermatum* from infected plants and *P. dimorphum* from soil was possible using the specific primers (Fig. 3.2). The specific amplifications enabled nine *Pythium* spp. to be differentiated (Wang et al. 2003).

Pythium intermedium, *P. sulcatum*, *P. sylvaticum*, *P. viola* and *P. vipa* causing cavity spot disease of carrot were detected in soils using primers designed based on ITS sequences of rDNA. In soils known to produce cavity spots on cropped carrots, relatively strong signals were obtained and in several soils more than one of the five *Pythium* spp. could be detected. In a field where the infection level was 79.7%, all the five *Pythium* spp. could be detected in soil samples by the PCR format developed in this investigation (Fig. 3.3). Utilization of this diagnostic PCR assay, may be useful to reduce the loss of carrot crops due to cavity spot disease (Klemsdal et al. 2008). Many studies on molecular detection and identification of *Pythium* spp. have focused on the use of ITS sequences of rDNA, since the ITS regions within *Pythium* spp. are conserved, but variable between species. However, these techniques are not quantitative or designed for identification directly from soil or plant samples. Variable regions of the ITS regions of rDNA were used to design species-specific primers for detection and quantification of nine species of *Pythium* from soils in eastern Washington. These primers were subsequently used to develop an assay to identify and quantify species of *Pythium* from artificially and naturally infested soils using a soil DNA extraction kit and

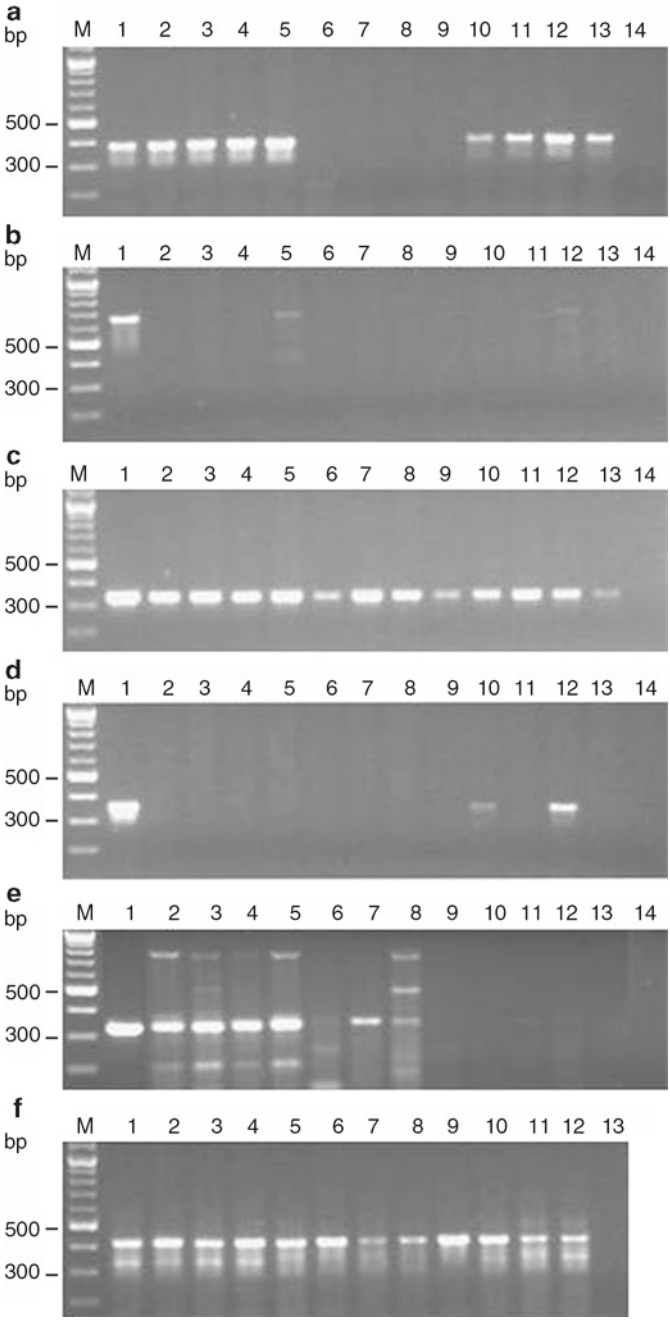

Fig. 3.3 Detection of *Pythium* sp., in soil samples using pathogen-specific primer pairs in the PCR assay'. Primer pairs PINTERf/r for *P. intermedium* (**a**), PSULCf/r for *P. sulcatum* (**b**), PSYLf/r for *P. sylvaticum* (**c**), PVIOLf/r for *P. violae* (**d**) and PVIPAf/r for *P. vipa* were employed

real-time PCR assay. Standard curves for *P. abappressorium*, *P. attrantheridium*, *P. heterothallicum*, *P. irregulare* group I, *P. irregulare* group IV, *P. paroecandrium*, *P. rostratifingens*, *P. sylvaticum* and *P. ultimum* were generated, using SYBR Green I fluorescent dye for detection and amplification. The standard curves were used to correlate population of each species in soil with quantities of DNA amplified from the same soil sample (Table 3.1). This new real-time PCR format was found to be rapid and accurate and hence, it can be a useful tool for assessing soil populations of pathogenic *Pythium* spp. (Schroeder et al. 2006).

Table 3.1 Amplifcation of indigenous *Pythium* spp. DNA from natural field soils using species-specific primers and real-time PCR assay (Schroeder et al. 2006)

	DNA quantification from field soils (fg/g of soil)[a]				
	Winter	Winter	Spring	Spring	Farm[b]
Pathogen species	Wheat	Fallow	Barley	Fallow	Spring wheat
Wet soils					
P. abappresssorium	2375	6065	680	–	–
P. attrantheridium	–	–	1595	–	1025
P. heterothallicum	6000	7290	1565	–	(440)
P. irregulare group I	–	–	7980	–	–
P. irregulare group IV	–	?	?	–	?
P. paroecandrum	–	–	–	–	–
P. rostratifingens	5810	4,505	7,960	–	1,730
P. sylvaticum	–	–	–	–	–
P. ultimum	–	–	(130)	–	–
Total (ppg)[c]	1210	1,130	1,300	570	2,070
Dry soils					
P. abappressorium	2,775	2,505	580	(490)	-
P. attrantheridium	–	–	4,445	–	3,045
P. heterothallicum	3,705	4,730	3,740	–	550
P. irregulare group I	–	–	5,360	–	–
P. irregulare group IV	–	–	?	–	–
P. paroecandrum	–	–	–	–	–
P. rostratifingens	7,945	2,895	1,890	(70)	2,870
P. sylvaticum	–	–	–	–	–
P. ultimum	–	–	(225)	–	–
Total (ppg)[c]	410	650	1,280	200	960

[a]Dash (–) *Pythium* was not detected
[b]Agricultural Research Service Palouse Conservation Farm
[c]Population in propagules per gram (ppg)

Fig. 3.3 (continued) for amplification of respective target DNA fragments. Lane 1: pathogen DNA; Lanes 2 to 13: 12 soil samples analyzed (**e**) Lane 14: Negative control (water), (**f**) primer pair ITS3 and ITS4 used as a control reaction for 12 samples (lanes 1–12) and negative control (lane 13), M: 100 bp DNA ladder (Courtesy of Klemsdal et al. 2008; British Society for Plant Pathology, Hertfordshire, England)

A DNA microarray technique was developed for the detection and identification of more than 100 species of *Pythium.* With the exception of *P. ostracoides*, all other species exhibited positive hybridization reaction with at least one corresponding species-specific oligonucleotide. Hybridization patterns were distinct for each species. Important pathogens detected in soils include *P. aphanidermatum*, *P. ultimum* var. *sporangiferum*, *P. ultimum* var. *ultimum* and *P. sylvaticum*. Verification of the specificity of the DNA array was carried out with *Pythium* isolates obtained by soil dilution plating. The hybridization patterns obtained were consistent with the identification of these isolates based on morphology and ITS sequence analyses. Further, 13 species of *Pythium* detected by DNA array corresponded to the isolates obtained by a combination of soil dilution plating and baiting. The DNA array format is a reliable tool for identification and quantification of multiple species of soilborne pathogens such as *Pythium* spp. in environmental samples (Tambong et al. 2006). DNA array technology, a highly suitable method for high-throughput detection, identification and quantification of multiple pathogens in a single assay was employed for *Pythium ultimum* and *Fusarium oxysporum* in infested soils. The ability to discriminate single nucleotide polymorphisms (SNPs) was pursued in the diagnostic assay. High specificity can be obtained with DNA arrays, even allowing discrimination of single nucleotide differences. Thus by using appropriate oligonucleotide sequences, closely related fungal pathogens can be reliably identified and differentiated. (Lievens et al. 2006).

Phytophthora cinnamomi causes root rot in a wide variety of plants and it is implicated as a major causative agent of the devastating eucalypt die-back disease. The conventional baiting method involving the use of a soil-water slurry with material colonized by the pathogen followed by plating and examination under the microscope is tedious and time-consuming. Hence, a PCR-based assay using the DNA of the pathogen extracted from the soil was evaluated for the detection of *P. cinnamomi* in soil samples. A RAPD-PCR product was determined and pairs of primers were designed to sites at each end of the fragment. One of the several primer pairs tested (LPC2/RPC3) showed specificity for *P. cinnamomi*. Amplification of *P. cinnamomi* DNA resulted in the formation of a 281-bp product that was unique to the target pathogen and this product was not amplified from other *Phytophthora* spp. tested. The detection limit of the PCR assay was as little as 10 pg of pathogen DNA (O'Brien 2008).

Root and stem rot caused by *Phytophthora sojae* is an important soybean disease accounting for severe losses. A rapid and sensitive PCR-based assay was considered essential for detection of *P. sojae* from soil carried with transported soybeans or in production fields. Real-time PCR combined with fluorescent SYBR Green I dye was developed for the detection and quantification of *P. sojae* in the soil. The universal primers DC6 and ITS4 amplified the sequences of ITS regions of eight *P. sojae* isolates. After alignment of the amplified sequences, a region specific to *P. sojae* was used to design the specific primers PS1 and PS2. A product of 330-bp of PCR amplification was detected exclusively from the isolates of *P. sojae*, but not in any of the isolates of 25 species of *Phytophthora* tested. The PCR assay combined with a simple soil screening method, allowed the detection

of *P. sojae* from soil within 6 h, the detection limit being two oospores in 20 g of soil. A real-time fluorescent quantitative PCR using primers PS1 and PS2 was employed to assess the pathogen populations in two field soil samples and one soil sample collected from soybeans imported from USA (Wang et al. 2006) (Appendix 3).

Cucumber Phytophthora blight disease is caused by *Phytophthora melonis*. Polymerase chain reaction (PCR) assay was developed for the detection of *P. melonis* using the primers based on the sequences of ITS nuclear rDNA. The PCR amplification with these primers resulted in the amplification of a ca. 545-bp product exclusively from the *P. melonis* isolates, but not from 26 species of *Phytophthora* and 29 other species of pathogens tested. The detection limit of the assay was 100 fg of genomic DNA. A nested PCR procedure with DC6 and ITS4 as first-round primers, followed by Pm1 and Pm2 primers, enhanced the detection sensitivity to 100 ag level representing 1,000-fold increase. The detection sensitivity of the pathogen present in the soil was 100 zoospores in 0.5 g of artificially inoculated soil. The PCR assay with Pm1 and Pm2 could be employed to detect the pathogen in naturally infested soil, irrigation water and plant tissues (Wang et al. 2007).

Detection and quantification of *Phytophthora capsici* oospores present in soil samples was accomplished by combining a sieving-centrifugation and a real-time quantitative PCR (QPCR) format. Five soil samples representing three different soil textures infested with oospores of *P. capsici* were suspended in water and then passed through 100-, 63-, 38-μm metal sieves and finally through a 20 μm filter. The material retained in the filter was resuspended in water and centrifuged. After subjecting the pellet to sucrose extraction process, the concentration of oospores was determined using hemacytometer. A QPCR method using primers based on the sequence of rDNA was applied for quantifying the DNA extracted from the oospores recovered from the soil samples. The relationship between the quantity of DNA of *P. capsici* oospores recovered from the soil and the number of oospores incorporated into the soil was determined by a regression equation (Pavon et al. 2008).

A PCR-based ‘molecular tool box’ was developed to detect and identify 15 *Phytophthora* spp. that seriously damage forests and trees and other natural ecosystems. The primers employed were designed based on the sequences of the ras-related protein gene *Ypt1*. Conventional PCR format was successful in detecting the pathogens in infected plant tissues. Nested PCR format had to be developed for the detection of *Phytophthora* spp. in infested soils. Amplification with *Phytophthora* genus-specific primers before amplification with various species-specific primers (nested PCR) increased the sensitivity of detection over amplification with species-specific primers only. *P. utricola* was the most frequently encountered pathogen among *Phytophthora* spp. detected in soil samples from natural Scottish ecosystems (Schena et al. 2008).

Phytophthora nicotianae is an important soilborne pathogen capable of causing economically important diseases in tobacco and other crops. A species-specific PCR assay for the detection of *P. nicotianae* in the soil and infected plants was developed by employing a pair of a primer pair based on the sequences of a

Ras-related protein gene *Ypt1* (a single-copy gene) which provided high level of sensitivity and specificity for the tests. Isolates (115) representing 26 species of *Phytophthora* and 29 phytopathogenic fungal species were used. Single round amplification by PCR using the primer pair *Ypt1*-F/*Ypt1*-R resulted in the detection limit of 1 ng pure DNA of *P. nicotianae*/25 μl PCR reaction. In order to enhance the sensitivity of detection of *P. nicotianae* in soil and irrigation water samples which contained very low quantities of pathogen DNA, a pair of *Phytophthora* universal primer pair *Ypt-1*F/*Ypt1*-R was employed for the first round amplification in the nested PCR format. The amplicons were then amplified with species-specific primer pair Pn1/Pn2. the detection limit of the nested PCR was 10 pg DNA of *P. nicotianae*. The protocol developed in this investigation was very sensitive for the detection of *P. nicotianae* in the soil samples and it has the potential for tracking the pathogen movement in soil and irrigation water (Meng and Wang 2010).

A rapid, specific and sensitive real-time PCR assay was developed for reliable detection of *P. nicotianae* in soil samples. Primers targeting ITS regions of rDNA genes of *Phytophthora* spp. were designed. Based on the difference in the nucleotide sequences of ITS2 of 15 different *Phytophthora* spp. primers specific for amplification of DNA fragment from *P. nicotianae* were designed for use in the real-time PCR assay. The detection limit of standard PCR was 10 pg/μl of pathogen DNA. On the other hand, SYBR Green I PCR format could detect as little as 1.2 fg/μl, while TaqMan PCR detected 1.2 fg/μl of *P. nicotianae* DNA. The real-time PCR assays were 10^4–10^5-fold more sensitive than the standard PCR assay. The protocol developed in this investigation maximized the yield and quality of DNA recovered from the soil samples in a short time. The real-time PCR procedure detected *P. nicotianae* in the soil with a detection limit of 1.0 pg/μl. This procedure was useful for survey of tobacco fields in China with history of *P. nicotianae* infection (Huang et al. 2010).

Nested PCR has been shown to be effective in detecting *Plasmodiophora brassicae*, an obligate parasite, causing club root disease of crucifers. Conventional PCR protocol was employed to amplify a section of rDNA repeat and the PCR products were sequenced. Primers were designed based on the sequences of the PCR product and they were directed at rRNA genes and ITS regions. The primers PbITS1 and PbITS2 amplified the predicted product of approximately 1,100 bp, whereas the nested primers PbITS6 and PbITS7 gave a product of 620 bp. The primers were specific to *P. brassicae* and did not amplify the DNA from more than 40 common soil organisms and host plants. The nested PCR assay could detect the pathogen in all soils where the inoculum was adequate to induce the disease (Faggian et al. 1999).

In an another investigation, a simple, one-step PCR assay was developed to detect *P. brassicae* in soil samples. The primers used were TC1F and TC1R designed using the partial sequence of 18S ribosomal rRNA gene. This primer pair yielded a 548-bp product. Another primer pair TC2F and TC2R amplified a fragment of the 18S and ITS1 regions of the rDNA repeat yielding a 591-bp product. Both primer sets were specific in amplifying only the DNA of *P. brassicae*, but not from the DNA of noninfected host plant or noninfested soil or common soil fungi and bacteria. The detection limit of the assay was 100 fg of *P. brassicae* DNA or 1×10^3 resting spores

per gram of soil. This protocol detected the pathogen consistently in the soil when the disease index was about 11% as determined by bioassay. This protocol has the potential for providing routine detection of *P. brassicae* in soil samples specifically and rapidly (Cao et al. 2007).

Spongospora subterranea causative agent of potato powdery scab disease was detected and quantified by employing a PCR-based assay using the primers SsF and SsR designed from the ITS regions of pathogen DNA. The PCR format was modified with improved soil DNA extraction methods to detect *S. subterranea* in soil. DNA was directly extracted from aqueous extracts of field soil samples and a non-infested field soil sample using a Bead-beating/CTAB method and also using the UltraCleanTM Soil DNA Kit. Following PCR amplification, *S. subterranea* was detected in the infested soils, but not from the negative control soil. A competitive PCR was employed to quantify *S. subterranea* in naturally infested soil samples. The ratios of *S. subterranea* product of 434-bp and competitor product of 541-bp was determined for each reaction. The amounts of amplicons (434-bp) of pathogen origin increased with increasing numbers of spore balls of *S. subterranea* in the reaction mixture (Fig. 3.4). Negative trend was seen in the case of competitor product (541-bp). The levels of spore balls in the soil samples determined by competitive PCR assay showed positive correlation with the extent of powdery scab disease incidence. The PCR formats have the potential for routine use to detect and quantify *S. subterranea* in infested soils and also in infected plants (Qu et al. 2006) (Appendix 4). A real-time PCR capable of detecting early and quantifying precisely *S. subterranea* in soil was developed. This assay could reliably detect and quantify DNA from spore balls, zoospores and plasmodia/zoosporangia of *S. subterranea.* This assay was able to determine the viability of spore balls in soil and it was combined with tomato bait plant test to study zoospore release and infection levels in potato tubers under different environmental conditions (Lees et al. 2008).

Synchytrium endobioticum, causative agent of potato wart disease, produces thick-walled resting spores that can survive in the field soil for about 20 years. According to the European Plant Protection Organization (EPPO), the fields infested with the resting spores of *S. endobioticum* should be prohibited for potato

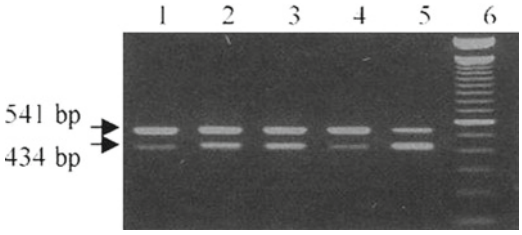

Fig. 3.4 Detection of *Spongospora subterranea* in field soil samples for competitive PCR assay. Lanes 1–5: soil samples from Ireland. Note the presence of the bands specific to pathogen DNA fragment (434-bp) and competitor DNA fragment (541-bp). Lane 6: 100 bp DNA ladder (Courtesy of Qu et al. 2006; The Potato Association of America, Orno, USA)

production for 20 years or more, until the presence of the pathogen can no longer be demonstrated. Hence, a sensitive and reliable method had to be developed for detection and quantification of *S. endobioticum* in plant tissues and the soils. A zonal centrifugation method designated Hendrickx centrifuge technique was tested for routine detection of winter sporangia of *S. endobioticum* present in the soil samples. Naturally and artificially contaminated soil samples were used for determining the recovery percentage of and variation in the numbers of winter sporangia. With the Hendrickx centrifuge procedure, recovery percentage was higher than the method recommended by the Dutch Plant Protection Service (van den Boogert et al. 2005; Wander et al. 2007). Specific primers and a TaqMan probe designed from the ITS region of the multicopy rDNA gene of the pathogen were tested using the extracts from artificially and naturally infested soils. Coamplification of target DNA along with an internal competitor DNA fragment resulted in a more reliable diagnosis of *S. endobioticum*, avoiding possible false-negative results (van Gent-Pelzer et al. 2010).

Fusarium oxysporum found frequently in all natural and cultivated soils was detected by a hybridization assay and PCR-based assay, using probe and primers based on the sequences of 28S rDNA. Two primers PFO2 and PFO3 and one 20-mer oligonucleotide probe were designed. The oligonucleotide probe HFO1 hybridized exclusively with DNA from *F. oxysporum* strains only. PCR assay resulted in amplification of DNA only from *F. oxysporum* strains, but not from any of the 80 other strains tested. Both PCR and hybridization protocols were validated with 53 additional isolates from four different soils. These assays provided reliable results for the identification of *F. oxysporum* in soils (Edel et al. 2000). *Fusarium oxysporum* f.sp. *niveum* causing Fusarium wilt disease and *Mycosphaerella melonis* causing gummy stem blight disease in water melon crops are soilborne pathogens. The two pathogens were detected in the soil and plant tissues by two species-specific PCR assays. Two pairs of specific primers Fn-1/Fn-2 and Mn-1/Mn-2 were designed based on the differences in the ITS sequences of *Fusarium* spp. and *Mycosphaerella* sp. Using ITS1/ITS4 as the first round primers, combined with either Fn-1/Fn-2 and or Mn-1/Mn-2, two nested PCR protocols were formulated. The detection sensitivity of the nested PCR format increased by 1,000-fold to 1 ag (attogram) level, compared to amplification with species-specific primers alone. The detection sensitivity for the pathogens in soil was 100 microconidia/g of soil. A real-time fluorescent quantitative PCR assay was useful to detect and monitor these pathogens directly in soil samples (Zhang et al. 2005).

A reliable and sensitive method of detection of Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *vasinfectum (Fov)* was found to be necessary to implement the disease prevention program. PCR amplification of ribosomal intergenic spacer (IGS) regions combined with digestion of three restriction enzymes *Alu*I, *Hae*III and *Rsa*I resulted in three unique restriction profiles (IGS-RFLP haplotypes) for Australian *Fov* isolates, which could distinguish them from other *formae speciales* of *F. oxysporum*. Further, two specific real-time PCR-based assays were developed for the absolute quantification of genomic DNA from isolates from soil

substrates and infected cotton tissues. The limit of detection of *Fov* was 5 pg/µl of genomic DNA. The detection sensitivity for inoculum spiked into sterile soil was lower than 10^4 conidia/g of soil. In the plant tissues, detection limit was 30 pg to 1 ng/100 ng of total plant genome DNA for quantification of pathogen DNA (Zambounis et al. 2007).

A method was developed for extraction of DNA from *Verticillium dahliae*, causing Verticillium wilt disease in many economically important crops, without the need for culturing the pathogen and DNA purification for use in PCR-based assay. The pathogen cells in soil are disrupted by grinding in liquid nitrogen with natural abrasives in soil and losses due to degradation and adsorption are largely eliminated by the addition of skim milk powder. The DNA from disrupted cells is then extracted with SDS-phenol and collected by ethanol precipitation. The DNA thus extracted after suitable dilution, can be used directly for PCR amplification. This method is rapid, cost-effective and by including the appropriate internal controls, it can be applied for detection of *V. dahliae* directly from soils. Similar results were obtained for conventional and nested PCR format employed for the detection and quantification of microsclerotia of *V. dahliae* present in soil samples (Volossiouk et al. 1995) (Appendix 5).

In a later study, by applying PCR assay, three species of *Verticillium*, *V. dahliae*, *V. albo-atrum* and *V. tricorpus* were detected in the field soils and the relative efficiency of detection with plating and PCR assays was compared. Although the plating assay could detect *V. dahliae* and *V. albo-atrum*, it failed to detect *V. tricorpus* whose presence was detectable by PCR assay. Furthermore, PCR assay was more rapid and efficient requiring only 1–2 days for positive identification of the three *Verticillium* spp., while plating assay needed 4–5 weeks. However, the plating assay provided a quantitative measure of pathogen propagules in the soil ranging from 0 to 21,625 colony forming units (CFUs)/g of soil. PCR assay on the other hand, could not provide a quantitative estimation of pathogen propagules, but it could differentiate between the weakly pathogenic *V. albo-atrum* strain 2 and the more aggressive *V. albo-atrum* strain 1 in addition to detection of the third species *V. tricorpus.* The major advantage of PCR assay is that when the symptoms of the disease appear, the major Verticillium wilt pathogen present in the field soils can be rapidly and reliably detected by the PCR assay (Platt and Mahuku 2001).

A competitive PCR format was developed for the detection and quantification of *Verticillium tricorpus* directly from infested soil samples. Co-amplification of *V. tricorpus* DNA with competitor DNA provided accurate quantification in the range of 10^2–10^6 spores and 1–500 microsclerotia in the inoculated soil. A strong correlation ($r = 0.99$) was found between number of spores added to pathogen-free soil under controlled conditions and the number of spores estimated by competitive PCR assay. The number of propagules determined at harvest time was not correlated with initial amount of inoculum spiked at planting time, as determined by the competitive PCR format (Heinz and Platt 2000). A quantitative PCR assay based on the competitive PCR technique was applied to estimate *V. dahliae* DNA with a

Table 3.2 Detection of microsclerotia of *Verticillium dahliae* in different years 1994–1997 (Mahuku and Platt 2002)

	Soil dilution method		PCR assay	
Year	Mean	SE	Mean	SE
1994	0.25[a]	0.125	7.05[a]	1.198
1995	0.39[a]	0.215	5.85[a]	1.571
1996	0.18[a]	0.043	8.64[b]	1.942
1997	0.07[b]	0.020	11.62[c]	1.225
Mean	0.22			8.29

Means followed by the same letter in the column are not significantly different according to Duncan's multiple range test (DMRT) ($P < 0.05$)

competitor DNA, provided accurate quantification in the range of 10^2–10^7 spores and 1–100 microsclerotia/g of soil. The results of soil dilution method and PCR assay were strongly correlated ($r = 0.97$) (Table 3.2). The competitive PCR format was found to be reliable, fast and did not depend on the subjectiveness of soil dilution method (Mahuku and Platt 2002). A real-time PCR assay was employed for the detection of *Verticillium dahliae* in soils on which pepper (*Capsicum annuum*) and tomato crops were raised. It was possible to detect the pathogen in plants, soil and irrigation water before the appearance of visible symptoms of infection. It was suggested that screening for the presence of *V. dahliae* should be carried out before the transport of plants or soil material, if the existence of the pathogen is suspected (Gayoso et al. 2007).

DNA array technology was designed for the simultaneous detection of multiple pathogens and optimized for quantification over at least three orders of magnitude of *V. dahliae* and *V. albo-atrum*. A strong correlation was noted between hybridization signals and pathogen concentration for standard DNA added to DNA from different origins and also for infested samples. In addition, quantitative assessment of other tomato pathogens *Fusarium oxysporum*, *F. solani*, *Pythium ultimum* and *Rhizoctonia solani* in the environmental samples was taken up by employing DNA array procedure. Assessments made by real-time PCR assay and DNA array technology were highly correlated, indicating the reliability and robustness of DNA array procedure (Lievens et al. 2005).

The soilborne pathogen *Phialophora gregata*, the causative agent of brown stem rot of soybean is difficult to isolate and the pathogen has a long latent period in the infected plants. Hence, a TaqMan probe-based quantitative, real-time PCR assay was applied to provide sensitive and specific detection and quantitative estimation of the two genotypes, A and B of *P. gregata* in plant and soil samples. The detection limits of the assay were 50 fg of pure genomic DNA, 100 copies of the target DNA sequence and approximately 400 conidia. The QPCR format was about 1,000-fold more sensitive in detecting DNA and conidia of *P. gregata*. Conidia of *P. gregata* were detected in soil, using QPCR after extraction of DNA using the MoBio Power soil DNA extraction kit. *P. gregata* was detected using QPCR assay in field soils with a history of brown stem rot disease at population levels of 10^3–10^5 conidia.

This protocol was able to provide rapid, specific and sensitive detection and quantification of *P. gregata* in plants as well as in the soil samples (Malvick and Impullitti 2007).

3.1.4 Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

Matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) technique involves separation of small DNA molecules on the basis of their charge to mass ratio. The separation time is in the order of microseconds, although several analyses are typically performed for each sample which extends the overall time to a couple of seconds (Ross et al. 2000). Small DNA molecules of less than 100-bp and differing in size by a single base can be easily differentiated. The high-throughput capability of the technique, as up to 800 samples can be processed simultaneously and automated, makes it attractive as an alternative diagnostic tool for detection and differentiation of microorganisms. The MALDI-TOF MS method has been employed earlier for the detection of virus (Kim et al. 2005) and bacteria (Jackson et al. 2007). Each reaction may be multiplexed, allowing several loci for each species or several different species to be detected in each reaction. In this technique, a dideoxynucleotide triphosphate (ddNTP) is added to a genotyping primer annealed by PCR-amplified DNA. The products (extended and unextended primers) are separated by MALDI-TOF MS. The ddNTP is complementary to a single nucleotide polymorphic (SNP) site in the template. The different mass of each of the four DNA bases can be used to identify which base has been added to the primer. Hence, four potential products can be obtained and detected from each SNP site (Siricord and O'Brien 2008).

Root rot diseases affecting eucalypt forests of south eastern Australia, are caused by different *Phytophthora* spp. that spread through the movement of infested soil or plant materials. The location of the pathogen has to be mapped to prevent the spread of the disease to new areas. As precise mapping involves testing a large number of soil samples, application of MALDI-TOF MS procedure was found to be effective. Appropriate sites for the design of genotyping primers for *Phytophthora cinnamomi* were located by visually scanning an alignment of the sequences of the ITS1 and ITS2 regions of *Phytophthora*. Two prominent peaks, one for the unextended primer and one for the extended primer were seen. The extension product was noted only when *P. cinnamomi* was present. The peaks were clearly differentiated. A similar profile was obtained for DNA extracted from the mycelium-soil mixture, demonstrating that the profile was not altered by contaminants coextracted with DNA from the soil. The results obtained by employing MALDI-TOF MS method were reproducible and rapid, showing the potential for wider application. The presence of PCR inhibitors in the soil extracted, did not affect the reactions (Siricord and O'Brien 2008).

3.2 Detection of Fungal Pathogens in Water

Soilborne fungal pathogens are primarily disseminated through irrigation or rain water that carries the fungal structures from field to field. Spread through transportation of infested soil may occur to a limited extent. Hence, detection of fungal pathogen in water has a bearing on the development of effective disease management systems aimed to restrict the spread of the soilborne diseases which are difficult to control by the existing disease management practices.

3.2.1 Immunoassays

Antibody-based immunoassays have been developed for the rapid detection of fungal pathogens in water. A method for the detection of *Phytophthora* sp. and *Pythium* sp. in irrigation water using commercial ELISA test was developed. The commercially available ELISA tests were able to detect relatively small numbers of *Phytophthora* and *Pythium* zoospores in water samples. When zoospores were concentrated into filter pads and antigens were extracted by brief heating, the Phytophthora E Kits gave significant ($P< 0.05$) positive reactions with as few as 30–40 zoospores on a filter pad. A water sample collected in early spring from a southern California nursery contained 442 viable propagules of Pythiaceous pathogens per liter of water. The species recovered on agar media were *Phytophthora parasitica*, *P. citrophthora*, *P. cryptogea* and an unidentified *Phytophthora* sp., in addition to *Pythium coloratum*, *P. rostratum*, *P. middletonii*, *P. ultimum* var. *sporangiferum* and *Pythium L* group. All of the *Phytophthora* spp. recovered reacted positively with Phytophthora Kits and negatively with Pythium Kits. In contrast, all of the *Pythium* spp. recovered reacted positively also with Phytophthora Kits, but negatively with Pythium Kits. With a relatively simple filter-extraction procedure, ELISA kits effectively detected *Phytophthora* and *Pythium* in water samples (Ali-Shtayeh et al. 1991). The presence of viable zoospores of *Pythium* sp. in irrigation water was demonstrated by Wakeman et al. (1997). A detailed study was taken up for evaluating two antibody-based methods and comparing their efficacy with conventional methods for detection and quantification of viable and infective *Pythium* spp. and *Phytophthora* spp. inoculum in horticultural and irrigation water. In the *in vitro* conditions, zoospore trapping immunoassay (ZTI) was the most effective in detecting the zoospores of *Pythium ultimum* var. *sporangiferum*, *Pythium* Group F, *Phytophthora cactorum* and *P. cryptogea.* The conventional membrane filtration-colony plating gave similar results as ZTI which were highly correlated ($r^2 = 0.95$). The membrane filtration-colony plating was also very sensitive especially for the detection of *P. cactorum* and *P. cryptogea.* The dipstick assays and baiting methods were less sensitive than the other two methods. However, these methods also were effective in detecting propagules of *Pythium* group F and *P. ultimum*. The efficacy of these techniques was evaluated using water samples collected from horticultural nurseries and in *in situ* tests of infected root

zones of *Chamaecyparis*, tomato and Chrysanthemum. ZTI assay was again the most sensitive test for water samples, although membrane filtration-dilution plating proved to be a more consistent test. Dipstick and baiting assays were the best techniques for *in situ* testing. Dipsticks provided quantitative data on propagules that would be useful for epidemiological investigations (Pettitt et al. 2002).

3.2.2 Nucleic Acid-Based Techniques

Nucleic acid-based techniques have become more acceptable because of their higher levels of specificity and sensitivity as evidenced by the results obtained with plant samples infected by fungal pathogens. PCR-based methods provide distinct advantages of speed, sensitivity, specificity and flexibility. *Phytophthora nicotianae*, a common and destructive oomycete infects numerous agricultural and horticultural crops. A species-specific PCR assay was developed for rapid and precise detection of this pathogen in irrigation water, a primary source of incoulum and an efficient means of propagule dissemination. The PCR format involves the use of a pair of species-specific primers (PN) and customization of a commercial soil DNA extraction kit for purification of DNA from propagules present in the irrigation water. PN primers were designed based on the sequences of the elicitin gene *parA*. PN primers allowed greater efficient detection of *P. nicotianae* isolates, while the PP primers were specific to *P. parasitica*. PN primers were found to be specific in amplifying the DNA sequences of *P. nicotianae* isolates (131) only, but not other isolates (102) of 15 species of *Phytophthora* and 64 isolates of other oomycetes, true fungi and bacteria tested. The detection limit of the PCR assay was between 80 and 800 fg DNA/µl of *P. nicotianae*. The assay was able to detect the pathogen in naturally infested water samples from nurseries more rapidly and accurately than the conventional standard isolation procedures. The PCR format has the potential for application under field conditions to assist the growers for making timely disease management decisions (Kong et al. 2003) (Appendix 6).

The presence of various *Phytophthora* spp. capable of infecting many crop plant species in the recycling irrigation water used in the nursery and other crop production systems has been detected by using bait plant species. In order to identify the pathogenic oomycetes present in the recycled water up to species level, a PCR-based single strand conformation polymorphism (SSCP) analysis was applied to analyze 907 *Phytophthora* isolates. Seven distinct SSCP patterns representing six morphologic species viz., *P. citricola*, *P. hydropathica*, *P. insolita*, *P. megasperma* (I and II) and an unidentified *Phytophthora* species were generated. By using the direct colony PCR-SSCP assay, it was possible to identify all subcultures resulting from each seasonal water sampling in a timely fashion. In this assay, the target DNA fragment was directly amplified from the subcultures without the need to extract the fungal DNA. However, morphological examination of selected cultures was found to be an essential step to confirm the identity of the isolate by its DNA fingerprint. This procedure facilitated identification of all other subcultures from

each sampling within a few days. The protocol developed in this investigation has the potential for detecting *Phytophthora* spp. in streams and rivers (Ghimire et al. 2009).

Forest trees are devastated by different species of *Phytophthora*. Primers based on a region of the ras-related protein gene *Ypt1* were employed for the detection and identification of 15 species of *Phytophthora*. Single amplification with species-specific primers was not successful for the detection of the pathogens in water samples. Amplification with *Phytophthora*-genus-specific primers before amplification with various species-specific primers (nested PCR) was suitable, as the sensitivity of the assay was increased significantly by the nested PCR format. Eight out of 14 water samples tested contained at least one *Phytophthora* species. *P. citiricola* was the most commonly detected pathogen in water samples. Other pathogens detected included *P. inunda*, *P. cambivora*, *P. europa* and *P. pseudosyringae* (Table 3.3) (Schena et al. 2008).

Phaemoniella chalmydospora, the causative agent of Petri disease is responsible for serious problems in newly planted vineyards. The possibility of nursery procedures aiding in the dissemination of the pathogen was examined. The nucleic acid-based techniques single PCR, nested PCR, SYBR® Green quantitative PCR and TaqMan® protocol were employed for the detection and quantification of propagules of *P. chalmydospora* in water samples. The pathogen was detected in water used to wash grapevine cuttings sampled at collection in the field. All samples taken from rain water storage and cool down tanks, plus water used to soak buds during grafting, tested positive for *P. chlamydospora* in all sites. The quantitative real-time PCR TaqMan® assay was able to detect *P. chlamydospora* at a concentration of as low as 10 spore per 100 ml of water. The nested PCR was the most sensitive for detection followed by quantitative PCR assay. Single PCR was the least sensitive (Table 3.4) (Edwards et al. 2007).

Verticillium dahliae causing the Verticillium wilt disease in pepper (chilli) was detected by employing real-time PCR assay in the irrigation water and soil samples collected from greenhouses in Spain. Likewise, the presence of the pathogen was detected in irrigation water from three of eight farm samples. The results of the PCR amplifications of different samples with specific primers of

Table 3.3 Detection of *Phytophthora* spp. in naturally-infested water samples from forests of Scotland by nested PCR (Schena et al. 2008)

Location	Sample No	*Phytophthora* spp. detected
Dunkeld (Central Scotland)	1	None
	2	*P. citricola* and *P. inundata*
Clyde valley	3	*P. citricola*, *P. inundata*, *P. europaea* and *P. pseudosyringae*
	4	None

Table 3.4 Comparative efficacy of PCR formats for detection of *Phaemoniella chlamydospora* (Edwards et al. 2007)

PCR formats	No. of samples that tested positive/total	Range of spore concentration
Single PCR-Pch primers	16/39	
Single PCR-Pmo primers	22/39	
Nested PCR-ITS/Pch primers	32/39	
Nested PCR-ITS/Pmo primers	30/39	
SYBR® Green quantitative PCR-Pmo primers	20/39	8–3,452 spores/ml
TaqMan® quantitative primers	24/39	1–17,394 spores/ml

pepper and *V. dahliae* correlated well with the proportion of pepper plants infected by *V. dahliae* (Gayoso et al. 2007). *Fusarium solani* complex (FSSC) Group1, a phylogenetic species is synonymous with *F. solani* f.sp. *cucurbitae* race 2, a pathogen of cucurbit fruits. The environmental sources of FSSC1 are considered to be important for understanding the epidemiology of both human and plant diseases caused by this organism. FSSC1 was detected in sewage influent and community shower drains in California with a concentration ranging from 75 to 413 colony forming units (cfus). The pathogen was detected by culturing and real-time PCR assay. However, FSSC1 was rarely detected in cucurbit fruits after harvest (Mehl and Epstein 2008).

3.3 Detection of Fungal Pathogens in Air

Dissemination of various kinds of spores produced by fungal pathogens infecting aerial parts of host plants occurs through air. The spores formed in the spore-bearing structures such as conidiophores, acervuli, pycnidia, sporodochia, sori etc., are released into the air which carries them to different locations. Airborne inoculum plays an important role in the incidence and spread of diseases, making the monitoring and determination of air spora essential for disease risk assessment. The incoulum potential in the orchards is an important factor affecting both blossom blight and brown rot caused by *Monilinia fructicola* in stone fruits. Determination of inoculum potential, particularly the amount of spores in the orchard air in early- and mid-season is critical for predicting and managing brown rot disease.

Classically detection and enumeration of airborne spores has been achieved by microscopic examination of surfaces on which spores were impacted. Spore traps (Hirst spore trap) have been traditionally used to determine the spore density for airborne plant pathogens. Monitoring of the concentration of airborne conidia has been used as an aid for effectively controlling the diseases affecting

aerial plant parts/organs by applying fungicides. This approach was studied for the management of late blight disease of tomato caused by *Phytophthora infestans*. Airborne sporangia concentration monitored at several sites increased rapidly 1–2 weeks before disease onset. The airborne sporangial concentration was used as the basis for initiation of fungicide programs (Bugiani et al. 1995). A similar attempt was made in the case of *Botrytis squamosa* causing onion leaf blight disease in Canada. Airborne conidial concentration (ACC) of *B. squamosa* was measured in the center of each experimental plot using rotating-arm impaction samplers (Rotorod type) placed at 10–15 cm above the canopy. A linear relationship was established between ACC and number of lesions per leaf. Fungicide application was recommended when the ACC reached 10–15 conidia/m^3. By following this procedure fungicide application was reduced by 75% and 56% in 2002 and 2003 respectively, resulting in saving of fungicide cost considerably (Carisse et al. 2005).

Examination and identification of the spores trapped by isolation and plating is a very time-consuming and laborious method, in addition to the requirement of considerable experience in taxonomical studies. However, only few attempts appear to have been made to develop alternative and more efficient techniques for the detection and identification of airborne microorganisms. Serological techniques have the potential for detection of fungal spores and other bioparticles present in the air (Burge and Solomon 1987). But only few applications to plant pathogens have been reported. The possibility of identifying spores of *Botrytis cinerea* deposited on the trapping surface of a Burkard spore sampler was indicated by employing an immunoassay (Dewey 1996). A commercial immunoassay for the detection of airborne ascospores of *Venturia inaequalis* causing apple scab disease was developed by Chaparral Diganostics Burlington, USA. The ascospores released by the pathogen from the infected overwintered leaves could be detected on a card support medium (Kennedy et al. 1999).

An immunofluorescence assay was employed to detect ascospores of *Mycosphaerella brassicola* using Burkard spore trap with Melinex spore tape. A polyclonal antiserum generated against whole ascospores was used to detect by immunofluorescence, the ascospore and mycelial wall of *M. brassicola*, following reaction with anti-rabbit IgG FITC conjugate. Autofluorescence of spores and mycelial components of other fungal species was eliminated by applying counterstains Evan's blue and eriochrome black at 0.2% and 0.5% respectively in phosphate buffered saline (pH 7.2). The ascospores of *M. brassicola* on artificially inoculated Melinex spore tape and the ascospores impacted on spore tape coated with bovine serum albumin blocking agent were detected by the immunofluorescence protocol (Kennedy et al. 1999) (Appendix 7).

A new micrometer immunospore trapping (MTIST) device that uses a suction system to directly trap air particulates by impaction in microtiter wells was developed. This device has been shown to be useful for rapid detection and immunoquantification of ascospores of *M. brassicola* and conidia of *Botrytis cinerea* by an ELISA format under controlled environmental conditions. A positive relationship was observed between the number of MTIST device-trapped ascospores of

M. brassicola per liter of air sampled and the amount of disease expressed on *Brassica oleracea* (Brussels sprouts) plants. Likewise, a significant correlation (r = 0.8797) was noted between the number of conidia of *B. cinerea* per microtiter well and the absorbance values generated by ELISA test. The MTIST device was shown to have the potential for rapid differentiation and quantification of target pathogen accurately, in addition to the device being portable, robust and inexpensive. This system that can be employed to perform multiple tests in a single sampling period (Kennedy et al. 2000; Wakeham et al. 2000).

In a further study, the collection and retention of a range of common airborne fungal spores in the microtiter immunopore trap (MTIST) was investigated in wind tunnel experiments. Concentrations of spores of *Botrytis cinerea*, *Cladosporium cladosporioides*, *Lycopodium clavatum*, *Erysiphe cruciferum* and *Penicillium roqueforti* were determined using MTIST and miniature suction traps. A clear positive relationship was observed between spore concentration determined by MTIST and those measured by the mini-suction traps. *Botrytis*-specific antiserum was employed in an ELISA format. The microtiter plate wells were coated with either distilled water, poly-L-lysine or petroleum jelly and paraffin mixture. A high level of positive correlation was noted between the numbers of *Botrytis* conidia trapped by the MTIST and corresponding absorbance values (Fig. 3.5) (Wakeham et al. 2004).

Onion downy mildew disease caused by *Peronospora destructor* is considered as the most serious disease in bulb and salad onions. The disease spreads through airborne spores. An immuno-monitoring system for conidia of *P. destructor* involving the use of specific MAb in lateral flow device was employed. A competitive lateral flow format was able to detect the sporangia of *P. destructor*. A monoclonal antibody (MAb) that could recognize components of the wall of sporangia of *P. destructor* was generated. Five microliter of gold anti-mouse IgM solution premixed with 10 μl of pathogen specific MAb (EMA242) was found to be optimum for the detection of sporangia of *P. destructor* when applied to sample pads of lateral flow device. Using a Milipore HiFlowTM240 membrane competitive lateral flow device and EMA242 conjugated to gold spheres at a dilution of 1:150, test line formation was observed for all spore samples tested. The limit of detection was 500 sporangia of *P. destructor* based on the absence of a test line on the lateral flow device within test samples. The sensitivity of detection could be substantially increased by employing a scanning densitometer (Kennedy and Wakeham 2008).

Leptosphaeria maculans, causing Phoma stem canker disease and *Pyrenopeziza brassicae* causing light leaf spot disease in oilseed brassicas account for significant losses frequently. It is essential to monitor the spore contents of air around brassicas to provide reliable information on the possibility of disease breakout. The possible use of DNA-based techniques for detection of airborne inoculum of these two pathogens was explored. This fungal DNA could be extracted from spores deposited on pieces of Burkard spore trap tapes. The PCR-based assay detected both pathogens with a similar level of sensitivity. The smallest number of spores detected was ten. Specific detection of *P. brassicae* DNA was accomplished using nested PCR format employing primers Pb1 and Pb2 for the first round and primers PbN1 and PbN2 in the nested PCR assay. *L. maculans* DNA was amplified with specific

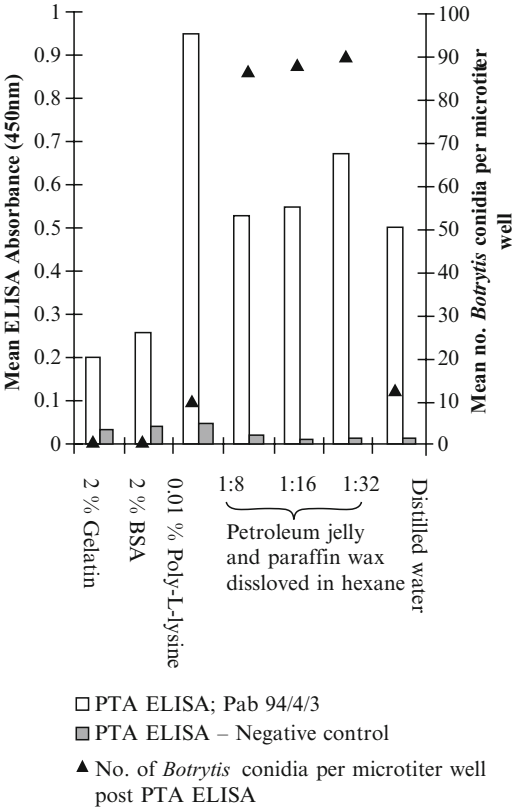

Fig. 3.5 Detection of *Botrytis cinerea* using plate-trapped antigen (PTA)-ELISA technique. Note the positive correlation between the numbers of *Botrytis* conidia trapped by MIST and ELISA absorbance values (Courtesy of Wakeham et al. 2004; Elsevier, Oxford, UK)

primers D1 and D2. The two target pathogens could be detected even in the presence of a large number of nontarget spores and other biological materials. This investigation revealed the potential of PCR-based assay for the detection of two important fungal pathogens in the air (Calderon et al. 2002).

Monilinia fructicola, the causative agent of blossom blight and brown rot of stone fruits produces spore inoculum in the mid and late season in stone fruit orchards originating from thinned fruits left on the orchard floor (Hong et al. 1997; Luo et al. 2005). These spores carried by the wind reach susceptible immature fruits on trees under favorable weather conditions. A nested PCR assay was developed based on microsatellite regions for detection of *Monilinia fructicola*. The nested PCR primers specific to *M. fructicola* were developed based on the sequence of a species-specific DNA fragment amplified by microsatellite primer M13. The external and internal primer pairs EMfF + EMfR and IMfF + IMfR amplified a 571-bp and a 468-bp fragment respectively from *M. fructicola* only. Using the nested PCR format, the spore density in the air was estimated employing the Burkard spore trap (Table 3.5). This study showed that about 170 spores per slide (about 0.01 spore/l) could be used as a spore density threshold for making a decision on fungicide application to reduce the risk of blossom infection (Ma et al. 2003).

Table 3.5 Detection of spores of *Monilinia fructicola* from the spiked air samples by nested PCR assay (Ma et al. 2003)

Number of spores used for extraction of DNA[a]	DNA dilution added for PCR amplification (equivalent number of spores/PCR)	Positive results obtained/total number of tests
1×10^2	1:100 (0.1 spore)	0/6
2×10^2	1:0 (20 spores)	1/6
2×10^2	1:10 (2 spores)	6/6
2×10^2	1:100 (0.2 spore)	0/6
2×10^3	1:10 (200 spores)	6/6
2×10^3	1:0 (20 spores)	6/6
2×10^3	1:100 (2 spores)	6/6
2×10^3	1:1000 (0.2 spore)	0/6

[a]Final DNA content from each sample was dissolved in 50 μl of sterile water

In a further study, a real-time PCR assay was developed to efficiently quantify the dynamics of spore density in six orchards where the spore densities of *Monilinia fructicola* in the air was monitored using Burkard spore traps. When the DNA template of ten isolates each of *M. fructicola*, *M. taxa*, *Botryosphaeria dothidea* and *Alternaria alternata* was employed to test the specificity of species-specific primer pair RTMfF/RTMfR. Only *M. fructicola* isolates showed the expected 390-bp PCR product, revealing the specificity of the primer pair. A strong linear relationship was observed between standard curves CT values and spore quantity in a range from 20 to 20,000 spores per sample. In addition, a linear relationship could be established between the number of spores counted with the light microscope and the corresponding number of spores determine with the real-time PCR assay (Fig. 3.6). Real-time PCR assay was used to quantify *M. fructicola* spores on spore trap tapes. High spore densities in the air were recorded in March and again in July and spore densities were low during April–June. The real-time PCR assay provided distinct advantages over the conventional method such as avoidance of errors in identifying spores along with speed, specificity and sensitivity in providing results. The cost of real-time PCR assay per sample was estimated to be US $3 for consumables excluding labor cost (Luo et al. 2007) (Appendix 8).

Oilseed rape (*Brassica napus*) crops suffer heavily due to the sclerotinia stem rot (SSR) disease caused by *Sclerotinia sclerotiorum*. As this disease primarily spreads through airborne spore, a specific diagnostic technique to accurately detect and quantify airborne inoculum of *S. sclerotiorum* was developed. The airborne ascospores were trapped using Buckard (Hirst-type) spore traps and rotating-arm traps. The DNA was extracted from the spores trapped in wax-coated plastic traps. A SYBR Green quantitative PCR (qPCR) method was applied to determine the pathogen population. A linear relationship between number of ascospores and *S. sclerotiorum* DNA (mean 0.35 pg DNA/spore) was observed. The assay was able to detect as few as two ascospores present in the spore trap. The specificity of the assay was revealed by the absence of amplification of DNA of *B. napus*, and other

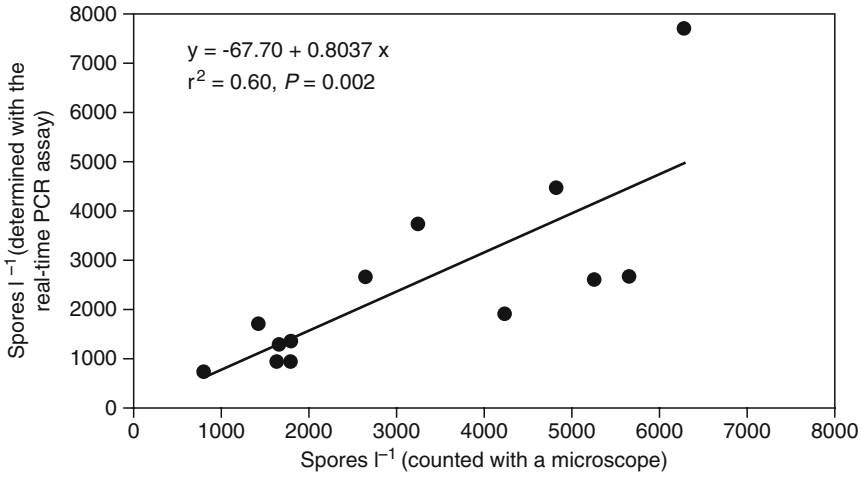

Fig. 3.6 Correlation between the number of spores of *Monilinia fructicola* estimated by microscopy and spores determined by real-time PCR assay (Courtesy of Luo et al. 2007; with kind permission of Springer Science and Business Media, Heidelberg, Germany)

fungal pathogens including *Sclerotinia minor*, *S. trifolium* and *Botrytis cinerea* and common airborne fungal genera like *Cladosporium* and *Penicillium*. No relationship between rainfall and airborne ascospores of *S. sclerotiorum* present at Rothamsted was evident. The qPCR protocol developed in this investigation may be useful for detection and quantification of *S. sclerotiorum* in seeds, other plant tissues such as stem or petals as well as in the soil (Rogers et al. 2009).

A DNA-based method for quantification of airborne inoculum of *Botrytis squamosa*, the causative agent of Botrytis leaf blight disease of onions was developed. The DNA from the conidia trapped in the rotating-arm samplers was purified and analyzed using the quantitative TaqMan real-time PCR (qPCR) procedure. The sensitivity of the qPCR assay was high with a detection limit of 2 conidia/rod. There was a positive correlation between the numbers of conidia counted using a light microscope and the quantity of DNA estimated by qPCR assay. Field samples (142) from commercial onion fields were analyzed to assess the possibility of predicting disease risk being low or above damage threshold (Dth) level. At the damage thresholds of five or ten lesions/leaf, conidia quantification with qPCR assay was more reliable at predicting disease risk than by microscopical counting of conidia. The results indicated that the qPCR protocol was reliable for quantifying *B. squamosa* airborne inoculum in commercial onion fields, providing a dependable basis for disease risk assessment (Carrise et al. 2009).

Pinus radiata grown in several countries for its timber, is affected seriously by pine pitch canker caused by *Fusarium circinatum* (*Hypocreales*). A novel method of combining the use of filter paper (Whatman No.1) for trapping the spores of *F. circinatum* in the air and real-time PCR format for analyzing the spores trapped was devised. The primer pair CIRC1A/CIRC4A was designed on the basis of alignment of the intergenic sequences (IGS) regions of 31 *Fusarium* strains. The primer

pair amplified specifically a 360-bp fragment only from the isolates of *F. circinatum*. The petriplates containing Fusarium selective medium (FSM) and filter paper were collected at 1 or 2 weeks after exposure to air and spore concentrations were quantified by SYBR Green method using SYBR-Green I as fluorescent dye. The filter paper method allowed prolonged and more sensitive spore sampling in the field compared to traditional traps using petridishes containing FSM. In a field assessment carried out at two sites in coastal California where trees were infected by pine pitch canker disease showed that spore counts were in the range of ca. 1×10^3 to $1 \times 7 \times 10^5$ m^{-2} with the highest spore counts in the fall, indicating seasonal variations in that location (Schweigkofler et al. 2004).

An integrated air sampling and PCR-based methods for detecting airborne fungal spores, using *Penicilliun roqueforti* as a model fungus in the presence of plant pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum* was evaluated. Single-step PCR, nested PCR and PCR followed by Southern blotting and probing were compared for their relative efficacies. It was possible to detect the DNA from one spore by nested PCR format using the crude disrupted spore preparation and the small scale DNA extraction method (Williams et al. 2001). The air borne fungal spores in boreal forest sites viz., clear-cut, young and old-growth forests were assessed by applying a PCR-based assay. The primers were designed based on the sequences of ITS regions of rDNA. The spore-trapping approach was utilized for the first time for identifying the spores of fungi and determining the phylogenetic relationships of the fungi identified. *Cladosporium* spp. was the most frequently observed in the air samples from the clear-cut and young forest site. A step-wise decline in the number of spores of *Cladosporium* spp. was recorded from the clear-cut forest site to the young and the old-growth forest site. The spore-trapping approach appears to have the potential of targeting and studying unknown fungi (Kauserud et al. 2005).

Cladosporium is one of the most common mold found in indoor and outdoor environments. The abundance of the airborne conidia is due primarily to their ability to grow in a wide array of substrates such as plants, wood, paper, leather and foods. Several species of *Cladosporium* are plant pathogens and some are allergenic to humans. Detection and quantification of *Cladosporium* in aerosols have relied on microscope/culture-based techniques which are time-consuming and laborious. Molecular techniques complementary to the conventional methods have the advantage of detecting the presence of the fungi regardless of their culturability. Conventional PCR format does not allow for accurate quantification of spores. Two real-time PCR formats, SYBR Green I and TaqMan chemistry were applied to quantify *Cladosoporium* in aerosols of five different environments including fruit and storage facilities. The numbers of *Cladosporium* spores determined by SYBR Green I and TaqMan probe ranged from 4.7×10^2 to 6.9×10^3 and from 5.0×10^2 to 2.4×10^3 respectively in four samples from vegetable storage facilities. These methods could facilitate accurate detection and quantification of *Cladosporium* spp. in different environments providing reliable data for public health related risk assessment (Zeng et al. 2006).

Erysiphe (Uncinula) necator, an obligate pathogen causes the powdery mildew disease, one of the most destructive diseases of grapevine. A PCR-based assay was developed, using species-specific primers (uncin 144 and uncin 511)

based on the unique sequences of the ITS regions of *E. necator*. A 367-bp fragment was amplified exclusively from *E. necator* DNA, but not from other powdery mildew pathogens infecting 48 different host plant species. The limit of detection was five conidia of *E. necator* directly added into the reaction mixtures or 100–250 conidia placed on glass rods coated with silicon grease. Under field conditions, the PCR protocol detected *E. necator* inoculum in air samples within hours of sample rod collection and prior to disease onset. When the grapevines were dormant, no amplification of the pathogen DNA occurred. The initial PCR detection of *E. necator* of the season occurred during seasonal ascospore releases caused by precipitation. Detection of the pathogen (ascospores) was possible several days before the appearance of visible symptoms of the disease. The development of this protocol may be considered as the initial step for making reliable disease risk assessment (Falacy et al. 2007).

Infection of hop by the downy mildew pathogen *Pseudoperonospora humuli* is common in most hop production regions. A PCR assay specific to *P. humuli* and the related pathogenic species *P. cubensis* was used to monitor the airborne inoculum in the hop yards to facilitate initiation of fungicide application at right time. The PCR assay could detect as little as one pg of the genomic DNA of the pathogen and yielded a specific amplicon in 70% of the reactions, using the DNA extracted from single sporangia. During the 9 years of testing, *P. humuli* could be detected in air samples not later than 8 days after the appearance of trace levels of disease symptoms and/or detection of airborne spores in a volumetric spore sampler. Inoculum was detected by PCR assay 4.5 days (average) before the first appearance basal spikes in six commercial hop yards. First fungicide application based on the PCR detection of *P. humuli* enhanced the effectiveness of disease control or reduced the fungicide use in four of the 6 yards compared to growers' standard practices. Diagnosis of *P. humuli* using the PCR assay may enhance the efficiency and efficacy of hop downy mildew disease management (Gent et al. 2009).

3.4 Detection of Fungal Pathogens in Alternative Host Plants

Fungal plant pathogens may either infect other plant species (alternate hosts) required for completion of their life cycle or infect other plant species (alternative hosts) especially weeds and sometimes other crop plants in the absence of principal crop plants for their survival. The weeds, wild plants and self-sown volunteer plants when infected serve as important sources of inoculum for the crops that are sown or planted in the next season. The importance of alternative host plants that can provide inoculum has been well realized, since epidemiological studies have emphasized the need for eradication of all sources of inoculum during the off-season to restrict the incidence and spread of the diseases to newly planted crops.

As discussed in the sections dealing with detection of fungal pathogens in the soil, water, air and crop plant species, application of conventional methods involving isolation and examination of the pathogen structures under the microscope will

be time-consuming and laborious for the detection and identification of fungal pathogens infecting alternative host plant species. As a first step in the detection and identification, pathogenicity of the isolates of pathogens infecting alternative host plants has to be established, before proceeding further. The isolates of rice blast pathogen *Magnaporthe grisea* from rice and grass hosts were first differentiated into four distinct races by inoculation on Korean rice differential varieties. They were then analyzed by RFLP using the repetitive probe MGR 586 and by restriction fragment length polymorphism (RFLP) technique. It was concluded that *M. grisea* populations from nonrice hosts such as *Digitaria sanguinalis*, *Eleusine indica*, *Lolium boucheanum*, *L. multiflorum* and *Festuca elatior* could serve as sources of inoculum for rice crops (Han et al. 1995).

The roots of *Solanum nigrum* were actively colonized by *Phytophthora capsici*, causing Phytophthora blight disease of pepper resulting in plant mortality. *Geranium carolinianum*, *Portulaca oleracea* and *Solanum americanum* were also found to be alternative hosts for *P. capsici* under natural field conditions by pathogenicity tests. Isolates of *P. capsici* were tested using PCR to validate morphological identification. The primer PCAP was employed in combination with the universal primer ITS1 and amplification of a 172-bp product confirmed the identity of the isolates as *P. capsici.* This investigation demonstrated the potential of weeds functioning as the reservoirs of inoculum that may help pathogen survival in the absence of crop plants (French-Monar et al. 2006).

Appendix 1: Detection of *Plasmodiophora barassicae* from Soil (Orihara and Yamamoto 1998)

A. Preparation of antiserum

(i) Homogenize infected roots and hypocotyls of turnip in distilled water using a blender for 5 min; filter through eight layers of gauze; centrifuge the filtrate at 3,000 rpm for 20 min; resuspend the pellet in distilled water and repeat the process of pelleting and resuspending five times.

(ii) Overlayer the final suspension on a sucrose density gradient column (20% and 40%) set up in the centrifuge tube; centrifuge at 3,000 rpm for 20 min; collect the layer containing the resting spores; wash them five times with distilled water and store at –20°C.

(iii) Immunize male New Zealand white rabbit by injecting intravenously 0.5 ml of antigen suspension (6×10^7 spores/ml in 0.85% NaCl solution); inject 1 ml of antigen emulsified with 1 ml of Freund's complete adjuvant intramuscularly twice at 2-week intervals and inject intravenously an additional dose of 0.5 ml antigen (5.4×10^7 resting spores/ml) at 2-week interval after intramuscularinjection.

(iv) Collect the blood 2 weeks after the final injection and separate the antiserum and estimate the antibody titer by microprecipitation interface test (1/2048).

(v) Purify the antibodies by adopting ammonium sulfate precipitation and DEAE-cellulose column chromatography procedures.

B. Detection of the fungal pathogen in infested soil

(i) Sterilize the soil by autoclaving followed by drying at 160°C for 10 h; grind and sift the soil through a 1 mm sieve and infest the soil with resting spores obtained from infected plant tissues at the density of 1×10^8 spores/g of dry soil.

(ii) Suspend 2 g soil sample in 40 ml of distilled water and stir well for 60 min filter through sieves of 60, 200, and 635 mesh sieves to remove the organic matter.

(iii) Centrifuge the filtrate at 3,000 rpm for 10 min and suspend the pellet in a predetermined volume of distilled water for performing immunoassays.

C. Indirect enzyme-linked immunosorbent assay (ELISA)

(i) Suspend the infested soil samples in coating buffer and dilute it serially by tenfold.

(ii) Dispense 200 µl of each diluted suspensions into two wells of microtiter plates and incubate the plates overnight at 4°C.

(iii) Wash the plates thrice with PBS containing 2% polyvinyl pyrrolidone (PVP) and 2% bovine serum albumin (BSA); incubate for 1 h at room temperature and wash the plates as done earlier.

(iv) Transfer to each well 2 µg/ml of anti-resting spore IgG in PBS; incubate for 4 h at 37°C and wash the wells as done earlier.

(v) Add to each well PBS containing goat-anti-rabbit IgG-alkaline phosphatase conjugate (Chemicon, USA) at a 1/2,000 dilution; incubate for 4 h at 37°C and wash the wells as done earlier.

(vi) Add 10% diethanolamine (v/v, pH 9.8) containing 1 mg/ml *p*-nitrophenylphosphate enzyme substrate to each well and incubate in the dark at 37°C for 20 min.

(vii) Record the absorbance values for the wells at 405 nm using a microplate autoreader (Tosoh Ltd., Japan).

D. Dot immunobinding assay (DIBA)

(i) Serially dilute samples by tenfold; spot the samples (2 µl each) onto a 40 cm^2 nitrocellulose membrane sheet (Trans-Blot, BIO-RAD, USA) and air dry the sheets.

(ii) Block overnight at 4°C in a buffer solution of 20 mM Tris-HCl, 500 mM NaCl and 0.05% Tween 20 (TTBS, pH 7.5) containing 2% PVP and 2% BSA.

(iii) Treat the membrane with 0.1–0.2 µg/ml anti-rabbit resting spore IgG in TTBS containing 2% PVP and 0.2% BSA (TTBSPB) for 1 h at room temperature.

(iv) Treat the membrane with alkaline phosphatase-conjugated goat anti-rabbit IgG in TTBSPB for 1 h; apply the buffer (0.1M Tris-HCl, 0.1M NaCl

and 5 mM $MgCl_2$, pH 9.5) containing 0.33 mg/ml nitroblue tetrazolium substrate and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine salt prediluted with *N,N*-dimethylformamide.

Appendix 2: Detection of Resting Spores of *Pythium myriotylum* from Soil by PCR (Wang and Chang 2003)

A. Preparation of soil samples

(i) Collect soil samples at depths between 3 and 30 cm from fields; air-dry them separately, sieve and mix before assay.

(ii) Collect oospore from fungal cultures grown on artificial medium and incubate in 1% nonsterile soil extract.

(iii) Add dormant oospores to sieved nonsterile soil; incubate at room temperature; examine under the microscope to confirm the presence of oospores; determine the oospore density using the hemocytometer and adjust the oospore concentration to 1,000 oospores per gram of soil.

B. Separation and extraction of oospores

(i) Suspend 5 g of soil samples in aliquots of 30-ml sterile distilled water (SDW); stir for 5 min; sonicate for 5 min; add 0.3 ml 1% Tween-80 and stir again for 5 min.

(ii) Pour the soil suspension into 40 ml of 70% sucrose solution in 250 ml centrifuge tube and centrifuge at 2,500 rpm for 5 min at 20°C.

(iii) Dilute the supernatant with SDW (1:3); filter through cellulose nitrate membrane (pore size 10 μm, 47 mm diameter) and use the oospores on the membrane for DNA extraction.

(iv) Extract the DNA from oospores by CTAB method.

C. Polymerase chain reaction (PCR) assay

(i) Use the 19-nt primer Pmy5 designed from variable sequence of ITS region of rDNA in *P. myriotylum.*

(ii) Apply an annealing temperature of 57°C for amplification of a specific 150-bp fragment.

Appendix 3: Detection of *Phytophthora sojae* in Soils by PCR Assay (Wang et al. 2006)

A. Extraction of the target pathogen from the soil

(i) Use 200-, 300-, and 400-mesh screens of 20 cm in diameter to remove/eliminate most soil and a 600-mesh, 20 cm diameter screen to collect the oospores.

(ii) Macerate 20 g of soil in 300 ml of water in a 500-ml beaker for 30 min; stir with a glass rod for 10 min and transfer the macerate to a series of screens

of 200-, 300-, 400- and 600-mesh; rinse the screens with large volumes of water and collect the material remaining on the 600-mesh screen for extraction of DNA and staining of oospores.

(iii) Grind the concentrated material from soil to a fine powder in liquid nitrogen with a mortar and pestle; transfer approximately 0.4 g of the powder to a 1.5 ml centrifuge tube and suspend in 0.5 ml of 0.4% skim milk powder solution by vigorous vortexing and centrifuge at 12,000 × g for 15 min.

(iv) Transfer the supernatant (approximately 0.4 ml) to a centrifuge tube; add 0.4 ml of proteinase K extraction buffer containing 50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate (w/v) and incubate for 1 h at 55°C.

(v) Add 0.4 ml 7.5 M ammonium acetate (half volume) to each sample; precipitate the cell debris and remove by centrifugation at 12,000 × g for 15 min.

(vi) Precipitate the nucleic acids in the supernatant with 2 volumes ethanol at –20°C for 30 min or overnight; pellet the DNA, rinse with 70% ethanol; dry the pellet, redissolve in 5 μl of sterile water or TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and store at –20°C until required for PCR amplification.

B. Differentiation of live and dead oospores by staining

(i) Gather fungal materials from the 600-mesh screen (step Aii above); add 0.05% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny-2H-tetrazolium bromide) and incubate for 48 h at 35°C.

(ii) Observe under a microscope for the development of blue color in germinated oospores: pink color in dormant oospores or dark color or absence of stain in the dead oospores.

C. Quantitative detection by real-time PCR assay

(i) Perform the reactions in a volume of 50 μl consisting of 0.5 μM of each primer (PS1/PS2), 5 μl of DNA solution extracted from soil samples, a 2.5 μl mixture containing 12.5 μM each dNTP, 5 μl of 10 × PCR buffer, 2.5 mM Mg^{2+}. 1.25 U of *Taq* DNA polymerase (Promega), 2.5 μl of 20 × SYBR Green I (OPE Technology Development Co., China) and sterile distilled water to a final volume of 50 μl.

(ii) Use the thermal cycling conditions as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s and final extension at 72°C for 10 min and maintain negative controls lacking the template DNA for each experiment.

(iii) Perform quantitative PCR in an ABI Prism 7000 Sequence Detection System (Perkin-Elmer).

(iv) Prepare standard curve by plotting the log of a known concentration (tenfold dilution series from 100 fg to 10 ng in reaction volumes of 50 μl) of DNA from target pathogen against threshold cycle (Ct) values and melting curve.

(v) Determine the DNA content/g of soil in a 20-g sample from the standard curve and calculate the mean value for six replicates.

Appendix 4: Detection of *Spongospora subterranea* f.sp. *subterranea* from Soil by PCR Assays (Qu et al. 2006)

A. Extraction of DNA from soils by Bead-beating/CTAB method

(i) Mix 10-g sample with 20 ml CTAB buffer consisting of 2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 in polycarbonate bead-beater chamber (Bio-Spec Products, USA) containing 5 g each of glass beads with 0.1, 0.5, 1.0 mm diameters and homogenized for 5 min.

(ii) Transfer the glass beads and soil suspension to a 50 ml centrifuge tube; incubate at 60°C for 30 min and centrifuge at 3,000 g for 10 min.

(iii) Transfer the supernatant to a 50-ml centrifuge tube; add an equal volume of chloroform-isoamylalcohol (24:1, v/v) mixture and centrifuge at 3,000 g for 20 min.

(iv) Transfer the aqueous phase to a 50-ml centrifuge; add an equal volume of isopropanol; shake the mixture well and centrifuge at 3,000 g for 30 min.

(v) Wash the pellet twice with 250 µl sterile distilled water (SDW) and purify the crude DNA with a Wizard DNA Clean-Up System (Promega, USA) as per the manufacturer's instructions.

(vi) Store the purified DNA at –20°C.

B. UltlraClean™ Soil DNA Kit procedure

(i) Add 0.25 g soil sample to a 2-ml bead solution tube (MOBIO Laboratories Inc., CA, USA) and extract the DNA as per manufacturer's recommendations.

C. Amplification of DNA by PCR

(i) Use primer pari Ss F/R for amplifying the DNA from *S. subterranea* spore balls.

(ii) Perform PCR amplification with the following conditions in 50 µl reaction mixture containing 1 × PCR buffer (Applied Biosystems, CA, USA), 2.5 mM $MgCl_2$, 200 mM of each dNTP, 20 pmol of each primer, 1 U of AmpliTaq polymerase (Applied Biosystems) and 20–30 ng of DNA template.

(iii) Carry out the amplifications in a PTC–100–60 programmable Thermal Controller (MJ Research Waltertown, USA) with an initial cycle at 95°C for 2 min, 60°C for 30 s and 72°C for 1 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min and a final cycle of 72°C for 7 min.

D. Competitive PCR assay

(i) Prepare an internal heterologous control competitor from l DNA with a competitive DNA Construction Kit (Takara Shuzo Co., CA, USA) as per the manufacturer's instructions.
(ii) Extract DNA templates used for competitive PCR amplifications from 0.25 g dry soil using and UltraCleanTM soil DNA Kit.
(iii) Add 4 fg of competitor DNA and perform PCR amplification as described above and separate the amplicons by electrophoresis on 2% agarose gels containing ethidium bromide.
(iv) Visualize the bands with a UV transilluminator; analyze using GeneGenius Software (Syngene, UK) to quantify densitometrically and calculate PCR product ratio to generate a standard curve.
(v) Maintain three replicates for each soil sample.

Appendix 5: Extraction of DNA Directly from Soil Samples for PCR-Based Assays (Volossiouk et al. 1995)

Extraction of DNA from soils

(i) Grind 0.25 g of soil samples with liquid nitrogen using mortar and pestle for about 5 min to get a fine powder; suspend the soil powder in 0.5 ml of skim milk powder solution (0.1 g milk powder dissolved in 25 ml water) and vortex vigorously.
(ii) For quantitative assays add the internal control template DNA (500 pg).
(iii) Remove soil and debris by centrifugation at 12,000 × g for 10 min at 4°C and mix the supernatant with 2 ml of SDS extraction buffer (0.3 SDS in 0.14 M NaCl, 50 mM sodium acetate pH 5.1) by vortexing.
(iv) Add equal volume of water-saturated phenol solution and mix by intermittent vortexing for 2 min at room temperature and separate by centrifugation at 12,000 × g for 10 min.
(v) Remove the aqueous phase containing the nucleic acid; precipitate with 2.5 volumes of ethanol at –25°C for several hours or overnight and collect the precipitate by centrifugation 4°C.
(vi) Wash the pellet with ethanol; centrifuge; again wash with ethanol and dry.
(vii) Dissolve the pellet in 250 µl water and store at –20°C until it is required for assay.

Appendix 6: Detection of *Phytophthora nicotianae* in Irrigation Water (Kong et al. 2003)

A. Extraction of DNA from pathogen propagules in water

(i) Collect the propagules by filtration or centrifugation; pass a predetermined volume of zoospore suspension or naturally infested irrigation water

through a 47-mm diameter nylon membrane filter with 5 μm pores (Millipore Corp.) using a vacuum pressure of 5.33 Pa (41.5 cm/Hg) to capture propagules on the filter and centrifuge the samples at 10,000 g for 10 min to pellet the propagules.

(ii) Use UltraClean Soil DNA Kit for extracting the DNA of propagules; cut the filter into fine pieces while it is wet; place the pieces into the bead tube (from the kit for loading soil samples); or resuspend the propagule pellet with solution from the bead tube and pour back into the tube.

(iii) Follow the manufacturer's recommendation to complete the process of DNA extraction from the pathogenic propagules.

B. DNA amplification by PCR

(i) Use the PN primers based on sequences of elicitin gene parA1 and PP primers specific to *P. parasitica*.

(ii) Perform PCR reactions using a total volume of 25 μl containing 2 μl of DNA extract; 2.5 μl of 10 × PCR buffer, 2.5 μl of each of the 10 μm primer solutions, 2 μl of 2 mM dNTPs solutions, 0.1 μl (5 U/μl) of *Taq* polymerase and 13.4 μl SDW.

(iii) Perform the reactions using a thermal cycler (Perkin-Elmer 480) with initial denaturation at 96°C for 2 min, followed by 40 cycles of 94°C for 30 s, 65°C for 45 s, 72°C for 1 min and a final extension at 72°C for 10 min.

(iv) Resolve the PCR products using an aliquot of 5 μl by electrophoresis in 1.0–1.5% agarose or 3% Nusieve gels; stain with ethidium bromide and capture the images for analysis using a BioImaging Chemi System (UVP Inc., CA, USA).

Appendix 7: Detection of Airborne Inoculum of *Mycosphaerella brassicae* (Kennedy et al. 1999)

A. Production of polyclonal antibodies against the target pathogen

(i) Cultivate the pathogen on senescent sprout leaf decoction (SLD) agar; collect the ascospores released from the pseudothecia and prepare a 100-ml spore suspension containing 2.5×10^6 ascospores.

(ii) Concentrate the spore suspension by freeze-drying; rehydrate in 15 ml of sterile distilled water (SDW); sonicate with a Soniprep apparatus (MSE, Crawley, UK) at a micron amplitude of 20 for a total of 15 min; freeze-dry again and rehydrate again in 5 ml of phosphate buffer saline (PBS), pH 7.2.

(iii) Immunize a female New Zealand White rabbit with an intramuscular injection of 500 μl of Freund's complete adjuvant mixed with 500 μl of an ascospore suspension (5×10^4 spores/ml) and administer four additional injection of 500 μl of Freund's incomplete adjuvant mixed with an equal volume of ascospore suspension at weekly intervals.

(iv) Bleed the immunized rabbit at 4 and 6 weeks after initial injection; store the preimmune and immune bleeds at 36°C for 2 h; centrifuge at 100 g for 20 min; collect the supernatants and store at –20°C in 250 µl aliquots in vials until required.

(v) Assign code (like A,B,C) to indicate the preimmune and immune bleeds respectively.

B. Detection and quantification of ascospores of the target pathogen using Burkard trapping and immunofluorescence (IF)

(i) Place a sporulating culture of the target pathogen in a plant growth cabinet operating at 94% relaltive humidity with a 12-h dark/12-h light regime and lightly mist with distilled water for 10 min every 4 h.

(ii) Incubate a Melinex tape for 2 h at room temperature (approx. 25°C) in 5% (w/v) bovine serum albumin (BSA)/PBStinc (0.2% antibacterial tincture of merthiolate in PBS); air-dry the tape and vertically section the coated tape and overlay vaseline to one half of the tape.

(iii) Fix tape to a rotating drum; position inside a Burkard volumetric spore trap (Burkard Scientific, UK) and operate continuously for 4 days in the plant growth cabinet where spores in the air are impacted directly onto the coated tape.

(iv) Remove the tape; section into four 24 h periods; examine each of the four sections under bright field microscope at a magnification of 400.

(v) Process the section for immunofluorescence (IF) by attaching the section to a glass microscope slide using double-sided adhesive tape; dilute the final bleed serum to 1:100 in blocking buffer; add to cover each section of the divided tape and incubate at room temperature (25°C) for 60 min.

(vi) Carefully rinse the slides with distilled water; air-dry, dilute anti-rabbit IgG-FITC conjugate to1:100 in blocking buffer; add to cover each section of the tape and incubate in darkness at room temperature for 60 min.

(vii) Rinse the divided section with distilled water; air-dry, add counterstains 0.2% Evan's blue (Sigma) in PBS and 0.5% eriochrome black in PBS to cover each divided sections of the tape for 30 min at room temperature.

(viii) Rinse the slides; air-dry and detach them from the holding adhesive tape and affix directly onto glass slides with Gelvatol (Burkard Scientific, UK).

(ix) Mount the slides with PPDG and view under episcopic-fluorescence microscope.

C. Immunodetection of artificially and field-produced ascospores of target pathogen

(i) Place the Burkard volumetric spore trap into a (Brussels sprout) crop inoculated with the target pathogen and exhibiting severe symptoms induced by the target pathogen; operate the spore trap continuously for 3 days and remove the tape.

(ii) Divide the tape in half and section into 24-h periods and divide one half of the divided sectioned tape and store at 4°C.

(iii) Expose the other half under cool white fluorescence/black lighting to a lightly misted sporulating culture of the target pathogen over a 24-h period at 12°C and incubate.

(iv) Attach the divided sections of tape to a glass microscope slide using double-sided adhesive tape and immunoprobe.

Appendix 8: Detection of *Monilinia fructicola* Spores in the Air by Real-Time PCR Assay (Luo et al. 2007)

A. Extraction of DNA from spore trap samples

(i) Remove the tapes from spore traps; combine the tape segments of 3 days into one sample; cut each segment into 1 × 2 cm pieces; place them in a 2-ml FastDNA tube containing 1.8 ml of 0.1 % Nonidet (Sigma-Aldrich, USA) and garnet matrix and incubate the tubes at 55°C for at least 20 min.

(ii) Shake the tubes in an Eppendorf mixer for 5 min; centrifuge at 14,000 rpm for 10 min and decant the supernatant.

(iii) Extract the DNA from the spores using the FastDNA Kit (Q-BIO Gene Corp. CA, USA) by adding 300 ml CellLysis/DNA solubilizing solution (for fungi) to each FastDNA tube containing centrifuged spores and shake the mixture in the PrepCell Disruptor for (QbioGene) 10 times for 40 s each at 4.5 m/s with 2 min cooling in ice after the fifth shaking cycle.

(iv) Follow the other steps as per the manufacturer's recommendations and dissolve the extracted DNA in 10 μl of H_2O for PCR amplification with real-time PCR assay.

B. Real-time PCR procedure

(i) Use the pathogen-specific primer pair (RTMfF/RTMfR) to generate the expected PCR product (390-bp from *M. fructicola*)

(ii) Perform amplifications in the DNA Engine Opticon2 System (BioRad Laboratories, USA), using the SYBRGreen I fluorescent dye in a total volume of 50 μl containing 25 μl of SYBR Green Supermix (BioRad), 4 μl template DNA from spores and 4 ml each of forward and reverse primers (4 μM each).

(iii) Provide the following conditions for amplification: an initial preheat for 3 min at 95°C followed by 50 cycles at 94°C for 15 s, 64°C for 25 s, 72°C for 30 s, and 73°C for 1 s in order to detect and quantify the fluorescence at a temperature above the denaturation of primer-dimers.

(iv) Obtain melting curves based on a standard protocol as per the manufacturer's recommendations for confirming the signal from the target product without inclusion of primers.

(v) Prepare a standard curve using different dilutions of target DNA from pathogen cultures.

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Chapter 4
Assessment of Variability in Fungal Plant Pathogens

Abstract Fungal pathogens show variations in their pathogenic potential (virulence) and physiological functions that support their survival and perpetuation in different environmental conditions. These variations may be largely due to genetic factors that may be conditioned by environmental factors. The existence of strains, races or biotypes may be recognized by differences in virulence of the isolates on differential species or cultivars. Assessment of variability may be performed by applying techniques based on the cultural, biochemical, immunological and nucleic acid characteristics. Attempts have been made to relate the variability in these characteristics to variability in pathogenicity of isolates of fungal pathogens. Information on the extent of variability of isolates of fungal pathogens is essential to plan effective methods of tackling the diseases caused by them. In addition, development of resistance to fungicides has been a serious concern to growers, researchers as well as the administrators, since the decision to continue application or withdrawal of a fungicide can affect both the growers and the industry economically.

Various studies on genetic variability of microbial plant pathogens have shown that variations may occur in characters which affect their ability to infect host plants and also other characters that do not alter the pathogenic potential, but they may be concerned with their survival. Knowledge of variation in pathogenic characteristics is valuable in planning effective measures for disease management. The pathogenic characters of the fungi may be subjected to selection pressure because of introduction of new resistance gene(s) through breeding or indiscriminate application of chemicals for controlling the pathogens. The microbial pathogens have to produce new strains/races to overcome the effects of newly introduced gene(s) or tolerate the adverse effects of chemicals applied on the plant tissues. Alterations in the virulence (aggressiveness) of fungal pathogens or tolerance to chemicals have been assessed by biological and molecular techniques.

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4.1 Methods of Assessment of Variability in Fungal Pathogens

Fungal pathogens exhibit variations in the cultural, physiological/biochemical, immunological and nucleic acid characteristics. Cultural and physiological characteristics are significantly altered by the media, pH and temperatures at which they are grown. Immunological characteristics may show distinct variations depending on the stages of life cycles of the fungal pathogen due to production of different spores that possess different antigenic determinants. On the other hand, genomic nucleic acid characteristics remain constant, unless mutations occur in the pathogen genome.

4.1.1 Assessment of Variations in Biological Characteristics

4.1.1.1 Use of Differential Host Species/Cultivars

Aadaptation of microbial plant pathogens to different host genotypes was first demonstrated by proposing the gene-for-gene concept based on experiments on flax rust (*Melampsora lini*) by Flor (1946, 1956). According to the gene-for-gene concept, a pathogen is avirulent on a plant species (or varieties), if it has an avirulence factor matching a specific host resistance factor. Thus, there is a matching pair of resistance and avirulence factor for a host–pathogen interaction to be incompatible. By inoculating the pathogen isolates onto a set of host varieties with different specific resistances, it is possible to identify the virulence/avirulence factors present in the pathogen isolates under investigation. A host variety susceptible to all strains/races has to be included in the set of differentials to check the success of inoculation. An isolate is considered as virulent, if it is compatible with a differential variety, with high infection type (IT) or avirulent if the interaction is incompatible and the IT will be low. The levels of resistance/susceptibility of different isolates have been determined by the classical studies on *Phytophthora infestans* causing potato late blight disease and *Puccinia graminis* f.sp. *tritici* causing wheat stem rust disease (Vanderplank 1963, 1978). The expression of specific resistances may be influenced by environmental factors. Hence, the comparison of results of experiments conducted at different time/location will be meaningful, only if the conditions under which the host differentials are maintained before and after inoculation with pathogen isolates, are uniform and controlled. For example, the expression of specific resistance of wheat cultivar Axona to the powdery mildew pathogen *Erysiphe graminis* f.sp. *tritici* was significantly influenced by day/night temperature regime (Clarkson and Slater 1997).

A differential interaction between host resistance gene and pathogen virulence gene is a characteristic feature of gene-for-gene relationship. The existence of gene-for-gene relationship was reported for several host–fungal pathogen interactions such as apple – *Venturia inaequalis*, barley – *Erysiphe graminis* f.sp. *hordei* and *Ustilago hordei*, coffee – *Hemileia vastatrix*, rice – *Pyricularia oryzae* and

maize – *Puccinia sorghi* (Day 1974; Person 1967; Person and Ebba 1975). The gene conferring resistance to the host against a race/strain of the pathogen is generally dominant, whereas the virulence gene of the pathogen matching the resistance is recessive. During the coevolution of hosts and pathogens, gene-for-gene interactions occur naturally, resulting in a natural balance in an ecosystem. When a gene for resistance is introduced into a cultivar to improve resistance level, the balance is upset. The pathogen reacts by producing populations of individuals (spores) with a virulence gene to match the newly introduced resistance gene for its survival and again the natural balance between the host and pathogen may thus be restored. The resistance shown by the cultivar is race-specific and its resistance breaks down, when a new race of the pathogen is produced (Parlevliet 1993).

4.1.1.2 Variability in Vegetative Compatibility

Assessment of variability by pathogenicity tests with appropriate host plant species or varieties is time-consuming and occasionally may give inconclusive results. Utilization of laboratory methods can contribute to differentiation of fungal pathogen isolates, as in the case of *Fusarium oxysporum*. In addition, they can also provide information on the genetic composition of pathogen populations (McDonald 1997). The ability to form 'heterokaryon' by fusion between genetically different strains within a morphologic species is the basis for grouping the strains as vegetative compatibility groups (VCGs). The vegetative compatibility groups (VCGs) were first recognized among the isolates of *Verticillium dahliae* and *Fusarium oxysporum* based on the ability or inability to utilize nitrates (Puhalla 1979, 1985). The VCGs were considered as good predictors of genetic relatedness and the vegetative compatibility of fungal isolates was shown to be related to the pathogenic potential of the isolates.

The strains of *Verticillium dahliae* isolated from wilt disease-infected cotton plants were consistently separated into different VCGs (Puhalla and Hummel 1983). In another study, the virulence of strains of *V. dahliae* isolated from several hosts growing in Africa, Asia, Europe and USA, was tested by inoculating on the cultivars of *Gossypium hirsutum*, *G. barbadense* and *G. arboreum*. The strains belonging to VCG1 were both cotton-defoliating pathotype and race 3 on cotton and tomato (Daayf et al. 1995). In a later study, *V. dahliae* isolated from cotton in Israel, were tested for vegetative compatibility using nitrate non-utilizing (*nit*) mutants. The isolates were assigned to VCG1, VCG2B, VCG4B of which the occurrence of VCG1 was recorded for the first time. VCG1 isolates were characterized as D pathotype (cotton-defoiating) and the isolates belonging to VCG2B and VCG4B were considered to be defoliating-like (DL) and non-defoliating (ND) pathotypes respectively based on the symptom type induced by them. The D isolates were more virulent than DL isolates on all tested plants including okra, cotton, watermelon and tomato. The pattern of virulence of ND isolates differed from that of D and DL isolates. The D isolates were highly virulent on eggplant, but mildly virulent on cotton. Tomato was resistant to all cotton *V. dahliae* isolates tested (Korolev et al. 2008).

Vegetative compatibility analysis of 77 isolates of *V. dahliae* isolated from 87 fields intended for potato showed that the isolates belonged to VCG4A group (present in 93% of the fields) or VCG4B group (present in 23% of the fields). It was suggested that preplant assessment of the nature of *V. dahliae* populations might be helpful for making the disease management decisions (Omer et al. 2008). Grouping of isolates of fungal pathogens into VCGs in the laboratory may be useful, if such a classification is corroborated by pathogenicity tests.

Vegetative compatibility groups (VCGs) can be used to evaluate the genotype diversity in fungal populations (Heilmann et al. 2006; Ramirez et al. 2006). The relative number of VCGs may be expected to be higher in a sexual population than in an asexual population. In asexual populations, VCG and pathogenicity may be correlated which allows VCG to be used as a surrogate for pathogenicity, whenever, the population is primarily asexual. Vegetative compatibility results for populations of *Fusarium oxysporum* were used to identify sub-specific groups that could be occasionally correlated with *formae specialis* and/or pathogenicity (Wang et al. 2006). Mango malformation is considered to be due to a new *Fusarium* lineage in the *Gibberella fujikuroi* species complex and *F. sterilihyphosum*. Six VCG groups were identified amongst the isolates of the new lineage from Brazil. Similar results were also obtained from AFLP analysis. FB-VCG1 was the most widespread group in the isolates obtained from 7 of the 13 sites sampled (Lima et al. 2009).

4.1.2 Assessment of Variations in Biochemical Characteristics

4.1.2.1 Isozyme Analysis by Electrophoresis

Isozyme analysis has been shown to be useful to resolve taxonomic problems, identify unknown isolates, analyze the extent of genetic variability in populations, trace the geographical origin of pathogens and also as a diagnostic tool. Highly virulent and weakly virulent strains of *Leptosphaeria maculans*, causative agent of black leg or stem canker disease of canola (*Brassica napus*) could be differentiated by isozyme anaysis. Isolates of *L. maculans* (92) from six countries were analyzed. A single isozyme of glucose phosphate isomerase (GPI) that moved to a longer distance (70 mm) after 11 h of electrophoresis, was detected in 68 of 92 isolates. The GPI isozyme in the other isolates could move only to 65 mm. The highly virulent strains contained the fast moving isozyme, while the weakly virulent isolates had slow isozyme. Based on the difference in the nature of isozyme that could be differentiated by electrophoresis, the virulent strains were placed in electrophoretic type 1 (ET1) and the weakly virulent strains were included in the ET2 group. By using this simple technique, it was possible to differentiate highly virulent and weakly virulent strains of *L. maculans* (Sippell and Hall 1995). In a later study, it was shown that the GPI electrophoresis on starch gels could be performed directly on leaf lesion extracts for reliably detecting *L. maculans* and for

differentiating it from *Pseudocercosporella capsellae* also infecting *B. napus*. Four distinct ET patterns were recognized. Highly virulent isolates (group A) of *L. maculans* showed ET1 and the weakly virulent isolates had ET2 pattern. ET3 allozyme was another enzyme recovered from a few typical and atypical leaf lesions caused by *L. maculans*. On the other hand, *P. capsellae* produced the fastest ET4 allozyme which could be detected in the lesions induced by *P. capsellae* (Braun et al. 1997). Both investigations indicated the possibility of differentiating the fungal pathogens based on the nature of enzymes produced by them during disease development.

Determination of electrophoretic types (ETs) was found to be useful for assessing the intraspecific population diversity in fungal pathogens such as *Colletotrichum gloeosporioides* causing anthracnose diseases in a wide range of host plant species. Isozyme variations in isolates from different plant species were assessed. Maximum number of electrophoretic phenotypes in the isozymes of nicotinamide adenine dinucleotide dehydrogenase (NADH) and diaphorase (DIA) were detected by electrophoresis on gels. The ETs clustered together on the basis of host origin. Three major ETs (I, II and III and four subgroups IA, IB, IIIA and IIIB) of *C. gloeosporioides* could be differentiated (Kaufmann and Weidemann 1996).

Cellulose acetate gel electrophoresis (CAE) detection of isozymes is possible even from very quantities of materials. Isozyme analysis by CAE was employed for assessing the variations in the isozyme patterns of *Plasmodiophora halstedii* causing sunflower downy mildew disease. Forty five isolates collected from sunflower, cocklebur and *Helianthus* x *laetiflorus* comprising 10 field isolates and 35 single-spore lines of an additional 30 field isolates representing 10 different virulence phenotypes, were examined. Three enzymes, isocitrate dehydrogenase, malate dehydrogenase and phosphoglucomutase providing sixteen isozyme systems were analyzed. Clear, reproducible banding patterns were generated revealing some polymorphisms among the isolates. Phosphoglucomutase differentiated into two groups among the isolates collected from cultivated sunflower, while the other enzymes were polymorphic between isolates from the different hosts. The CAE technique was found to be suitable for isozyme analysis requiring only very small amounts of tissues (31–100 μg of sample/reaction) for obtaining good resolution. The differences among the samples appeared to be associated with host origin rather than geographic location or virulence (Komjati et al. 2008).

4.1.2.2 Cellular Fatty Acid Analysis

Fungal pathogens included in the genera *Phytophthora* and *Pythium* produce several fatty acids, not generally produced by true fungi (Müller et al. 1994). Hence, the potential of using whole cell fatty acid methyl ester (FAME) profiles for the identification, characterization and differentiation of isolates of *Phytophthora infestans*, causative agent of potato late blight, was examined. All isolates of *P. infestans* produced eight prominent fatty acids which accounted for 97–99% of total fatty acid contents. Analysis of FAME profiles showed distinct differences

among *Phytophthora* species, as well as considerable variability among isolates of *P. infestans*. Isolates of the US-1 genotype, in particular, had profiles different from all other isolates of *P. infestans* and formed a separate group. FAME profiles of the oomycetes, *Pythium ultimum*, *Phytophthora capsici* and *P. erythroseptica* were drastically different from those of true fungi, *Rhizoctonia*, *Penicillium*, *Trichoderma*, *Verticillium* and *Fusarium*. FAME profiles could also be used for differentiating subgroups within species. As long as culture conditions were kept constant, profiles of individual isolates of *P. infestans* were consistent and reproducible over time. The FAME profiles may be potentially useful for establishing greater isolate-specificity due to the close relationships between profile characteristics and specific isolate traits such as pathogen aggressiveness, sporulation or other characteristics of epidemiological significance. FAME profiling represents a relatively rapid cost-effective and efficient approach useful for assessing pathogen variability (Larkin and Groves 2003).

Total cellular fatty acid analysis was found to be useful for the identification and differentiation of *Rhizoctonia* spp. (Stevens Johnk and Jones 1992). The MIDI method (Microbial Identification System, Microbial ID Inc., Newark, USA) was slightly modified and this procedure was effective to differentiate between *R. solani* anastomosis groups as well as between subgroups within anastomosis groups AG1, AG2-2, AG3 and AG4 (Stevens Johnk and Jones 2001). The comparative efficacy of total cellular fatty acid analysis and modified MIDI method permitted a clear differentiation of *R. oryzae* and *R. oryzae-sativae* which infect the leaf sheath or rice plants, causing sheath spot and aggregate sheath spot diseases respectively. The fatty acid profiles obtained by the modified MIDI protocol have the potential for diagnosis and differentiation of these two rice pathogens, regardless of the isolate origin (Lanoiselet et al. 2005).

4.1.3 Assessment of Variations in Immunological Characteristics

Characterization of antigens against fungal pathogens is a major problem, since fungi are complex antigens and different antigenic determinants (epitopes) may be present on different spore forms produced during different stages in the life cycle of the fungal pathogen. Hence, preparation of antisera containing polyclonal antibodies (PAbs) capable of reacting with the fungal pathogens at all stages of the life cycle has been difficult. However, the sensitivity, specificity and reliability of immunological reactions have been enhanced significantly following development of monoclonal antibodies (MAbs). Application of immunoassays for differentiation of strains/races of fungal pathogens has been successful in a few cases.

The pathogenic and nonpathogenic isolates of *Venturia inaequalis*, causal agent of apple scab disease were differentiated by immunocytochemical technique. A clear distinction based on the appearance of cell wall and distribution of fimbrial epitopes labeled with specific antiserum and immunogold complex could be observed (Svircev et al. 2000). Chicken egg yolk was used to raise antibodies (IgY)

against *Colletotrichum falcatum* and *Fusarium subglutinans* infecting sugarcane. The strains of these two sugarcane pathogens could be differentiated and also quantitatively estimated by employing the specific IgY which could be prepared less laboriously and more rapidly (at least tenfold larger quantify per time unit) than IgG prepared using rabbits (Vöhringer and Sander 2001).

Head blight or scab disease caused by *Gibberella zeae* (anamorph-*Fusarium graminearum*) produces the mycotoxin deoxynivalenol (DON). The mycotoxin-contamination of wheat grains leads to health hazards in humans and animals. The antiserum against exoantigens (ExAgs), a soluble mixture of extracellular pathogen products was employed for the detection and differentiation of pathogen isolates which showed distinct variations in host tissue colonization and presence of DON in infected tissues. The contents of ExAgs were used as the basis for measuring fungal biomass within host tissues. A linear relationship was established by employing an indirect ELISA format between ExAgs of *F. graminearum* and DON content in the host tissues. The progenies from segregating populations of *G. zeae* were inoculated onto a susceptible wheat cultivar. Extent of fungal colonization, ExAg contents and DON production were determined by applying ELISA tests. All traits examined showed significant genotype variations. A high correlation between DON production and ExAg contents across environments (year-location combination) was observed (Cumagun et al. 2004).

4.1.4 Assessment of Variations in Genomic Characteristics

Variations have been observed in the nucleotide sequences of genes governing pathogenicity and production of enzymes or toxins that are involved either host tissue degradation, thereby releasing food materials required for the pathogens or for survival as saprophytes in the environment. Nucleic acid-based techniques have been shown to be highly sensitive and specific to reveal variability in genomic characteristics of fungal pathogens.

4.1.4.1 Nucleic Acid Hybridization Methods

Differences in the virulence of isolates of *Gaeumannomyces graminis* were assessed by applying a slot-blot hybridization procedure using a specific DNA probe pG158. This probe hybridized strongly to pathogenic isolates of *G. graminis* var. *tritici* (*Ggt*) and moderately to *G. graminis* var. *avenae* isolates, but not to any of the nonpathogenic isolates of *Ggt* and other soil fungi. The probe pG158 was effective in detecting pathogenic isolates of *Ggt* both in the soil and wheat roots. This technique has the practical utility for differentiating pathogenic isolates of *Ggt* from the morphologically similar nonpathogenic isolates and for relating the soil population of *Ggt* to the incidence of wheat take-all disease caused by this pathogen (Harvey and Ophel-Keller 1996).

Dot blot hybridization procedure was employed for the detection and differentiation of *Rhizoctonia solani* which has a wide host range including rice and several vegetable crops. All isolates of *R. solani* AG-2-2-IV inducing large patch disease of *Zoysia* grass were detected by using a plasmid DNA fragment (PE-42) that was able to hybridize to the isolates of this pathogen. No hybridization occurred to the DNA of other pathogens infecting *Zoysia*, indicating the specificity of the probe. The probe PE-42 was employed as a marker in Southern hybridization procedure and this plasmid DNA could differentiate *R. solani* AG2-2-IV from other intraspecific groups of *R. solani* in addition to its ability to be used for the diagnosis of large patch disease of *Zoysia* (Takamatsu et al. 1998).

4.1.4.2 Restriction Fragment Length Polymorphism Technique

The extent of natural variations in the genomes of different species, biotypes, strains or races of fungal pathogens can be determined by applying restriction fragment length polymorphism (RFLP) procedure. The pathogen DNA is subjected to the action of different restriction enzymes that act on specific sites in the genomic DNA resulting in number of DNA fragments which form a definite pattern that can be identified on the gels by Southern blot analysis. The RFLP patterns may be useful to assess the genetic diversity of pathogen population and to estimate the extent of relatedness between the pathogen isolates/strains.

Pseudocercosporella herpotrichoides infecting rye exists as R, N, C or S pathotypes showing variations in the virulence. A pathotype-specific DNA probe was effective for the identification of R-type isolates of the pathogen. A 6.7-kb DNA fragment from R-type isolates hybridized with R-type isolates only, but not with N, C or S pathotypes of *P. herpotrichoides* or *P.anguioides*. Rye seedlings infected by R-type isolates could be identified by hybridization of the probe to DNA extracted from infected rye plants (Nicholson et al. 1994). The RFLP procedure was applied to assess the genetic variations in the clones of *P. herpotrichoides*. Polymorphic pathogen-specific probes were efficient in differentiating the clones directly in DNA preparations obtained from infected plants without the need for isolation of the pathogen (Frei and Wenzel 1993).

The virulent and avirulent isolates of *Phytophthora parasitica* var. *nicotianae* causing tobacco black shank disease differs in their ability to produce elicitin (TE) in inoculated tobacco plants. The isolates of avirulent strains (TE^+) produce elicitin resulting in the induction of resistance in tobacco against these isolates, whereas the isolates of virulent strain (TE^-) do not produce the resistance-inducing compound. Elicitin production (TE^+) was generally associated with low virulence on tobacco and frequently pathogenicity on tomato. RFLP analysis of both mictochondrial (mt) and nuclear DNA was found to be effective in differentiating isolates infecting tobacco (TE^+) from other *P. parasitica* isolates (TE^-). The results suggested that monitoring the loss of elicitin production (leading to virulence) may be an important factor in disease management programs (Colas et al. 1998).

A dispersed repeated sequence (known as MGR) was used as the probe to construct genotype specific *Eco*RI RFLP profiles (MGR-DNA fingerprints) from field isolates of rice blast pathogen *Magnaporthe grisea* (anamorph-*Pyricularia grisea*). The MGR DNA fingerprints were used as the basis for differentiation of major pathotypes of *M. grisea*, identifying the pathotypes precisely and defining the organization of clonal lineages within and among pathotype groups (Levy et al. 1991). A correlation between molecular and biological characters of the biotypes of *Stagnospora nodorum* from barley and wheat was established by RFLP analysis. The genetic similarity was very low (0.12) between the two types of *S. nodorum* (Ueng et al. 1995).

Anthracnose disease caused by *Colletotrichum* spp. affect several fruit and vegetable crops all over the world. By applying rDNA and mtDNA restriction patterns, variations in strains of *C. acutatum*, *C. fragariae* and *C. gloeosporioides* isolated from infected strawberry plants could be assessed. 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. On the other hand, all strains of *C. gloeosporioides* produced an identical pattern with each of the four enzymes used. The rDNA and mtDNA of strains of *C. gloeosporioides* from avocado, papaya and banana showed high levels of polymorphisms following digestion with restriction enzymes. In contrast, identical banding patterns with all restriction enzymes were obtained for all mango strains of *C. gloeosporioides* originating from USA, West Indies, Sri Lanka, Malaysia and Australia (Mills et al. 1998).

Four different forms, the slow-growing grey (SGG), the fast-growing salmon (FGS), the fast-growing olive (FGO) and the fast-growing grey (FGG) forms of *Colletotrichum gloeosporioides* infecting water yam (*Dioscorea alata*) were differentiated, based on cultural characterisitics. The FGG isolates produced unique ITS-RFLP banding patterns, whereas the isolates of the other three forms produced RFLP patterns identical to those of *C. gloeosporioides* reference isolates. Restriction patterns generated following digestion with endonuclease enzymes *Alu*I, *Hha*I and *Hae*III could be used as the basis for rapid differentiation of FGG isolates from other forms of *Colletotrichum* from yam (Abang et al. 2001, 2002). The FGG group considered as *C. gloeosporioides* based on morphological characters, formed a distinct ITS-RFLP group and showed only limited ITS sequence similarity (<86%) to reference isolates of *C. gloeosporioides* (Abang et al. 2003).

Based on the pathogenic potential, species, *formae speciales* and races have been recognized within the genus *Fusarium*. RFLP analysis of the total pathogen DNA was performed after digestion with endonucleases *Pst*I, *Hind*III and *Eco*RI, followed by Southern blotting and hybridization with a mtDNA polyprobe from *F. oxysporum* f.sp. *niveum* (*Fon*). It was possible to identify unique RFLP patterns for each *formae specialis*. Genetic divergence in *F. o*. f.sp. *cucumerinum (Foc)* was maximum, while *Fon* exhibited the least variations (Kim et al. 1993). In the case of *F. o.* f.sp. *cubense* causing Panama wilt disease of banana, a probe was designed from a 3.38-kb mtDNA fragment. By employing this probe, polymorphisms within and between different *formae speciales* were recognized based on the RFLP

patterns (Bridge et al. 1995). Several species of *Fusarium* infect cereal grains which become unfit for consumption by humans and animals, because of the presence of hazardous mycotoxins produced by these pathogens. By using appropriate genetic markers, the toxigenic isolates capable of producing fumonisins could be differentiated from nontoxigenic isolates. Two sets of primers VERT F-1 and VERT F-2 were employed in PCR-RFLP technique for specific detection of toxigenic isolates of *F. verticillioides* that produced fumonisins (Patiño et al. 2004).

Soybean seed decay is primarily due to *Phomopsis longicolla*, but *Diaporthe phaseolorum* is also found associated with this problem, leading to significant yield losses. Distinguishing these two fungal pathogens in latent and systemic infections is difficult by employing conventional isolation-based methods. RFLP analysis of PCR amplification products was shown to be effective in differentiating these fungal pathogens present in soybean seeds. Specific primers PhomI and PhomII were designed from the polymorphic regions of *P. longicolla* and *D. phaseolorum* isolates from soybean. Specific bands in PCR amplicons from ten pooled seed samples and also from individually infected seeds were detected. DNA extracts of tissues of asymptomatic soybean plants inoculated with *P. longicolla* and *D. phaseolorum* var. *sojae* revealed the presence of specific bands indicating the specificity of the RFLP analysis (Zhang et al. 1997).

Verticillium dahliae infects cotton and many vegetable crops. Host-adapted pathotypes of the pathogen have been differentiated based on the pathogencity of isolates to different host plant species. Two pathotypes, subgroup D adapted to cruciferous plants were examined for variations in polymorphisms by employing two specific probes for differentiating the subgroups (Okoli et al. 1994). In a later study, the sequences of entire intergenic spacer (IGS) region and the ß-tubulin gene were amplified and sequenced. In order to determine the genetic relationships among different isolates of *Verticillium* spp., the sequences of IGS region and ß-tubulin gene were used as the basis. Four distinct groups were formed to include isolates of *V. albo-atrum*, *V. tricorpus* and *V. dahliae* from cruciferous and noncruciferous crops. In addition, four subgroups were formed to differentiate isolates of *V. dahliae* from noncruciferous hosts based on the sequence similarity within the IGS region (Qin et al. 2006).

4.1.4.3 Polymerase Chain Reaction-Based Techniques

Polymerase chain reaction (PCR) either alone or in combination with other nucleic acid-based techniques has been applied not only for detection, but also for differentiation of fungal pathogens which are known to exist as strains, races or biotypes which may differ in their pathogenic potential. PCR can be used to amplify sequences of specific regions such as ITS or rDNA with universal primers for the differentiation of various species or races/strains within a morphologic species.

The fungal pathogens causing wilts and root rots have been distinguished by employing PCR-based assays. The primers Fov1 and Fov2 designed using the nucleotide difference in the ITS sequences between 18S, 5.8S and 28S rDNAs

unambiguously amplified a 500-bp DNA fragment of all isolates of *Fusarium oxysporum* f.sp. *vasinfectum* causal agent of cotton wilt disease. This protocol was found to be useful for disease diagnosis and also for disease monitoring (Morricca et al. 1998). Cell wall-degrading enzymes (CWDE) secreted by *Fusarium* spp. such as polygalacturonase (PG) have a role in disease development. By comparing the sequences of PG genes of isolates, it may be possible to assess the variability in the pathogenic potential of *Fusarium* species and *formae speciales*. A PCR-based technique targeting PG genes was applied to differentiate pathogenic types of *F. oxysporum* causing wilt symptoms in tomato under field conditions. The partial nucleotide sequences of endoPG (*pg1*) and exoPG (*pgx4*) genes from isolates of *F. oxysporum* f.sp. *lycopersici* (FOL) and *radicis-lycopersici* (FORL) were compared. By employing specific primers in PCR, the pathotypes of *F. oxysporum* pathogenic to tomato in Japan could be accurately identified by a combination of amplification with the primer sets developed in this investigation (Hirano and Arie 2006).

Ability to produce mycotoxin and molecular variability of 37 isolates of *Fusarium culmorum*, one of the principal causative agents of Fusarium head blight of wheat were assessed. Mycotoxin production was determined by thin layer chromatography (TLC) and PCR using primer pairs specific for the *Tri7* and *Tri13* genes was applied to assess molecular variability among the selected isolates. The presence of functional *Tri7* gene correlated well with nivalenol production. Thirty isolates belonging to chemotype I (producing deoxynivalenol) were more pathogenic in *in vitro* tests than those belonging to chemotype II (producing nivalenol and/or fusarenone X). A correlation was observed between the geographic origin of the isolates and their position on the cladogram produced based on the sequence data. However, no correlation could be established between mycotoxin-producing abilities and aggressiveness (virulence) and molecular characteristics of the isolates (Tóth et al. 2004).

Differentiation of fungal population may be accomplished based on the intergenic spacer (IGS) region of rDNA which has highly variable noncoding sequences. Appropriate primers were used for PCR amplification of the entire IGS region of *Fusarium verticillioides* infecting banana fruits. The partial sequences of the IGS region and the translation factor *EF-1*α gene of 48 representative samples of various host and geographic origin were analyzed. High genetic variability was revealed by comparison of both sequences. Two distinct clusters of strains of *F. verticillioides* were recognized to include a major population with wide geographical distribution, wide host preferences and the ability to produce fumonisins in the FP group. The group designated FNP enclosed strains associated with banana located in central America and these strains were unable to produce fumonisins (Miréte et al. 2004).

Genetic diversity of *Verticillium dahliae* capable of infecting many economically important crops such as cotton, potato, tomato and artichoke was studied by employing PCR and vegetative compatibility group (VCG) procedures. Isolates of *V. dahliae* from artichoke (109) and cotton (3) were characterized by PCR using three sets of primer pairs that could differentiate the cotton-defoliating (D) and non-defoliating (ND) *V. dahliae* pathotypes. Two subgroups of isolates were identified in VCG2B based on heterokaryon and amplification of 334- and 824-bp

DNA fragments which were used as markers of 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).

A multiplex-nested PCR method was developed for assessment of the variability of isolates of *Verticillium dahliae* in respect of the assessment of their vegetative compatibility grouping. PCR markers capable of reacting specifically with VCGs were identified. The multiplex-nested PCR assay was significantly superior in detecting the pathogen isolates belonging to different VCGs. In addition, this protocol allowed the VCG identification and detection of *V. dahliae* infections in asymptomatically infected plants which had yielded false negatives, when isolation procedures were adopted. This 'molecular tool box' protocol efficiently revealed the presence of several *V. dahliae* VCGs infecting the same artichoke plants in the Communidad Valenciana Region. The procedure developed in this investigation may provide data for predicting the severity of Verticillium wilt epdemics based on the VCGs detected in the geographic location concerned (Collado-Romero et al. 2009).

Plants infected by *Phytophthora* spp. were examined by PCR-based assays using primers directed to nuclear ribosomal DNA (rDNA) repeat sequences. The pathogens detected were differentiated and characterized on the basis of primer specificity as well as through extensive RFLP and sequence analysis of PCR-amplified rDNA. *Phytophthora capsici* was identified in diseased pepper and zucchini plants; *P. infestans* in tomatoes with late blight symptoms; *P. nicotianae* in tomatoes infected by buckeye rot and *P. cactorum* in strawberry plants. Declining Clementine trees showed the presence of *P. citrophthora* and *P. nicotianae* at almost equal frequency. These pathogens were fully characterized based on PCR-amplified nuclear rDNA sequences (Camele et al. 2005).

Guignardia citricarpa causes the citrus black spot (CBS) disease which is widely distributed in Asia, southern Africa and South America. The nonpathogenic *G. mangiferae* also colonizes the citrus fruits, the presence of which is often misidentified as *G. citricarpa* creating problems for countries exporting citrus fruits to Europe and USA, because of the regulatory protocols preventing the import of citrus from countries where the CBS disease is prevalent. Hence, a PCR procedure was developed using a primer set from ITS region that proved to be highly effective for the detection of *G. citricarpa* and differentiating it from *G. mangiferae*. The primer set NP-Br-ITS-Gc was able to detect *G. citricarpa* in various types of black spot lesions and was very sensitive and the falvedo extracts did not interfere with the detection of the pathogen. The primer set NP-Br-ITS-Gm specifically amplified the expected DNA fragment only from *G. mangiferae*. This procedure was found to be highly specific, rapid and less expensive and reliably differentiated *G. citricarpa* from *G. mangiferae* facilitating the early clearance of the consignments meant for export (Peres et al. 2007) (Appendix 1).

4.1.4.4 Random Amplified Polymorphic DNA Technique

Arbitrary primers are employed for amplification in the random amplified polymorphic DNA (RAPD) technique which can be applied for the differentiation of races and strains within a morphologic species, in addition to differentiation of pathogenic and

nonpathogenic isolates of fungal pathogens. Very short pieces of DNA (110 or less number of bases) from the appropriate source are selected as primers. These primers may be able to find suitable complementary sequences in the DNA of target pathogen isolates to be differentiated resulting in the formation of mixture of DNA fragments of different sizes. The amplified products may be analyzed on gels following electrophoresis and staining. The unique bands in patterns specific to species or strains or varieties may be detected in the gels. The unique bands representing the fungal species may be cut out of the gels and sequenced to produce specific primers for more accurate PCR analysis or for preparation of probes for dot hybridization procedure. As the RAPD technique is simple, sensitive, reliable and rapid, this approach has been followed for detection, differentiation and assessment of relatedness between isolates of a morphologic species of many fungal pathogens.

Pathogenic and nonpathogenic isolates of *Fusarium oxysporum* f.sp. *dianthi* causing wilt disease of carnations were detected and differentiated by employing RAPD technique. By using the primer OPA17 and genetic markers, four amplification groups were recognized among the isolates of the pathogen. However, no relationship could be established between the RAPD patterns and races identified based on the reactions on differential host varieties (Hernandez et al. 1999). RAPD procedure was applied to distinguish pathogenic and nonpathogenic isolates of *F. o*. f.sp. *phaseoli* (FOP), infecting common bean plants. The oligonucleotide primers (23) were employed for amplification of 229 polymorphic and seven monomorphic DNA fragments ranging from 234- to 2590-bp. The pathogenic isolates tended to cluster together in one group, whereas nonpathogenic isolates were placed in another group (Zanotti et al. 2006).

In order to differentiate the rust pathogens, a fragment of the mitochondrial cytochrome b (*cyt b*) gene 13 of economically important plant pathogenic Basidiomycetes was sequenced. The deduced amino acid sequences (residues 142–266) were used as the basis of relatedness of these pathogens. In addition, the ITS in rDNA was studied at nuclear level. On the basis of both cytochrome b and ITS sequences, *Puccinia recondita* f.sp. *tritici*, *P. g*. f.sp. *striiformis* and other rusts infecting cereals were found to be very closely related, whereas *P. arachidis* (peanut) was closely related to *Uromyces appendiculatus* (beans), but more distantly related to other *Puccinia* spp. Both rusts on soybean (*P. pachyrhizi*) and on coffee (*Hemileia vastatrix*) were outside the *Puccinia* cluster. All the rust pathogens were separated from the other Basidiomycete, *Rhizoctonia solani* causing root rots of several crops. Because of their high variability, ITS sequences were useful to discriminate *Puccinia* spp. which were identical based on cytochrome b amino acid sequence. Hence, ITS sequences could be used as the basis for detecting differences among as well as within species, whereas cytochrome b was more suitable for phylogenetic inference at family or genus level (Grasso et al. 2006a, b).

Verticillium dahliae isolates (27) from cotton in Israel were tested by RAPD and specific PCR assays. By using nitrate non-utilizing (*nit*) mutants, the isolates were assigned to VCG1 (13 isolates), VCG2B (8 isolates) and VCG 4B (6 isolates). DNA extracted from the isolates was used for RAPD and specific PCR assays. RAPD assays of VCG2B and VCG4B isolates consistently produced the DNA bards diagnostic of the ND pathotype. Likewise, assays of VCG1 isolates (except one)

yielded RAPD markers diagnostic for the D pathotype. Because of the lack of consistency, all isolates were further examined by specific PCR assays, using D and ND-specific primers. The results of the PCR confirmed that all VCG2B and VCG4B isolates belonged to ND pathotype as well as that of all VCG1 isolates except three isolates belonged to ND pathotype. The latter three VCG1 isolates did not yield any amplification products with either D- or ND-specific primers. The isolates of VCG2B and VCG4B from Israel appeared to be molecularly homogeneous, since all of the tested ones yielded the DNA bands characteristic of the ND pathotype in RAPD and specific PCR assays. But VCG1 isolates appeared to be molecularly heterogeneous, because three of them failed to amplify the D pathotype-associated marker (0.55-kb), although they induced defoliation symptoms in cotton as other D pathotype isolates (Korolev et al. 2008).

4.1.4.5 Amplified Fragment Length Polymorphism Technique

As the isozyme analysis, RFLP and RAPD are time consuming and unable to differentiate pathogen populations beyond species level, amplified fragment length polymorphism (AFLP) was developed to overcome these problems. The AFLP is a versatile and powerful technique and it has been used for performing fingerprint analyses, mapping and other genetic studies on several fungal pathogens. In the AFLP procedure, 50–100 fragments may be amplified simultaneously by PCR. Although AFLP is technically more difficult than RFLP method, it has become a very powerful marker system to study the genetic variation among strains/races of fungal pathogens. This technique involves selective amplification of restriction fragments from a digest of total genomic DNA using PCR. The DNA fragments are resolved on the polyacrylamide gel and designated AFLP markers. The principal advantages of AFLP technique are high levels of sensitivity and reproducibility of the results.

Development of isolates that can overcome the resistance incorporated in potato against *Phytophthora infestans*, the late blight pathogen has been reported from different countries. *P. infestans* isolates from potato cultivars carrying *R2* gene were characterized for pathogenicity. Molecular fingerprints were prepared to assess the possibility of development of virulent isolates of *P. infestans* populations, if *R2* gene had to be deployed extensively in French commercial potato crops. By applying AFLP procedure, 89 bands were detected within the collection of *P. infestans* isolates tested. Five AFLP genotypes were recognized based on the genomic fingerprinting patterns obtained from the isolates of *P. infestans*. Virulence in isolates of *P. infestans* was detected against all recognized resistance genes except *R5* tested. The number of virulences varied between three and eight and about 50% of the isolates exhibited seven or eight virulences. However, it was not possible to establish any relationship between AFLP genotypes and races identified based on the pathogenicity of isolates to differential potato cultivars (Pilet et al. 2005).

Several genotypes of *Phytophthora infestans* can infect potato and other cultivated crops and weed species of the family *Solanaceae*. It is essential to establish, if the pathogen strains from non-potato hosts represent distinct genotypes/

populations or can freely exchange with those on potato. Isolates of *P. infestans* from four solanaceous hosts, black nightshade, hairy nightshade, petunia and potato growing within and around fields of blighted potatoes in four US locations and one UK location were characterized. The isolates were characterized for mitochondrial DNA, haplotype, mating type, metalaxyl resistance, allozyme of glucose-6-phosphate isomerase and peptidase and DNA fingerprint with the RG57 probe. Close similarity could be observed between the isolates of petunia, hairy and black nightshade isolates and potato isolates. The results indicated that the weed and cultivated plants infected by *P. infestans* might serve as clandestine reservoirs of inoculum and they might escape detection and fail either to be eradicated or treated with effective fungicides. Hence, they may play a major role in the perpetuation and spread of *P. infestans* to new locations (Deahl et al. 2006).

Characterization of isolates of *Phytophthora cactorum* and 15 other species of *Phytophthora* was performed with respect to their genomic DNA, pathogenicity and sensitivity to mefenoxam. The AFLP analyses were carried out for 132 isolates of *P. cactorum* from almond (30 isolates), strawberry (86 isolates), walnut (five isolates) and 11 other host plant species, in addition to 22 isolates of 15 other *Phytophthora* spp. from various hosts. All 16 *Phytophthora* spp. could be differentiated by their respective AFLP banding patterns. High coefficiency of genetic similarities (>0.9) were observed among all California isolates of *P. cactorum*. Among all 132 isolates of *P. cactorum*, 30.8% and 24.5% of the AFLP variation was associated with hosts and geographical sources of isolates respectively. Sensitivity of all isolates of *P. cactorum* to mefenoxam was at the same level of 1 ppm. Populations of *P. cactorum* in California apparently were mefenoxam sensitive and exhibited host specificity with relatively minor variation in genomic DNA. Significant geographical and host origin components were also indicated in the genetic variation determined by AFLP analyses of *P. cactorum* (Bhat et al. 2006).

Five *Fusarium* spp. associated with sorghum and millets were analyzed for their ability to produce mycotoxins, fumonisins and moniliformin, their toxicity to ducklings and their ability to cause disease on sorghum seedlings *in vitro*. *F. andiyazi*, *F. nygamai*, *F. pseudonygamai*, *F. thapsinum* and *F. verticillioides* were distinguished by isozyme anlysis and AFLP banding patterns. Two species of *F. pseudonygamai* and *F. thapsinum* produced high levels of moniliformin, but little or no fumonisins. In contrast, *F. verticillioides* and *F. nygamai* produced high levels of fumonisins, but little or no moniliformin. The five species of *Fusarium* mentioned above were earlier considered to be one species *F. moniliforme* and the present study clearly brings out the differences between them facilitating the accurate identification of the *Fusarium* spp. (Leslie et al. 2005). Fusarium head blight (FHB) or scab disease of wheat has spread to large areas in the northern China. In order to assess the genetic diversity of *Fusarium graminearum* and *F. asiaticum* isolates involved in FHB, 437 isolates collected from wheat spikes were subjected to sequence characterized amplified region (SCAR) analyses which resolved both species of *Fusarium.* Twenty one percent of the isolates belonged to *F. graminearum* SCAR type 1, while 79% of the isolates belonged to *F. asiaticum* SCAR type 5. The AFLP profiles clearly resolved two groups A and B that were entirely

congruent with both species. In addition, AFLP analyses revealed several groups within each group. In many cases, isolates from the same location were included in different AFLP haplotypes showing marked variations. Phylogenetic analyses of multilocus DNA sequence data showed that all isolates of SCAR type 1, AFLP group A were *F. graminearum*, whereas isolates of SCAR type 5, AFLP group B were *F. asiaticum*, indicating that differentiation of these two species based on SCAR/AFLP analyses was reliable and efficient (QU et al. 2008).

In another study on the causal agents of mango malformation disease agents *Gibberella fujkuroi* and *F. sterilihyphosum* was investigated to determine the genetic diversity of the pathogens by employing AFLP and vegetative compatibility group (VCG) analyses. These two techniques confirmed the results of each other, since both techniques identified the same genetic groups. Six AFLP and VCGs were identified among the isolates of the new *Fusarium* lineage associated with mango malformation disease. AFLP I was the most widespread group, present in 7 of 13 sites sampled. The clonal nature of the observed populations suggested that these fungal pathogens either occurring naturally on indigenous hosts and jumped to the introduced new mango host or that they might have originated with mango introduced to Brazil from India or Southeast Asia (Lima et al. 2009).

The genetic variability within the VCGs of *Verticillium dahliae* was examined by applying AFLP technique. Isolates of *V. dahliae* from artichoke (53), from cotton plants (96), from cotton soil (7) and from olive trees (45) were subjected to AFLP and PCR procedures. Clustering of isolates within VCGs correlated with respective VCGs, irrespective of host plant species and geographical locations. However, VCGs exhibited molecular diversity, the variability being highest in VCG2B and VCG2A. In addition, VCG2B isolates from artichoke formed two distinct clusters based on PCR markers of 334-bp or 824-bp. Further, correlations between molecular differences and their virulences of VCG2B isolates pathogenic to artichoke and cotton cultivars were observed (Collado-Romero et al. 2006).

Pyrenophora teres has been differentiated into two forms of *P. teres* f. *teres* (PTT-net form) and *P. teres* f. *maculata* (PTM-spot form), based on the type of symptoms produced on barley. These two forms contain large numbers of pathotypes and resistance to disease is inherited independently (Arabi et al. 2003). Application of AFLP technique provided more reliable differentiation of the isolates of PTM and PTT than by leaf symptoms. The isolates of *P. teres* were divided into two strongly divergent groups corresponding to the net and spot forms of *P. teres* based on the AFLP patterns (Rau et al. 2002). Later the Czech isolates of *P. teres* were examined for their genetic diversity by employing AFLP procedure. The other cereal pathogens *P. graminea*, *P. tritici-repentis* and *Helminthosporium sativum* were also included in the analysis. The AFLP profiles were distinctly different for each fungal pathogen studied. Polymorphic bands (948) were scored based on the amplification reaction using 19 primer combinations. In *P. teres*, two distinct groups representing PTT and PTM forms were identified, using the diagnostic markers – 83 and 134 – specific for PTT and PTM forms of the pathogen respectively (Leišova et al. 2005).

Genetic variability of *Pyricularia oryzae* (*Magnaporthe grisea*) was assessed by applying AFLP procedure. Isolates of *P.oryzae* (114) collected from rice grown in the Red River Delta during 2001 and other isolates (9) in Vietnam were analyzed based on 160 polymorphic AFLP markers. Based on DNA similarity and cluster analysis, the isolates were classified into 12 different AFLP genetic groups. Two separate groups were formed to accommodate isolates of *P. oryzae* from *japonica* and *indica* cultivars which exhibited at least 60% dissimilarity with little evidence for gene flow between these two clusters. On the other hand, isolates from *indica*, forming the predominant group, showed significant gene flow between these populations found in south and north of Red River. Pathogenicity tests indicated the presence of two avirulent isolates and 23 pathotypes which were differentiated into 12 AFLP groups genetically (Thuan et al. 2006).

Claviceps purpurea infects rye and other grasses. The ergot sclerotia produced by the pathogen in place of grains, are used as a source of novel chemicals, alkaloids with medicinal value. Three groups within this species (G1, G2 and G3) have been recognized based on habitat association, sclerotia, conidial morphology and alkaloid production. Isolates (21) selected from a wide range of host plant species and large geographical area were analyzed by RAPD and AFLP techniques. The results supported the recognition of three discrete groups within *C. purpurea* and revealed high genetic variability between groups with less than 2% of polymorphic markers that shared across all isolates. In order to determine, whether *C. purpurea* complex was made up of one or multiple species, phylogenetic and population genetic analyses were performed. The G1 types were found to be more integrated with one another. The results suggested that little or no gene flow occurred between the different ecotypes and that this process might be driven by adaptations to ecological habitats (Douhan et al. 2008).

4.1.4.6 Heteroduplex Mobility Analysis

Anthracnose diseases caused by different species of *Colletotrichum* are responsible for substantial qualtitative and quantitative losses in agricultural and horticultural crops. Differentiation of *Colletotrichum* spp. based on the morphological characteristics alone is time-consuming and unreliable. The possibility of applying heteroduplex mobility analysis (HMA) of ITS regions of fungal pathogen genomic DNA was explored. The ITS regions of 29 isolates of Colletotrichum including *C. gloeosporioides*, *C. acutatum*, *C. musae*, *C. graminincola*, *C. capsici*, *C. dematium* and *C. lindemuthianum* were amplified using specific primers. A greater divergence of nucleotide sequences within the ITS1 region was observed compared to that of ITS2. The DNA distance and sequence identity within intraspecies ranged from 0.0% to 1.1% and from 98.9% to 100% respectively, whereas those within interspecies ranged from 1.46% to 13.43% and 90.02% to 98.56% respectively. Based on the DNA distance inferred from HMA, the isolates of *Colletotrichum* were classified into five distinct species groups as CG, CA, CC, CM and CL. The results of HMA could be obtained relatively rapidly and this approach was found to be convenient for species-specific identification of anthracnose pathogens (Huang et al. 2010).

4.1.4.7 Electrophoretic Karyotyping Technique

Pathogenicity and virulence of fungal pathogens are determined by specific genes in the genomic DNA. Pathotype-specific DNA fragments involved in host-recognition mechanisms of *Verticillium dahliae*, were cloned and three DNA fragments specific to tomato pathotype were identified (Usami et al. 2002). In a further study, the karyotypes of Japanese isolates of *V. dahliae* were investigated to identify the chromosomes on which tomato pathotype-specific DNA fragments are located. In a genomic Southern blot analysis of seven isolates probed with a telomere consensus sequence, 12–14 bands were observed. Pulsed-field gel electrophoresis (PFGE) of these isolates revealed five or six chromosomal bands. A band (approximately 3.5 Mbp) common to all isolates apparently contained more than two chromosomes. Although the chromosome sizes differed among isolates, karyotypes were similar within tomato and sweet pepper pathotypes. Subsequent PFGE-Southern hybridization analysis revealed that the three DNA fragments specific to tomato pathotype are located on the same chromosome (Usami et al. 2008).

4.2 Assessment of Variability in Sensitivity to Chemicals

Fungal pathogens have been reported to develop resistance to both non-systemic and systemic fungicides. Application of fungicides has undoubtedly contributed to reduction in damages caused by fungal pathogens to a variety of crops. However, excessive and indiscriminate use of fungicide(s) repeatedly has been responsible for the undesirable effects of development of resistance to fungicides in addition to the presence of chemical residues in harvested produce beyond permissible limits. Loss of fungicidal activity applied at recommended doses has occurred more frequently following application of narrow-spectrum systemic fungicides, when compared to non-systemic fungicides that act on the fungal pathogens through different pathways. Existence of fungicide resistant isolates/strains may be detected by employing tests based on the biological and molecular characteristics of the fungal pathogen species (Staub and Sozzi 1984; Staub 1991). Fungicide resistant isolates of several fungal pathogens affecting various crops have been detected in different countries (Table 4.1).

4.2.1 Assessment of Variations in Biological Characteristics

4.2.1.1 Cultural Characteristics

Isolates exhibiting resistance to fungicides can be detected by growing them on the artificial medium amended with different concentrations of test fungicide. The sensitive isolates are able to develop even at concentrations of the fungicide far higher than that is recommended for field application. A bioassay technique using Alamar Blue

Table 4.1 Detection of fungicide resistance in fungal pathogens

Fungicides	Pathogens	References
Acylalanines	*Pseudoperonospora cubensis*	Katan and Bashi 1981
Benomyl compounds	*Rhynchosporuim secalis*	Hollomon and Butters 1994
Benzimidazoles	*Venturia inaequalis*	Kiebacher and Hoffmann 1976
	Cercospora beticola	Georgopoulos and Dovas 1973
	Botrytis cinerea	Schüepp and Küng 1981
	Pseudocercosporella Herpotrichoides	Murray 1996
	Helminthosporium solani	McKay and Cooke 1997
Dicarboximides	*Botrytis cinerea*	Leroux and Besselat 1984
Dodine	*Venturia inaequalis*	Szkolink and Gilpatrick 1973
Dinocap	*Sphaerotheca fuliginea*	Georgopoulos 1969
Hexachloro-Benzene	*Tilletia foetida*	Georgopoulos 1969
QoI compounds	*Podosphaeria fusca*	Ishii et al. 2001
	Pseudoperonospora cubensis	
	Alternaria solani	Ishii 2006
	Erysiphe graminis f.sp. *tritici*	
	Mycosphaerella fijiensis	
	Plasmopara viticola	
	Uncinula necator	
	Corynespora casiicola	Ishii et al. 2007
	Pyricularia oryzae	Ishii et al. 2008
	Botrytis cinerea	Ishii et al. 2009
Thiophanate-	*Colletotrichum gloeosporioides*	Chung et al. 2006
Fentin hydroxide	*Cercospora beticola*	Giannopolitis 1978

as a growth indicator of fungal development can be applied to test large number of isolates for detecting fungicide resistance/sensitivity of pathogenic isolates. This high throughput assay was performed in microtiter plates to assess the sensitivity levels of *Septoria tritici* to fungicides. Metabolic activity of *S. tritici* in the presence of normally inhibitory concentrations of a fungicide could be determined rapidly by measuring color or fluorescence released after conversion of the growth indicator substrate Alamar Blue incorporated into medium kept in plate wells. Development of pink or blue color indicated growth or no growth respectively, revealing the resistance/sensitivity of the isolates of *S. tritici* (Rothamstead Research 2003–2004).

Citrus black spot (CBS) disease caused by *Guignardia citricarpa* (anamorph-*Phyllosticta citricarpa*) reduces the market value of fruits drastically, because of the presence of lesions on the fruits. The disease could be managed effectively by benomyl for a decade till the pathogen developed resistance to this fungicide in South Africa. The sensitivity of ten strains of *G. citricarpa* to benomyl and strobilurin (azoxystrobin) was assessed in Brazil. The fungus was grown on artificial medium amended with the fungicides at increasing concentrations in the range of 0.1–10 μg/ml. The efficacy of fungicides on the colony diameter and sporulation were determined. All ten strains were sensitive to benomyl as the growth of *G. citricarpa* was entirely inhibited at concentrations at and above 0.5 μg/ml. In contrast, the growth of the pathogen was not

inhibited by azoxystrobin, but the sporulation was completely arrested at 10 μg/ml. The finding on the adverse effect of azoxystrobin on sporulation might have practical utility, because dissemination of *G. citricarpa* occurs primarily through spores produced from lesions on infected plant tissues (Possiede et al. 2009).

Isolates of *Botrytis cinerea* obtained from grey mold lesions on citrus (Satsuma mandarin) plants were cultured on potato dextrose agar (PDA) medium. Mycelial discs (4 mm in diameter) were transferred onto plates containing PDA medium amended with QoI fungicides, azoxystrobin or kresoxim-methyl at 1 or 100 mg/l of active ingredient (a.i). The inhibition percentage was determined in comparison to the control without the fungicide supplement. The inhibitory activity of azoxystrobin or kresoxim-methyl alone on mycelial growth was low even at 100 mg/l. But inhibition by these chemicals on sensitive isolates increased dramatically in the presence of 1 mM n-propyl gallate (PG), because of synergistic action of PG. However, QoI resistant isolates of *B. cinerea* grew well on PDA even in the presence of PG. The results of this in vitro test were in good agreement with those of inoculation tests (Ishii et al. 2009).

4.2.1.2 Inoculation Tests on Detached Leaves/Whole Plants

Resistance to metalaxyl in *Peronospora tabacina* causing tobacco blue mold disease was tested using tobacco leaf disks for inoculation. The leaf disks were inoculated on the lower side with fresh sporangiospores at the rate of 5,000 spores/leaf disk and they were floated upside down on either water (control) or increasing concentrations of metalaxyl-M (pure compound in water) in the range of 0.01–100 μg/ml. The extent of fungal growth was recorded over a period of 14 days. The ratio of sporulating disks was calculated. Absence of sporulation marked the sensitivity of the pathogen isolate to the fungicide at concentration >0.1 ppm of metalaxyl-M, while resistant strain sporulated even at concentrations >10 ppm (Zipper et al. 2009).

Cucumber cotyledon paper disc method was followed to assess the sensitivity of *Botrytis cinerea* to QoI fungicides, azoxystrobin or kresoxim-methyl. The fungicides at recommended doses were sprayed on the cotyledons of cucumber plants raised in a phytotron. After air-drying, the inoculum (50 μl of conidial suspension) was pipetted on the filter paper discs which were placed at the central portion of the cotyledons treated with fungicide or water for control plants. Alternatively mycelial discs of pathogen were placed upside down on the cotyledons, followed by addition of a drop of water to the mycelial disc. The inoculated plants were kept at 20°C in a dark dew chamber for 3 days. Inhibition of lesion development (in percentage) was calculated in comparison to the lesion size on controls (Ishii et al. 2009).

4.2.2 Assessment of Variations in Genetic Characteristics

Resistance to fungicides in the fungal pathogens may be due to changes in the nucleic acid sequences which may be detected by applying molecular techniques. Resistance to benzimidazoles may be acquired in a single step by the fungal

pathogens and it appears to be controlled by genes of major effects. By employing PCR assay with allele-specific oligonucleotides, point mutations (one-step change) in fungal genome could be detected (Williams et al. 1990). Molecular markers linked to loci controlling resistance in *Phytophthora infestans* were identified by employing bulk segregant analysis (BSA). Six RAPD markers have been identified (Judelson and Roberts 1999). One dominant gene plus minor genes may be involved in phenylamide resistance in *P. infestans* (Lee et al. 1999). Benzimidazole fungicides bind to the fungal ß-tubulin molecule distrupting micro-tubular assembly and by interfering at the site of action, it inhibits this conformational change resulting in microtubule resistance to inhibition by thiabendazole (Davidse and Ishii 1995; Paluh et al. 2004).

Sensitivities of *Colletotrichum gloeosporioides* and *C. acutatum* infecting different fruit crops to three fungicides thiophanate methyl, diethanocarb and iminoctadinetriacetate were examined. The partial sequence of ß-tubulin gene of *C. gloeosporioides* highly resistant to thiophanate methyl showed a substitution of the amino acid Glu (CAG) with Ala (GCG) at codon 198. In the intermediate resistant isolates of *C. gloeosporioides*, Phe (TTC) was substituted with Tyr (TAC) at codon 200. The sequence in *C. acutatum* isolates showed variation at codon 198 (GAG or AAG), but the substitution at codon 200 was similar as in *C. gloeosporioides* (Chung et al. 2006). Sixty four isolates of *C. gloeosporioides* obtained from *Limonium* spp. (ornamental perennial plant species) were examined. Twelve selected isolates were subjected to arbitrarily primed PCR and ITS-1 sequence analysis. Benomyl resistant- and sensitive-populations formed two distinct genotypes. Sequence analysis of the ß-tubulin genes *TUB1* and *TUB2* of the five sensitive and five resistant representative isolates showed amino acid substitutions. Resistant isolates had an alanine substitute instead of glutamic acid at position 198 in *TUB2*. The results suggested that the resistant and sensitive genotypes were two independent and separate populations (Maymon et al. 2006).

Application of fungicides for the management of grey mold disease of grapes caused by *Botrytis cinerea*, has been a simple strategy to protect grapes. However, emergence of phenotypes exhibiting resistance to fungicides has become a serious problem in the production zones. A nested PCR-RFLP technique was developed to identify the fungicide resistant isolates rapidly and reliably at the early growth stages of grapes in the grapevine fields. For the nested PCR-RFLP procedure, primer pair Bctub-F1/Bctub-R1 was designed based on ß-tubulin gene for the first round PCR and for the second round PCR, the primer pair Bctub-F2/Bctub-R2 or BcOS-F2/BcOS-R2 was employed. The PCR products were digested with the restriction enzymes *Bmg*B1 and *Taq*1. The nested PCR-RFLP method successfully detected and differentiated *B. cinerea* isolates exhibiting resistance to benzimidazole, phenylcarbamate and/or dicarboximide present in grape berries and leaves. This protocol required only 8 h from tissue sampling to phenotyping of fungicide resistance of *B. cinerea* isolates. The nested PCR-RFLP procedure was found to be a high-speed sensitive and reliable tool for early diagnosis of fungicide resistant *B. cinerea* isolates contributing to significant improvement in the integrated disease management system (Saito et al. 2007).

Isolates of *Botrytis cinerea*, the incitant of onion grey mold disease, exhibit multiple resistance to two fungicides, benzimidazole and dicarboximide. A fluorescence resonance energy transfer (FRET)-based real-time PCR assay was applied to detect benzimidazole- and dicarboximide-resistant mutations – ^{198}Glu to Ala (E198A), F200Y and E198K – in ß-tubulin *BenA* were detected using a single set of fluorescence-labeled sensor and anchor probes by melting curve analysis. Likewise, three dicarbomide-resistant mutations – 1365S, V368F plus Q369H and Q369 – in the histidine kinase *BcOS1* were successfully distinguished. A new benzimidazole-resistant *BenA* E198V mutation was identified by *BenA* genotyping assay. Among the 210 *B. cine*rea field isolates, most of the benzimdazole-resistant isolates possessed the E198V or E198A mutation in the *BenA* gene and the 1365S mutation in the *BcOS1* gene was also frequently noted on the Japanese isolates. The method of genotyping based on *BenA* and *BcOS1* gene sequences was shown to be rapid and reliable to detect and identify the resistant isolates and to have the potential for monitoring the fungicide-resistant field populations (Banno et al. 2008).

Isolates of *Peronospora tabacina*, incitant of tobacco blue mold disease could be differentiated in metalaxyl-sensitive and -resistant phenotypes based on bioassay tests. A PCR-based assay was employed for determining sensitivity or resistance of *P. tabacina* isolates to metalaxyl fungicide. A total of 44 strains of *P. tabacina* collected from different regions of Germany during 2002–2006 were subjected to PCR fingerprinting with SSR primers for detecting and assessing genetic diversity of isolates. DNA samples from *P. tabacina* produced characteristic fingerprints which distinctly differed from the patterns of other oomycetes. DNA fingerprints using SSR and minisatellite primers allowed separation of the samples into two groups. Fingerprints with $(GACA)_4$ primer amplified a prominent product of 830-bp only from the resistant isolates. A faint product of similar size was present in the sensitive strains also. However, high resolution capillary electrophoresis revealed the presence of a slightly bigger sized (844-bp) fragment. Hence, this fragment was not identical with the 830-bp fragment amplified from resistant isolates. The differences, in the amplification pattern coincided with the sensitive and resistant reaction of the isolates determined in metalaxyl bioassays. The primers allowed PCR-based detection of *P. tabacina* and differentiation of metalxyl-sensitive and metalaxyl-resistant strains. In Germany during 2002–2004, resistant isolates were exclusively detected (Zipper et al. 2009).

Melanin biosynthesis inhibitors (MBI) have been demonstrated to be effective against rice blast pathogen *Magnaporthe grisea* which forms melanized appressoria essentially required for penetration of epidermal cells of rice plants to establish infection successfully. Resistance to MBI-D fungicides (inhibitors of polyhydroxy naphthalene dehydratase of the pathogen) was shown to be due to a point mutation in the gene encoding the targeted protein which resulted in a single amino acid substitution in scytalone dehydratase by employing primer-introduced restriction enzyme analysis-PCR (PIRA-PCR) assay (Kaku et al. 2003). A novel PCR-Luminex detection system was employed based on hybridization between a fluorescent PCR product and an oligonucleotide probe that specifically recognized the resistance-type mutation in *P. grisea*. Two wild (parent) type and two MBI-D resistant isolates of *M. grisea* were detected efficiently by

this procedure which has the potential for a high throughput analysis of single nucleotide polymorphisms (SNPs) (Ishii et al. 2005). The PCR-Luminex detection was applied for the detection of four representative species causing Fusarium head blight disease of wheat and barley and isolates showing resistance to thiophanate-methyl and two sensitive isolates of *F. culmorum* and *F. graminearum* (Ishii et al. 2008).

Appendix 1: Identification of *Guignardia citricarpa* by PCR-Based Technique (Peres et al. 2007)

A. Extraction of DNA from fruit lesions

(i) Dissect out very small citrus fruit lesions (approximately 2 to 4 mm in diameter); discard the surrounding healthy flavedo, the pigmented outer tissue of the fruit rind and disrupt the tissue using alkaline lysis extraction method.

(ii) Follow the dipstick method by adding 150 µl of 100% ethanol and a small piece of cellulose thin-layer chromatography plate (dipstick) to the 2-ml tube; place the tubes on their sides on ice and shake for 30 min.

(iii) Aspirate off the liquid from the tube; add 500 µl of wash buffer [10 × (Tris Na_2-EDTA and NaCl, pH 7.0)] diluted to 25%; invert tubes to mix the contents well; repeat washing twice; place the dipstick in a new tube and dry under vacuum.

(iv) Place the tubes on their sides; add 50 µl Tris-EDTA buffer; incubate for 5 min; spin the tubes for 10 s; discard the dipstick and recover the DNA.

B. PCR amplification

(i) Carry out the amplification in a total volume of 20 µl in the mixture containing 8 µl 2.5 × Eppendorf MasterMix (*Taq* DNA polymerase at 0.06 U/µl, 2.5 × Taq reaction buffer (125 mM KCl,75 mM Tris-HCl, pH 8.4, 4 mM Mg^{2+} and 0.25% Nonidet-P40, 500 µM each of dNTP and stabilizers), 0.8 µl of each primer at 10 µM and 2 µl of template DNA.

(ii) Provide the following conditions: 94°C for 2 min, followed by 39 cylces of 94°C for 30 s, annealing at 64°C for 30 s, 72°C for 1 min and a final extension for 10 min at 72°C

(iii) Separate the amplicons by electrophoresis in 1% agarose gels in 1 × Tris-borate-EDTA buffer.

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Chapter 5
Diagnosis of Fungal Diseases of Plants

Abstract Crop plants are infected by numerous fungal pathogens from seedling stage to the seed maturing stage under the natural conditions, either singly or in combination with other kinds of microbial plant pathogens. Diagnosis of the disease problem observed for the first time, involves isolation of the fungi in pure cultures using appropriate media and identification of the putative pathogen based on morphological, physiological/biochemical, immunological and genomic nucleic acid characteristics, followed by inoculation onto the natural plant host for reproducing the symptoms observed earlier. The characteristics of the fungus reisolated from the inoculated plants are then compared with original description of the fungus isolated from the diseased plants earlier. Adoption of all steps in Koch's postulates is essential. Precise identification of the pathogens up to subspecies, varieties, races/biotypes of the fungal pathogen species is accomplished by applying immunological and/or nucleic acid-based techniques. Correct diagnosis of the disease (s) is considered to be the corner stone of the development of effective management of the disease concerned. The need for establishing disease diagnostic centers and the role of plant quarantines in preventing the introduction of new plant diseases and principle of certification of seeds and propagative plant materials to reduce disease incidence and spread is highlighted.

Crops cultivated in different ecosystems are exposed to various fungal plant pathogens, in addition to different kinds of other biotic and abiotic agents that can adversely affect their growth and yield potential to varying magnitudes, depending on the duration for which the disease-inducing agents have access to the susceptible plant species. Any deviation from the normal functions and development leads to disease in the affected plant species. The nature of the agent(s) involved in observed diseased condition, has to be ascertained immediately. Fungal plant pathogens are able to cause distinct macroscopic symptoms that can be visually seen in most cases and hence, these diseases can be separated with some experience from the problems caused by insect pests or adverse environmental conditions or industrial pollutants injected into the atmosphere. Disease diagnosis pertains to the identification of the nature and cause of the disease, while detection relates to establishment of the

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identity of the causative agent present in the infected plants. Plant Pathogen detection and disease diagnosis are inseparable processes, the results of one process complimenting the other resulting in reliable identification of the disease-causing agent precisely for initiating follow up action for the measures to minimize disease spread and the consequent yield losses to the growers.

Disease diagnosis is based on the postulates enunciated by Robert Koch and it is needed essentially to recognize the primary pathogen causing the disease. In a disease complex, two or more fungal pathogens may be involved, making it difficult to identify the primary pathogen and the secondary invaders of the affected plant tissues. Symptom expression may not be clear or may take long time for the development of symptoms in tissues far away from the site of infection as in the case of smut and downy mildew diseases. In such cases the infection may take place in the roots or basal portions of the stem and the symptoms are expressed only at the time of heading/flowering. By the time symptoms become observable, the pathogen might have well established in the internal tissues of the plants, making it difficult, if not impossible, to apply any effective measures to reduce disease spread. However, reliability on the development of external symptoms of infection alone may lead to erroneous identification of the pathogen and consequently the control methods that are suggested may prove to be ineffective. Hence, accurate identification of the disease-causing organism(s) is the basic requirement for planning effective disease management strategies. In addition, precise identification and differentiation of the cause of the disease-causing agent enables more informed decision to be made about the choice of cultivar (showing resistance, if available) and the appropriate application of chemicals in time.

5.1 Choice of Diagnostic Tests for Fungal Pathogens

5.1.1 Conventional Methods

Fungi (including oomycetes) based on their ability to infect one or more plant species, are the earliest to be recognized as pathogens among the microbial plant pathogens. Conventional methods of disease diagnosis involves the study of symptomatology, isolation of the fungi in suitable culture media and determining the morphological characteristics of aexual and sexual structures and the spores produced which are used for taxonomic classification of fungi. Determination of morphological characteristics alone may not result in correct identification of the fungal pathogens. Characterization of fungal pathogens based on the phenomena of heterothallism and vegetative compatability (Korolev et al. 2008) and other physiological functions that are responsible for the production of toxins and enzymes has been demonstrated to be useful for the identification of incompatible groups/isolates or chemotypes within a morphologic species of a fungal pathogen. Furthermore, physiologic races differing in their pathogenic potential have to be identified by using a set of differential host plants species or crop cultivars.

The various characteristics of fungal pathogens based on which they can be precisely identified have been described in the Chapter 2 of this volume.

The process of disease diagnosis commences with the isolation of the presumptive disease-causing agent from the diseased plants, and careful descriptions of the characteristics of the fungus isolated by various methods. Some fungi may need special/differential media that may favor their faster development in the presence of fast-growing saprophytic contaminants. For example, isolation of *Gaeumannomyces graminis* var. *tritici* (*Ggt*), causative agent of wheat take-all disease was difficult, because of the presence of *Pythium* spp, *Fusarium* spp. or secondary fungal colonists that could overgrow the slow-growing pathogen. A semi-selective medium R-PDA amended with rifampicin and tolclofos-methyl was used for the successful isolation and detection of *Ggt*, based on the color change that occurred from orange to purple only in the presence of *Ggt* (Duffy and Weller 1994). Esca is the complex disease and considered as one of the most important grapevine trunk diseases causing severe decline in grape production in European countries. An etiological plurality has been suggested, because of the involvement of the fungi *Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia mediterranea* (*Fomed*) as primary causes of Esca. Due to their slow growth, other fungi overgrew and it was difficult to isolate them in pure cultures. Further, suitable selective media are not available. Since isolation and differentiation of these fungi based on the conventional methods was not reliable, application of more sensitive and reliable technique(s) like nucleic acid based methods became necessary for resolving the components of this complex disease of grapevine plants (Romanazzi et al. 2009).

Consistent association of the fungus isolated has to be ascertained by isolating the fungus from as many samples as possible from different locations and times. The fungus whose identity is tentatively determined is then inoculated onto healthy plants of the same species in which the incidence of the disease has been reported. After observing the similarity of symptoms induced on the test plants, the fungus is reisolated from the inoculated plants. The characteristics of the fungus reisolated are compared with the original descriptions of the fungus. All steps of Koch's postulates mentioned above, may not be adoptable in certain groups of fungal pathogens causing rust, downy mildew and powdery mildew diseases in a wide range of crops, as they are obligate parasites requiring living cells for their existence. Consistent association of the suspected fungal species and their pathogenicity on the host plant species concerned can be ascertained and the descriptions of the asexual and sexual spore-bearing structures may be compared with the ones known to occur earlier and the identity of the test fungus may be confirmed.

Latent or quiescent infection of plants or fruits by fungal pathogens has been observed in horticultural crops. Symptoms of infection may be seen when the fruits mature either in the plants or when they are stored after harvest. Infection of grape berries by *Botrytis cinerea* may be difficult to recognize by visual inspection, because the pathogen remains dormant in the early stages of berry development. As no diagnostic tool was available for discriminating between quiescent and actively colonizing *B. cinerea* in developing berries, an effective method had to be developed. A bioassay involving berry surface sterilization, followed by killing of

berry tissues by freezing and incubation, led to activation of the pathogen and colonization of the dead tissues by *B. cinerea*. This simple bioassay was effective in detecting berry infection by *B. cinerea*. If quantitative estimation of the pathogen is required, PCR-based technique may be adotpted (Cadle-Davidson 2008).

After ascertaining the pathogenicity of the fungus isolated from the infected plants, by inoculation on the healthy test plants, the fungal pathogen has to be assigned to a particular taxon in an appropriate classification system and designated appropriately, indicating whether this pathogen is a new species/subspecies/variety or closely related to the already existing fungal pathogen species. The traditional procedures generally require skilled and specialized expertise in classical taxonomy which may need several years to acquire. In addition, isolation-based methods require several days or even weeks, if the pathogen is slow-growing, to provide results and they may not be suitable when results required rapidly during the occurrence of disease epidemics.

5.1.2 Molecular Methods

With rapid advancements achieved in plant disease diagnostics, dependence on the isolation-based conventional methods has been reduced to a great extent. Further, development of symptoms in infected plants reflecting interaction effects between the host plant and the pathogen may be influenced significantly by changes in the environmental conditions such as unusual dry summer or cold spring seasons. Molecular technique-based diagnosis do not depend on the symptoms induced by the pathogen in the infected plants. But this approach reveals the presence and quantity of pathogen in the infected samples which may or may not exhibit the symptoms of the disease in question. These techniques rely on the inherent pathogen properties such as immunoreactivity of the proteins of pathogen origin or genomic nucleic acids of the fungal pathogens. The identity of the fungal pathogen present in the infected plants may be established even at the early stages of pathogen development when no asexual or sexual are formed. The comparative efficacy of conventional and molecular methods in identifying the fungi involved in the Esca complex disease of grapevine that is widespread in European countries is presented in the Table 5.1. The PCR-based technique was shown to be more sensitive and reliable than the conventional methods in detecting and identifying them precisely (Romanazzi et al. 2009).

The imperative need for rapid and reliable diagnostic methods for detecting and identifying microbial plant pathogens is underscored by the continuing globalization of trade and the large scale movement of people and goods which enhance significantly opportunities for introduction of new pathogen species or strains of pathogen species present already in the particular geographical location. The introduced pathogen may find conditions for their survival and perpetuation, resulting in devastating epidemics when favorable environmental conditions prevail. Not only introduction of new pathogen(s) may pose threats to profitable crop production, but

Table 5.1 Efficacy of conventional and molecular methods in detecting fungal pathogens in the wood of 30-year-old rootstock mother plants (Romanazzi et al. 2009)

Method of detection/ fungal pathogens	Rootstocks				Positive detection/total
	Kober 5BB	1103 P	420A	S04	
Conventional method					
Pch[a]	2/3[b]	2/2	3/3	2/4	9/12
Pal	2/3	0/2	0/3	2/4	4/12
Fomed	0/3	2/2	0/3	2/4	4/12
Bot	2/3	2/2	2/3	0/4	6/12
Molecular method					
Pch	2/3	2/2	3/3	4/4	11/12
Fomed	0/3	2/2	0/3	2/4	4/12

[a]Pch – *Phaeomoniella chlamydospora*; Pal – *Phaeoacremonium aleophilum*; Fomed – *Fomitiporia mediterranea*; Bot – *Botryosphaeria* spp.
[b]Positive detection/total number tested

evolution of new races or biotypes of the indigenous fungal pathogens may also lead to such problematic situations. Hence, development of necessary infrastructure and adequate technical manpower for constant and consistent monitoring of pathogen movement and evolution of more virulent strains has to be developed to link the diagnostic facilities available nationally and internationally.

Fungal pathogens such as *Colletotrichum gloeosporioides*, *Botrytis cinerea* and *Rhizoctonia solani* have a wide range of host plants which include crops and weed host plant species. The crucial role played by additional host plant species either crops or weed hosts in pathogen survival and perpetuation has been well demonstrated (Volume 1, Chapter 3). Genetic variability of *C. gloeosporioides* has been studied by applying molecular methods to focus on the need to establish the relationship between isolates from a large number of cultivated and wild plant species, many of which were found in close proximity with water yam (*Dioscrorea alata*). The possibility of wild hosts serving as sources of inoculum for yam was indicated. Eighteen virulence phenotypes of *C. gloeosporioides* among 217 isolates were identified by employing microsatellite-primed (MP)-PCR assay. *C. gloeosporioides* isolates from yam were vegetatively compatible with weed isolates. Hence, it was hypothesized that genetic exchange might occur between isolates from yam and non-yam hosts under field conditions (Abang et al. 2004, 2005).

A step-by-step procedure of diagnosing a new disease of maize causing grey leaf spot (GLS) disease occurring in southern Africa was applied. In order to establish *Cercospora zeina* as the primary cause of GLS of maize, single conidial cultures were generated by transferring single conidia from lesions on maize leaves to V8 medium (containing 200 ml of V8 juice, 15 g of agar and 2 g of $CaCO_3$). Maize plants (hybrid PAN7624B) were inoculated with the conidial suspension prepared from the culture grown on the agar plate (3×10^4 conidia/ml), using a brush to transfer the inoculum to the maize leaves. Conidia were reisolated from the lesions on inoculated maize leaves, transferred to V8 medium and the characteristics of the fungal culture developed, were verified with the original descriptions of *C. zeina*. PCR amplification of histone gene fragment from the genomic DNA of *C. zeina*

was accomplished using pathogen-specific primer. An expected 284-bp histone gene fragment was amplified from all single conidial cultures of *C. zeina*. The ITS1/ITS4 region of the rDNA operon and the EF gene fragment were sequenced. The sequence alignment of these DNA fragments revealed no differences between the single conidial cultures. All 71 isolates collected from different locations in southern Africa were classified as *C. zeina*. The results of this investigation confirmed the presence of *C. zeina* and the absence of C. *zeae-maydis* in commercial maize plantations in these areas in southern Africa (Meisel et al. 2009).

The possibility of detecting two or more plant pathogens simultaneously is being explored, since simultaneous detection and identification of more than one pathogen at a time can be expected to save time and costs of testing them individually. *Didymella bryoniae*, the fungal pathogen causing stem blight disease and *Acidovorax avenae* subsp. *citrulli*, the causal agent of bacterial fruit blotch (BFB) are transmitted through the cucurbit seeds. A combination of magnetic capture hybridization (MCH) and multiplex real-time PCR was applied for the detection of these fungal and bacterial pathogens simultaneously. The MCH real-time PCR protocol facilitated detection of both target pathogens in watermelon and melon seed samples (n = 5,000 seeds/sample) in which 0.02% (2 seeds in 1,000 seeds) were infested with *D. bryoniae* and 0.02% were infested with *A. avenae* subsp *citrulli* (Ha et al. 2009). As attempts are being made to simplify the test procedures and to reduce the cost of testing, it may be possible to enhance the level of application to provide results of diagnosis for large number of new diseases rapidly.

5.2 Agencies Involved in Disease Diagnosis

Disease diagnosis may be the primary responsibility of the personnel of diagnostic centers, plant quarantines and certification programs. In addition, diagnosis of diseases may be required for epidemiological and breeding for disease resistance programs to some extent. Precision, sensitivity, rapidity, reproducibility, simplicity and cost-effectiveness of diagnostic methods may determine the quality and utility of diagnosis. The speed of providing reliable results may have high priority, while choosing the method suitable for epidemiological investigations, since large number of samples may have to be analyzed during field surveys. The conventional methods, although simple, are labor-intensive and require large greenhouse space and longer time to give the results. The conventional methods are being largely replaced by more sensitive methods involving the use of antibody-dependent immunological and nucleic acid-based techniques which can provide results rapidly.

Disease diagnostic centers have the responsibility of analyzing the samples received from the growers and communicating the results with necessary advice for follow up action by the growers. Plant Pathology departments attached to the Universities/Research stations are entrusted with this function of offering advisory service to the clientale group. Plant quarantines, both domestic and international, have the primary to responsibility of preventing the introduction of new pathogens/pests

into the country by enforcing specific regulations for the import of plants and plant materials. They are able to employ serodiagnostic methods more frequently compared to nucleic acid-based techniques. Certification personnel have the responsibility of certifying seeds and other propagative plant materials for freedom from all microbial plant pathogens. It may suffice to employ generic diagnostic methods for pathogen detection for plant quarantine and certification personnel, whereas the epidemiologist may need more specific and sensitive diagnostic methods that can identify the fungal pathogens up to subspecies/varieties of fungal species causing epidemics in one area. Such specific information may be needed to verify whether the same pathogenic strain already exists in other areas of the country and to take up necessary measures to restrict the spread of the disease to other parts of the country. The higher level of sensitivity and specificity of detection methods will be useful to assess the distribution of different strains of a pathogen species with varying levels of pathogenic potential (virulence). In addition, evolution of new races/biotypes of indigenous pathogens has to be monitored by epidemiologist by employing discriminative methods. Information on the population structure of the pathogen gathered will be useful for developing disease forewarning system to alert the farmers on the possible occurrence of a more aggressive pathogen strain, if necessary (Narayanasamy 2002, 2008). Development of resistance to fungicides in fungal pathogens has been observed in several countries. It is essential to rapidly detect, differentiate and identify the fungicide resistant isolate(s) to apply effective strategies for the management of the diseases that may be induced by these fungal variants as in Botrytis cinerea (Ishii et al. 2009).

5.2.1 Disease Diagnostic Centers

Accurate and reliable identification and differentiation of fungal pathogens and their strains with different pathogenic potential is the basic requirement of the development of effective disease management systems. Plant disease diagnostic centers (DDCs) are expected to provide organized, systematic and professional service for rapid detection and precise identification of plant pathogens as the primary cause(s) of the disease problems observed in the region where DDC is located. Any disease management system will be ineffective, if it is not based on the proper diagnosis of the disease to be controlled. Hence, DDCs have the responsibility of making accurate identification of the disease problems and suggesting suitable changes in the disease management strategies with the aim of reducing both qualitative and quantitative losses that have to be otherwise incurred by the cultivator. Stem rot disease of oilseed rape caused by *Sclerotinia sclerotiorum* has the potential to cause serious losses. The ascospores released from germinating sclerotia can infect only rape flowers and petals which support initial development of the pathogen. The fungus then invades the leaves and stems from the fallen petals. Hence, early detection of *S. sclerotiorum* on the petals is important for the growers to make timely decision on the fungicidal application to avoid the practice of fungicidal

treatment during blooming, irrespective of the magnitude of disease risk. The real-time PCR assay was demonstrated to be very efficient in diagnosing early infection of petals by *S. sclerotiorum*. This protocol could help growers avoid needless fungicide application, saving the cost of fungicides directly and possible development of fungicide resistance in the pathogen indirectly (Yin et al. 2009). The credibility of the DDCs depends on the availability of adequate facilities to analyze large number of samples within short time and to offer corrective measures without any loss of time, based on the results obtained.

Adoption of molecular techniques for the detection of pathogens and diagnosis of diseases caused by them, will enhance the sensitivity and reliability of results and validate the false positive or negative results. Among the immunodiagnostic methods, enzyme-linked immunosorbent assay (ELISA) formats have been extensively employed to obtain results with greater reliability and reduction in time required for analysis compared with isolation-based methods. Polymerase chain reaction (PCR)-based techniques have been demonstrated to provide improved detection and differentiation of the fungal pathogen isolates which exhibit differences in their virulence and other characteristics. The results from PCR-based techniques are more precise and can be obtained much earlier compared to the immunodiagnostic tests. Many DDCs in North America and Europe are capable of applying highly technical diagnostic procedures. Access to computers for maintenance and retrieval of information has significantly enhanced the quality of diagnostic services offered by the DDCs. On the other hand, disease diagnostic centers in the developing countries, on the other hand, have to be upgraded and they should be established in as many locations as possible to cater to the needs of more areas, so as to bring larger number of farmers under the protective umbrella. Thus the aim of establishing the DDCs to record the extent of disease incidence, diagnose the disease problems accurately and to offer effective suggestions to keep the pathogens at bay, can be satisfactorily realized.

The DDCs have also been involved in field surveys on regional or national basis to assess the distribution of crop diseases and monitor the 'pathogen path' to indicate the vulnerability of adjacent states/regions for possible attack by the disease(s). During field surveys, recognition of the latent infection by fungal pathogens may be difficult to recognize, unless sensitive diagnostic methods are employed. The failure to diagnose infections may facilitate the infected plants to become sources of inoculum for the newly planted crops. Stripe rust of wheat due to *Puccinia striiformis* f. sp. *tritici* (*Pst*) has worldwide distribution. A rapid and reliable diagnosis of the pathogen in latently infected wheat leaves during overwintering of the fungus in the dormant stage may avoid this latent inoculum to contribute to the initial inoculum potential and this assessment will be useful to predict early outbreak of the rust disease and improve effectiveness of the management of the stripe rust disease. A PCR-based method was applied to diagnose infection by testing the extracts from *Pst*-infected wheat leaves before symptoms appeared. The stripe rust pathogen could be detected in the dormant stage by the PCR procedure in samples of wheat leaves collected during the winter season (Wang et al. 2008).

A plant biosecurity system was visualized for early detection, precise diagnosis and rapid follow up action to minimize disease spread. The National Plant Diagnostic

Network (NPDN) has been established in the USA, with specific the objective of (i) establishing a national communications system linking plant diagnostic centers; (ii) upgrading infrastructure for effective diagnosis; and (iii) providing training to facilitate rapid reporting of outbreaks of pathogens/diseases. A nationwide network aims to function as a cohesive system to rapidly detect and diagnose high consequence pathogens in the agricultural and natural ecosystems. The network ensures that all participating diagnostic facilities are alerted to possible outbreaks and are technologically equipped to rapidly detect and identify high risk pathogens. NPDN has established an effective communication network and developed standardized diagnostic and reporting protocols. Cataloguing of disease and pest occurrence has been taken up to widen the national database (Stack et al. 2006). The concept of establishing similar national network with suitable modifications may be considered by other countries for exchange of information on the occurrence of new pathogens in the respective countries.

5.2.2 Plant Quarantines

Exclusion of plant pathogens by adopting different approaches is one of the principles of crop disease management. Plant quarantines play a vital role in excluding plant pathogens by preventing their introduction through infected plants and plant materials. Domestic and international plant quarantines have been established by most countries to exercise statutory control of plant health through legislation under which appropriate actions can be taken by the country concerned to prevent introduction or spread of the notified plant pathogen(s) that are not present or insignificant in that country concerned. The principles of establishing plant quarantines recognize the sovereignty of the country which has the right to impose the conditions and phytosanitary measures to be taken by the exporting country. The need for establishing modern quarantine laboratories has been well realized by several nations, after the implementation of the General Agreement on Tariffs and Trade (GATT), since there is a spectacular increase in the movement of people and plant materials that are likely to carry and introduce the plant and human pathogens into another country where they may be absent. This situation necessitated the enforcement of sanitary and phytosanitary measures at global level.

The International Plant Protection Convention (IPPC) was established in 1991, following the acceptance of GATT by majority countries. Basic principles required for formulating standards for plant quarantine procedures in relation to the international trade have been formulated by an expert committee (FAO 1991). Accordingly, an organism is considered to be of quarantine significance (QS), if its exclusion is perceived as important enough to protect agriculture and natural vegetation of the importing country. Sixteen basic principles have been suggested by the expert committee with the expectation that judicious application of these principles might lead to reduction or elimination of the use of unjustified phytosanitary measures as a barrier to trade. Eight regional plant protection organizations have been set up

to advise and assist member governments on the technical, administrative and legislative measures concerned with plant quarantine activities. The QS organisms have been divided into two groups designated List A and List B organisms. List A includes dangerous organisms and List B encloses organisms of importance for individual countries. List A is subdivided into List A1 containing pathogens absent in all member countries (exotic pathogens) and List A2 enclosing pathogens present only in some countries in the region (Narayanasamy 2002). Some of the representative fungal pathogens included in the List A1 and List A2 by European and Mediterranean Plant Protection Organization (EPPO) are as follows: *Alternaria mali*, *Certocystis fagacerum*, *Cronatium fusiformae*, *Diaporthe vaccinii*, *Giberella circinata*, *Guignardia citricarpa*, *Gymnosporangium juniperi-virginianae*, *Melampsora yamadae*, *Mycosphaerella gibsonii*, *Ophiostoma wageneri*, *Phoma andigena*, *Phyllosticta solitaria*, *Phytophthora lateralis*, *Puccinia himerocallidis*, *Septoria lycopersici-malagutti*, *Thecophora solani* and *Tilletia indica.*

Tilletia controversa, causing wheat dwarf bunt disease is a pathogen of international quarantine importance. The pathogen has close similarity in morphological and genomic characteristics to *T. caries*. Hence, it became necessary to distinguish these two pathogens accurately. A random amplified polymorphic DNA (RAPD) primer-mediated asymmetric PCR (RM-PCR) procedure was applied to detect and differentiate *T. controversa* and *T. caries* efficiently. SYBR Green I and TaqMan probe real-time PCR assay provided a more rapid and precise detection of different strains of *T. controversa* and *T. caries*, in addition to its ability to detect *T. controversa* in asymptomatic wheat tissues efficiently (Yuan et al. 2009).

The other approach of excluding fungal pathogens is through certification of seeds and asexually propagated plant materials such as tubers, suckers, corms, setts and budwood materials. Onion seeds carry the fungal pathogens *Botrytis aclada*, *B. allii* and *B. byssoidea* which cause neck necrosis disease. Agar assay involving isolation of these pathogens requires long time to provide the results. Standard PCR assay cannot distinguish between seed lots with large populations of pathogens in a few seed versus seed lots with low amount of *Botrytis* spp. present in many seeds. These two categories of seeds may have different epidemiological significance when planted into the fields (Beever and Weeds 2004). In contrast, the real-time PCR assay can provide the results within 24 h as against 2 weeks required for agar assays. In addition, *Botrytis* spp. can be distinguished precisely and large number of samples can be processed at a time by this protocol. The real-time PCR assay may prove to be a valuable tool for disease management decisions such as whether or not to treat specific onion seed lots and identification of appropriate regions in which to plant onions seed lots based on the relative risks of *Botrytis* seed transmission associated with regional environmental conditions (Chilvers et al. 2007). In addition postharvest produce fruits and vegetables that are exported, may carry the fungal pathogens which may be in the quiescent phase. Rapid and reliable methods have to be employed to detect and identify the fungal pathogens which may induce symptoms after the clearance of the consignments from the ship (Narayanasamy 2006). The imported plants and plant materials are checked carefully by the plant quarantine personnel. Post-entry quarantines (PEQs) are involved in this exercise.

Disease-free materials are released after testing the plants and plant materials using appropriate techniques. In order to check the spread of the fungal pathogens within the country, the certification personnel inspect the seed lots and mother plants from which propagative materials are taken. Necessary tests are performed before certifying them for freedom from plant pathogens. Various kinds of detection techniques based on the biological, immunological and genomic nucleic acid characteristics have been employed for detection and differentiation of fungal pathogens present in test materials. The relative usefulness and applicability for large scale use of the detection and identification methods have been indicated in Volume 1, Chapter 2 to enable the technical personnel to choose the ones that suit their requirements.

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