

## 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 compatibility 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

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. tritici-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
<i>Alternaria yaliinficiens</i>	Potato carrot agar (PCA)	20	Roberts 2005
<i>Botrytis cinerea</i>	Potato dextrose agar (PDA)	20–22	Mirzaei et al. 2008
<i>Botryodiplodia theobromae</i>	PDA	28	Fu et al. 2007
<i>Botryosphaeria dothidea</i>	PDA broth (Difco)	25	Ma et al. 2003
<i>Cladosporium fulvum</i>	PDA	24	Yan et al. 2008
<i>Colletotrichum acutatum</i>	PDA	20	González et al. 2006; Yoshida et al. 2007
<i>Fusarium oxysporum cucumerinum</i>	PDA	22	Lievens et al. 2007
<i>F. oxysporum</i> f.sp. <i>niveum</i>	PDA/lima bean agar	25	Zhang et al. 2005
<i>F. oxysporum</i> f.sp. <i>cucurbitae</i>	Fusarium selective medium (FSM)	25–37	Mehl and Epstein 2007, 2008
<i>Monilinia fructicola</i>	PDA broth (Difco)	25	Ma et al. 2003
<i>Mycosphaerella melonis</i>	Lima bean agar/PDA	25	Zhang et al. 2005
<i>Phaeomoniella chlamydospora</i>	Malt extract agar	25; exposure to UV light for sporulation	Martin and Cobos 2007
<i>Phytophthora</i> spp.	Difco corn meal agar	25	Polashock et al. 2005
<i>Phytophthora cactorum</i> , <i>P. infestans</i> and <i>P. phaseoli</i>	PDA	17–24	Causin et al. 2005
<i>P. capsici</i>	V8 agar	24	Larkin et al. 1995
<i>P. bisheria</i>	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 sub-cultured 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 ultraviolet (NUV) region of light is a very effective method of inducing sporulation 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 sclerotia on incubation at  $8 \pm 1^\circ\text{C}$  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 (Roebroek 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 characteristics (Clear and Patrick 1992).

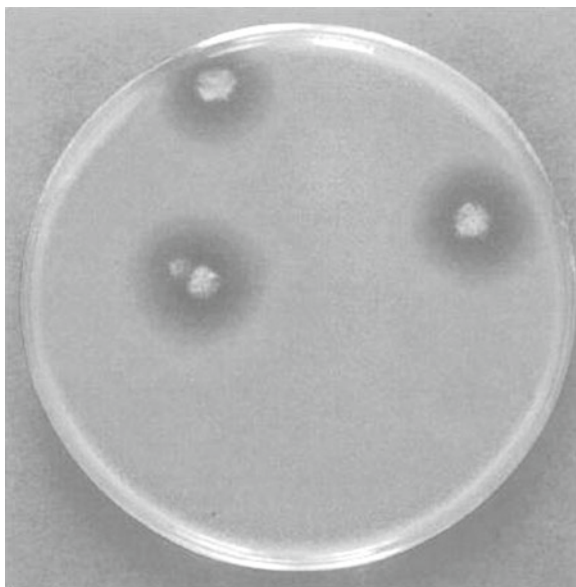
Isolation of *Gaeumannomyces graminis* var. *tritici* (Ggt) was facilitated by the use of a semi-selective and diagnostic medium (R-PDA) containing dilute PDA amended with 100 µg/ml of rifampicin and 10 µg/ml of tolclofomethyl. In addition, the presence of Ggt could be detected by its ability to alter 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 tolclofomethyl, differentially favoring the growth of Ggt (Duffy and Weller 1994) (Appendix 1).

For the isolation of *Lasidiplodia theobromae* from the woody tissues of *Pinus ellioti*, 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



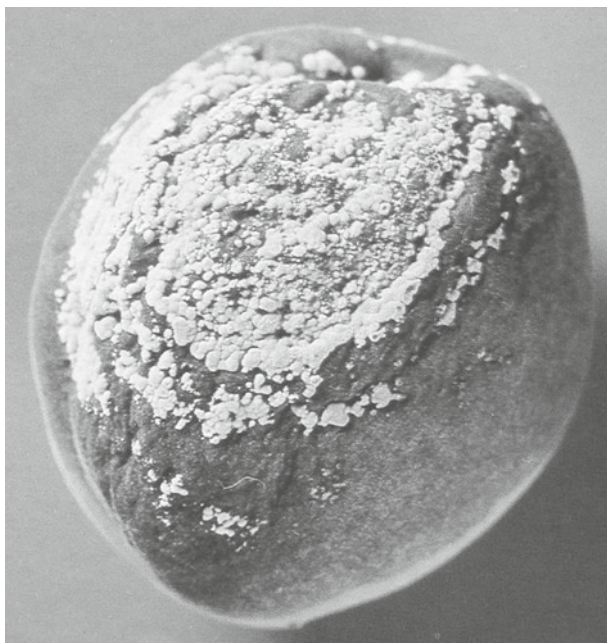
**Fig. 2.4** Symptoms of rice sheath blight disease (Courtesy of International Rice Research Institute, Manila, Philippines)

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 diseased 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/ <i>Plasmodiophora</i>
Straminipila	Oomycota	Oomycetes/Pythiales Peronosporales	Pythiaceae/ <i>Pythium</i> Peronosporaceae/ <i>Peronospora</i> <i>Peronosclerospora</i> <i>Plasmopara</i> <i>Sclerospora</i> Albuginaceae <i>Albugo</i>

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

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

(continued)

**Table 2.3** (continued)

Phylum	Class	Order	Genus
		Uredinales	<i>Cronartium</i> <i>Hemileia</i> <i>Melampsora</i> <i>Puccinia</i> <i>Uromyces</i>
		Exobasidiales	<i>Exobasidium</i>
		Ceratobasidiales	<i>Thanatephorus</i>
		Agaricales	<i>Armillaria</i>
		Aphyllphorales	<i>Ganoderma</i>

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 mutants, 19 strains of *V. dahliae* were classified into four VCGs. Later, 86 strains of *V. dahliae* isolated from several host plant species and geographical locations were 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* mutants were identified among the 126 *nit* mutants characterized. Eighty two *nit* mutants (65%) were able to utilize all nitrogen

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

In 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. arboreum*, 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 portion 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. *melons* (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 pimarin (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 pathogen 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  $\beta$ -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  $\beta$ -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<sub>4</sub> (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-β-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-β-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
<i>Erysiphe graminis</i> f.sp. <i>hordei</i>	0	Newton et al. 1985
<i>E. graminis</i> f.sp. <i>secalis</i>	20	Koch and Kohler 1990
<i>E. graminis</i> f.sp. <i>tritici</i>	20	Koch and Kohler 1990
<i>Fusarium oxysporum</i>	24	Bosland and Williams 1987
<i>Peronosclerospora sorghi</i>	23	Bonde et al. 1984
<i>Phytophthora cinnamomi</i>	23	Old et al. 1984
<i>P. infestans</i>	54	Tooley et al. 1985
<i>Puccinia graminis</i> f.sp. <i>tritici</i>	0, 61	Burdon et al. 1983, 1986
<i>P. recondita</i> f.sp. <i>tritici</i>	9	Burdon et al. 1986
<i>P. striiformis</i> f.sp. <i>tritici</i>	0	Newton et al. 1985
<i>Pyricularia oryzae</i>	55	Leung and Williams 1986
<i>Rhizoctonia solani</i>	38	Liu et al. 1990
<i>Rhynchosporium secalis</i>	38	Newton 1991
<i>Stagonospora nodorum</i>	11	Newton 1991
<i>Tilletia indica</i>	44, 52	Bonde et al. 1985, 1989
<i>Uromyces appendiculatus</i>	67	Lu et al. 1987
<i>Ustilago zeae</i>	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 inter-specific 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 *Peronosclerospora* 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 maublans* 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 *Pseudocercospora 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),  $\beta$ -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- $\beta$ D-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).

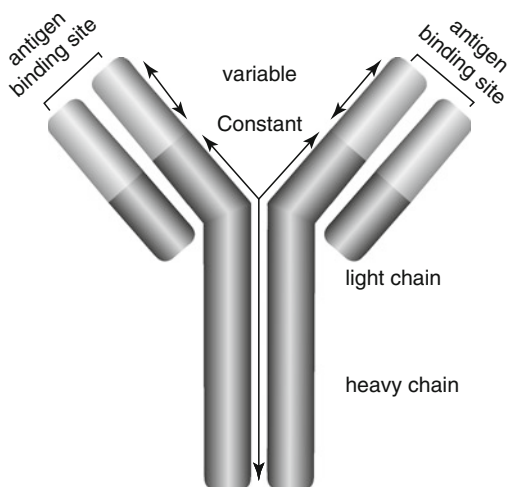
Antigenicity of the antigen is due to the existence of specific parts of the antigen known as epitopes or antigenic determinants. An epitope has a three-dimensional structure that is complementary to the binding site present in the antibody molecule. The epitopes may be of a continuous determinant type 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  $\beta$ -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 B-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 (B-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 B-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  $\beta$ -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 B-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<sup>125</sup>-labeled  $\gamma$ -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  $-20^{\circ}\text{C}$ . This procedure was applied for the detection of *Penicillium auran-tiogriseum* 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)-ELISA. 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  $\beta$ -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 exopolysaccharuronase (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 cinnamomi*. 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. cinnamomi* 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 plasmon 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  $2.2 \times 10^6$  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 *Pseudocercospora 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 *Pseudocercospora 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 eptopes 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 immunofluorescence 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  $1 \times 10^2$  to  $1 \times 10^3$  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  $1.5 \times 10^5$  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 *Plasmiodiophora brassicae*, causing club root disease of crucifers. Samples (2  $\mu$ l) 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  $1 \times 10^2$  to  $1 \times 10^3$  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 arundinacea*). 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)

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

<sup>a</sup>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 endopolysaccharuronase (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. cinnamomi*. 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. meibomia* 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 immunofluorescence 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 epitopes 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  $^{32}\text{P}$ , 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 *EcoRI*. 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 *EcoRI* 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* I 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-blot 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 digoxigenin (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 reilianum*, 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. parasitica* 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 elicitor which is involved in the development of resistance to the disease. The avirulent isolates and nontobacco isolates of *P. parasitica* produce elicitor (TE<sup>+</sup>). The TE<sup>-</sup> 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<sup>+</sup>) from other isolates (TE<sup>-</sup>), incapable of infecting tobacco (Colas et al. 1998). Identification of isolates that cannot produce elicitor 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 pepper 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 *EcoRI* restriction fragment length profiles (MGR-DNA fingerprints) from field isolates of rice blast pathogen, *Magnaporthe grisea* 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, *HaeIII* 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 (González 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* I, *EcoR* V and *Mbo* I. 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 denaturation of the target ds-DNA is followed by hybridization of a pair of synthetic oligonucleotide primers to both strands of the target DNA, one to the 5' end of sense strand and one to the 5' end of the antisense strand by an annealing step. A thermostable *Taq* DNA polymerase from *Thermus aquaticus* (*Taq*) is used for 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 compared 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 (Netherlands) and root rot of raspberry (Australia) was identified based 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 I*) 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 targeting induced local lesions in genomes (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 (approximately 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 fruticola*. 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/ $\mu$ l 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<sub>242</sub>) 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<sub>242</sub> 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 pathosystem (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 (Radisšek 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 specialized 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 *Plasmodiophora 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 uninfected 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 restriction 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 solani* 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. korrae* 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 *AluI* (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. *Phaemoniella 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 pini* (*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* (*Mycosphaerella 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  $\beta$ -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  $\beta$ -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 *Rhynchosporium 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/ $\mu$ l from *M. fijiensis*, *M. musicola* and *M. eumusae* respectively. Further, TaqMan real-time quantitative PCR assays were developed based on the  $\beta$ -tubulin gene and detected quantities of DNA as low as 1 pg/ $\mu$ l for each *Mycosphaerella* spp. from pure cultures and DNA at 1.6 pg/ $\mu$ l/mg of dry leaf tissue for *M. fijiensis* that was validated using naturally infected banana leaves (Arzanlou et al. 2007). The presence of *Mycosphaerella fijiensis*, causing banan black Sigatoka disease was detected, using PCR assay to amplify, the simple sequence repeat 203 (GTT)<sub>7</sub> 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 electrophoresis (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 reilianum*, causing head smut and *Ustilago maydis*, causing common smut disease in maize. Primer pairs SR1 and SR 3 specific for *S. reilianum* and UM11 specific for *U. maydis* (= *U. zeae*) amplified the respective pathogen sequences. *S. reilianum* 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 pathogens was realized. A random amplified polymorphic DNA (RAPD) primer-mediated asymmetric-PCR (RM-PCR) was developed to screen differential sequences 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 I*). 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 (Bindselev 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 fuliginea*), *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).

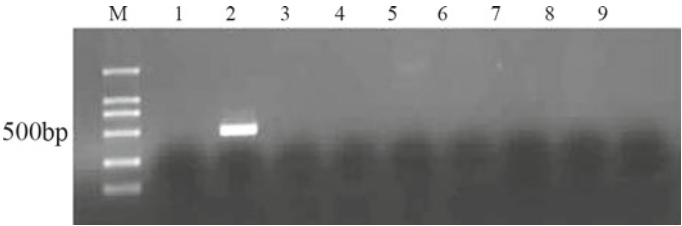
Sugar beet crops were reported to be infected by *Microsphaera betae*, later renamed *Erysiphe betae* in Europe and by *Erysiphe polygoni* in North America, creating confusion over the correct taxonomic identity of the pathogen causing powdery mildew diseases in these two well separated geographical 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
<i>Puccinia striiformis</i> f.sp. <i>tritici</i> (Pst)	Su11	+	+
Pst	Hy46	+	+
Pst	CYR-23	+	+
Pst	CYR-31	+	+
Pst	CYR-32	+	+
<i>P. graminis</i> f.sp. <i>tritici</i>	Pgt	+	—
<i>P. triticea</i>	Pt	+	—
<i>Blumeria graminis</i> f.sp. <i>tritici</i>	Bgt	+	—
<i>Fusarium graminearum</i>	Fg	+	—
<i>Rhizoctonia cerealis</i>	Rc	+	—
<i>Bipolaris sorokiniana</i>	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 almond 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)<sub>5</sub> and (GACAC)<sub>3</sub> 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 evaluated 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 anti-fluorescein 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  $\beta$ -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  $\beta$ -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  $\mu$ l of PCR solution in a 200  $\mu$ l 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 only 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 *Mycosphaerella* 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  $\beta$ -tubulin, actin and calmodulin gene regions identified *P. australiensis*, *P. krajdienii*, *P. scolymus*, *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 *Phaeoacremonium aleophilum* and *Phaeoacremonium 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  $\beta$ -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 PCR with the primer pair Psta/Psts. The second round PCR was performed using the amplified product from the first PCR as template and Nests/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 (*Humulus 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 *Verticillium 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 unaltered 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 regulatory (*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 multiplex 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 fluorogenic 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 fluorogenic 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.

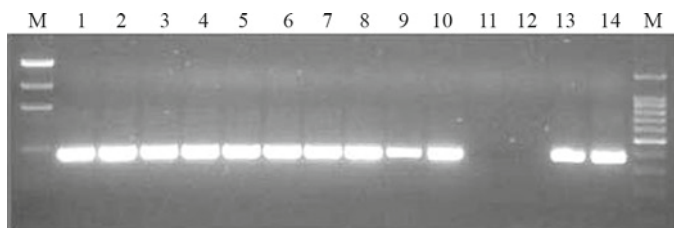
A specific Ct-value is worked out for each sample. The Ct value may be defined as that number of cycle at which a statistically significant increase in the reporter fluorescence can first be detected. The Ct-values are then used to calculate the starting copy number of the target for each sample. This 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 (Skena 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 cycles. 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. cryptogea* in naturally infected gerbera plants at 21 days after inoculation. The detection limit was  $5 \times 10^3$  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. erythroseptica* 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) fluorogenic 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,  $\beta$ -tubulin, and elicitor 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  $\beta$ -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 characteristics 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  $9.0 \times 10^{-5}$  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 euteichus*, 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  $\beta$ -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 Inoculation	Hypocotyl		Leaves	
	VL <sup>a</sup>	VD <sup>a</sup>	VL <sup>a</sup>	VD <sup>a</sup>
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)

<sup>a</sup>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 damaged 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).

*Fusicoporia 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 Scorpion-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-nineteenth 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 (Bearehell 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 ( $\beta$ -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  $\beta$ -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).

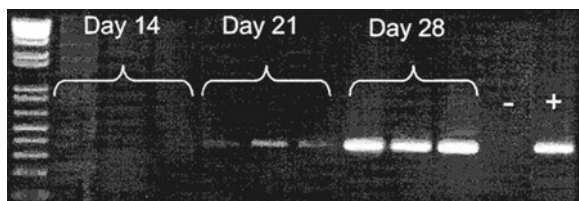
Crown rust of oat caused by *Puccinia coronata* f.sp. *avenae* accounts for serious losses. A quantitative method was developed for detecting and quantifying the



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 amplifies 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. tritici* 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 sclerotoides*, 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. sclerotoides*. A real-time PCR with greater sensitivity of detection, compared with conventional PCR assay was developed. Alfalfa isolates of *P. sclerotoides* 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).

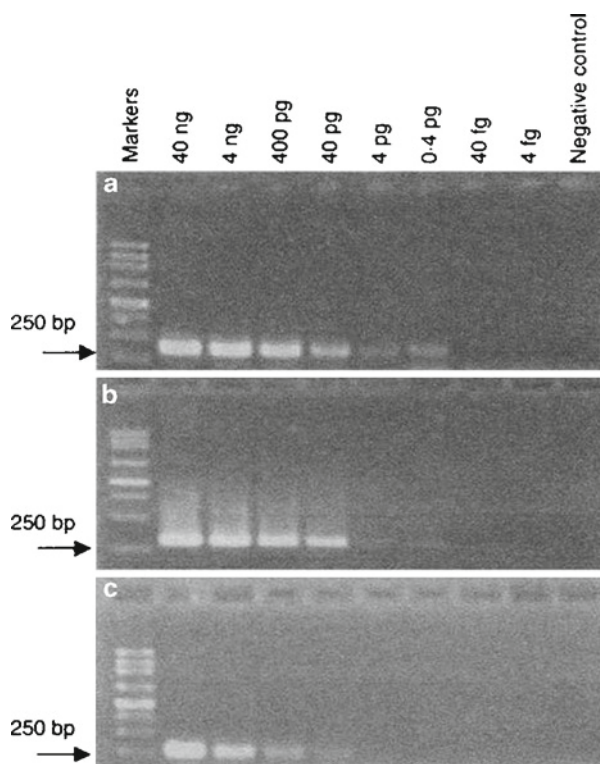


**Fig. 2.9** Detection of *Phoma tracheiphila* in artificially inoculated *Citrus aurantium* seedlings by standard PCR assay. Note the presence of the amplification product of 378-bp detectable at 28 days after inoculation (Courtesy of Demontis et al. 2008 and with kind permission of Springer Science 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  $\beta$ -tubulin. The comparative efficacy of the PCR primers designed based on sequences of  $\beta$ -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  $\beta$ -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).

Anthraxnose 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)  $\beta$ -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*  $\times$  *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).

Anthraxnose 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).

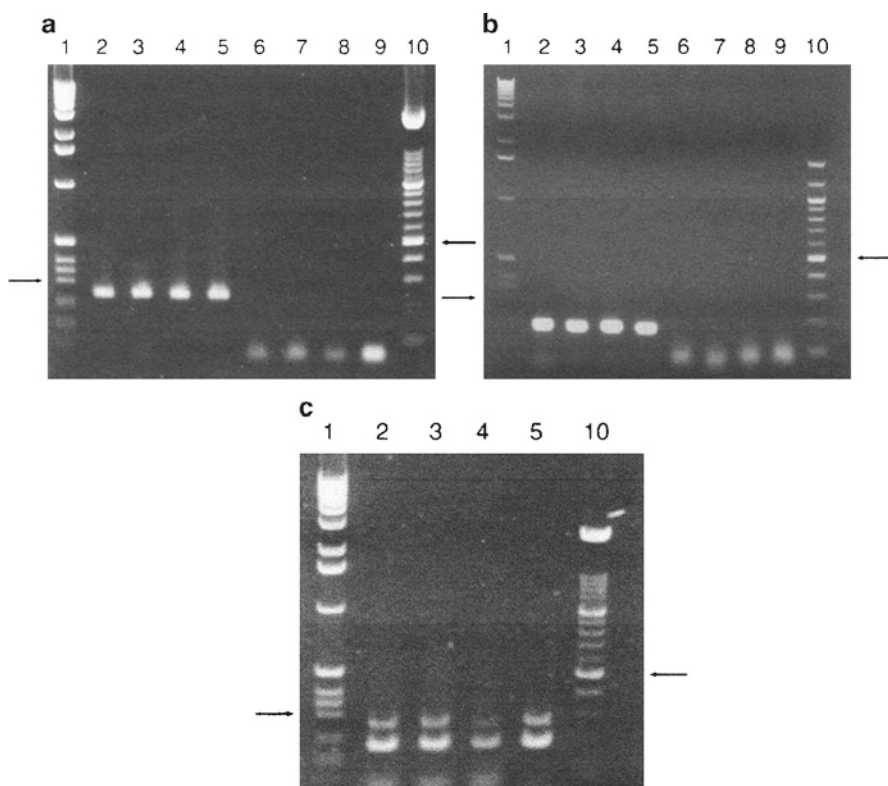
Grapevine downy mildew disease caused by a strictly biotrophic oomycete *Plasmopara viticola* has a worldwide distribution. A rapid high-throughput multiplex real-time quantitative PCR assay with TaqMan chemistry was developed. This method allows simultaneous amplification, but independent detection 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 species (Fig. 2.11). The rust fungi infecting pasture 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  $\geq 1.0\%$  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. triticultura*; 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 monitoring 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. *deltoideae* 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)

<i>P. aphanidermatum</i> <sup>a</sup>	<i>P. ultimum</i> <sup>a</sup>	<i>P. irregulare</i> <sup>a</sup>	Clade I	Clade II
55 <sup>b</sup>	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				

<sup>a</sup>Species diagnostic fingerprints  
<sup>b</sup>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 multiple 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 *Fusarium mangiferae*, *F. proliferatum*, *F. sacchari*, *F. sterilihyphosum* and *F. subglutinans*. The isolates of *Fusarium* spp. were evaluated through analyses of AFLPs and DNA sequences of the genes encoding  $\beta$ -tubulin (*tub2*) and translation elongation factor 1- $\alpha$  (*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 electrophoresis 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 speciales*-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–teleomorph 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  $\beta$ -tubulin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) genes were used to examine the relationship between *Botrytis* isolates causing apple decay. The combined G3PDH and  $\beta$ -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  $\beta$ -tubulin gene and part of the translation elongation factor 1- $\alpha$  gene (EF 1- $\alpha$ ) 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 highly 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* spot 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 complementary 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  $\beta$ -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 molecule 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 *EcoRI*, *HindIII* and *BamHI* 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  $\mu\text{m}$  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  $\mu\text{m}$  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  $55^{\circ}\text{C} \pm 5^{\circ}\text{C}$  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- $\alpha$  (TEF-1- $\alpha$ ). A consensus PCR amplification of a part of the TEF-1- $\alpha$  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, resulting 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 desthiobiotin 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/ $\mu$ l 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 Sinclair 1996; 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  $\times 2$  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  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$  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 *Leptosphaeria 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

After 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 (Roebroek 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 structures 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<sup>®</sup> 4.01 and calculations were made using MS Excel<sup>®</sup>. 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

Comparatively 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 characteristics 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<sub>1</sub> 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 comparative efficacy was assessed. By applying seed immunoblot assay (SIBA) for detection of the pathogen,



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

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

<sup>a</sup>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.

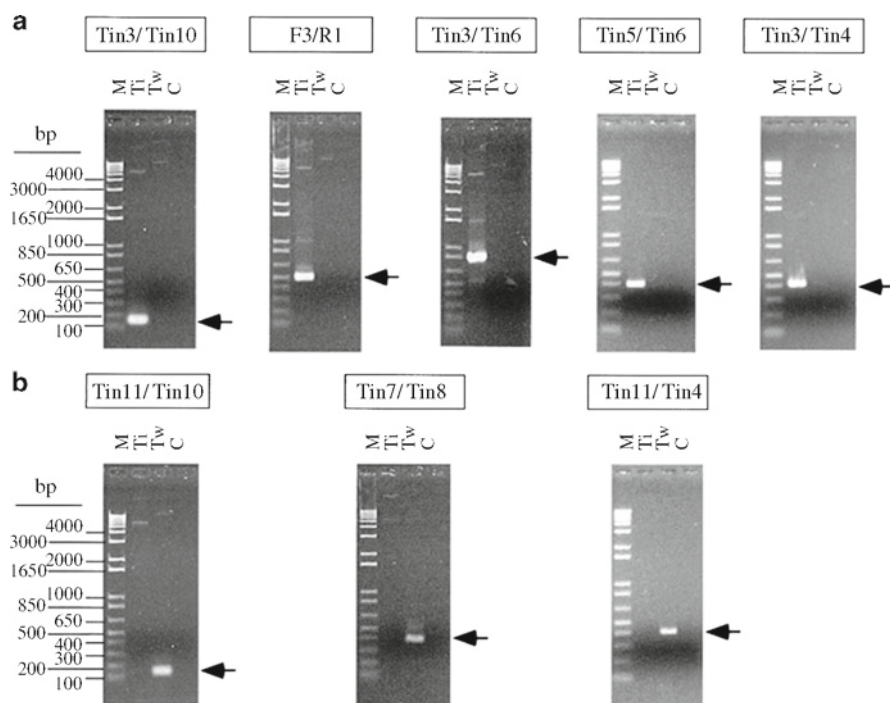
*Peronosclerospora sorghi* causing sorghum downy mildew disease was detected in seeds using dot blot hybridization. The target DNA from this pathogen was extracted by grinding up sorghum seeds. Hybridization of probes occurred only with the DNA of *P. sorghi*, but not with the DNA of any other fungi 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 detected 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  $\mu$ m pore size nylon screens. The material retained in the 20  $\mu$ m 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 fluorogenic 5' (prime) nuclease for PCR assay by employing the TaqMan® system to detect and differentiate *T. indica* and *T. walkeri* (Frederick et al. 2000).

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).

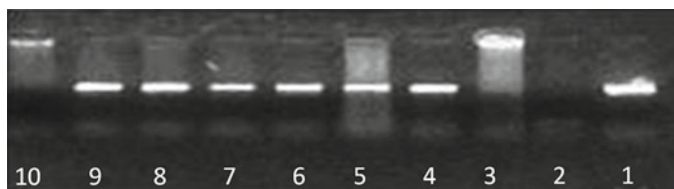
Barley scald disease pathogen *Rhynchosporium secalis* overwinters in plant debris and it can infect seeds symptomlessly or induce typical scald symptoms. Lima bean agar medium amended with Bengal rose and streptomycin was used to isolate the pathogen. A species-specific primer set based on sequence 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 incidence 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 advantage 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. graminearum* and *F. verticillioides* in field-collected barley and corn samples were accomplished by employing a fluorogenic (TaqMan®) real-time PCR assay. Primers and probes were designed from genes involved in mycotoxin biosynthesis (*TRI6* and *FUM1*). In addition, primers and probes were prepared based on the rDNA sequences of *Fusarium* to provide a genus-specific internal positive control. Barley and corn samples infected by *F. graminearum* and *F. verticillioides* tested positive for the presence of trichothecene and fumonisin (Bluhm et al. 2004). A real-time PCR assay useful for monitoring and quantifying the major *Fusarium* spp. involved in FHB complex was developed. TaqMan® primers and probes showing high specificity to *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale* var. *majus* were designed. By maintaining an internal PCR control and using proper dilutions of pure genomic DNAs of the pathogens, the DNA contents of each pathogen in leaves, ears 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/ZE. 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 toxigenic 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)

Pathogen	dPCR		qPCR	
	Chaff	Grain	Chaff	Grain
<i>Fusarium avenaceum</i>	46	29	19	4
<i>F. culmorum</i>	20	13	2	0
<i>F. graminearum</i>	43	47	18	8
<i>F. poae</i>	54	36	16	4
<i>Microdochium majus</i>	50	28	25	9
<i>M. nivale</i>	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*), sterigmatocystin-*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 <sup>a</sup>	Nebali <sup>a</sup>
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 *Alternaria* 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

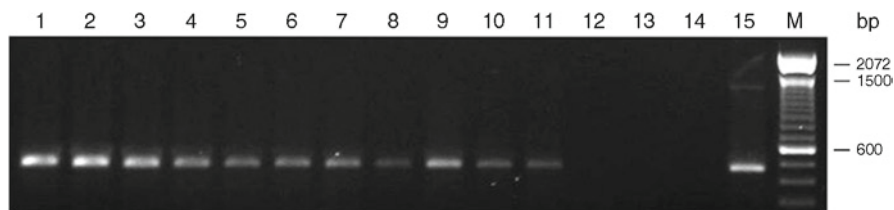
methods were similar, PCR assay offers the advantage of providing early and reliable results, making it the method of choice, when results are needed rapidly (Konstantinova et al. 2002).

*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 clustered 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. byssoides*. 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 identification 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  $2.5 \times 10^5$  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).

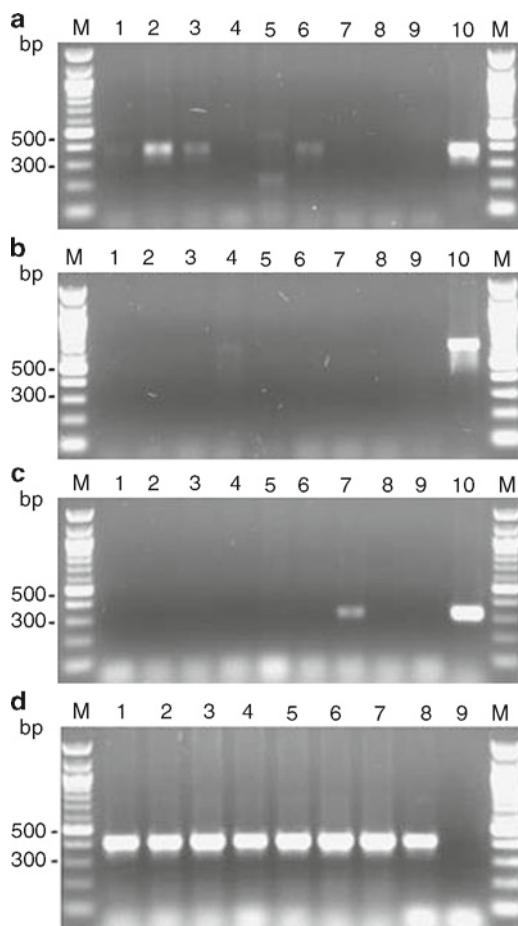
Potato wart disease pathogen *Synchytrium endobioticum* has a worldwide distribution and exists as at least 30 biotypes. The resting spores carried by the tubers may be introduced into new areas or into a new field in the same area where it can survive in the soil for 30–70 years (Hampson 1993). The 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  $\text{CsCl}_2$  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 multi-copy 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 sensitivity 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/ $\mu\text{l}$  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 during 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. chlamydospora* 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 harvest 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 Cerkaskas 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 *Botrytis* 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

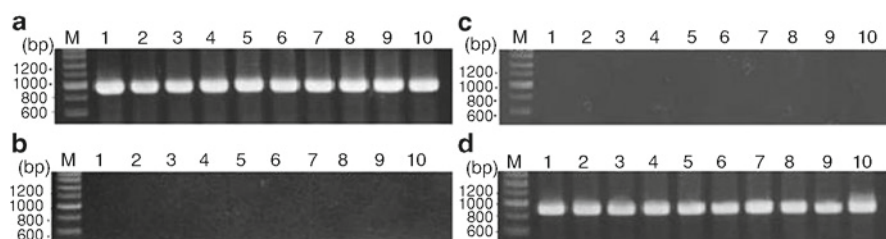
detection methods, as in the case of seeds and clonal propagative plant materials. The isolates (39) of *Colletotrichum gloeosporioides*, causing anthracnose diseases of various fruits were detected and differentiated using the probes designed from the ITS regions of rDNA of this pathogen (Mills et al. 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 fruticola* 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. fruticola* and differentiated related species of *M. fructigena* and *M. laxa*. These primers amplified this PCR product also from plum tissue infected by *M. fruticola*, but not from the other related fungal pathogens, indicating the specificity and reliability of the PCR protocol (Fulton and Brown 1997). *M. fruticola* 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. fruticola* 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. fruticola* 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. fruticola*, 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. fruticola* 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  $\beta$ -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 citrus 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 natsudaiddai 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 detected 64.3% incidence of infected pea-sized berries, but qPCR formats failed to detect 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

<b>Czapek Dox agar</b>	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
Oxoid 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
<b>Malt extract agar</b>	
White bread malt extract	20 g
Oxoid agar No.3	20 g
Tap water	1 l
<b>Oat agar</b>	
Oat meal ground	30 g
Oxoid agar No.3	20 g
Tap water	1 l
<b>Potato carrot agar</b>	
Grated potato	20 g
Grated carrot	20 g
Oxoid agar No.3	20 g
Tap water	1 l
<b>Potato dextrose agar</b>	
Potatoes	200 g
Oxoid agar No.3	20 g
Dextrose	15 g
Tap water	1 l
<b>V8 agar</b>	
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 <sub>3</sub>	0.1 g
K <sub>2</sub> HPO <sub>4</sub>	0.1 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	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 instead of Rose Bengal used in 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 <sub>2</sub> HPO <sub>4</sub>	1 g
KCl	0.5 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	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
<b><i>Fusarium</i> selective (FS) medium for <i>Fusarium circinatum</i></b> (Schweigkofler et al. 2004)	
Bacto peptone	15 g
Agar	20 g
KH <sub>2</sub> PO <sub>4</sub>	1 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 g
Streptomycin sulfate	0.3 g
Ampicillin	0.1 g
Pentachloronitrobenzene	0.2 g
PDA	39 g
Water	1 l
<b>R-PDA medium for <i>Gaeumannomyces graminis</i> var. <i>tritici</i></b> (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  $1 \times 10^4$ /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* mutants.

#### 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;  $\text{KH}_2\text{PO}_4$  – 1 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  – 0.5 g; KCl – 0.5 g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  – 10 mg; agar – 20 g; trace element solution 0.2 ml; water 1,000 ml (Trace element solution:  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  – 5.0 g;  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  – 1.0 g;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  – 0.25 g;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  – 50 mg;  $\text{H}_3\text{BO}_3$  – 50 mg;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  – 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;  $\text{NaNO}_3$  – 2 g

D. Minimal agar medium with chlorate (MMC): Minimal medium 1 l; L-asparagine 1.6 g;  $\text{NaNO}_3$  – 2 g;  $\text{KClO}_3$  – 15 g

E. Potato dextrose agar medium with chlorate (PDC): Potato dextrose broth (Difco) – 24 g; agar – 20 g;  $\text{KClO}_3$  – 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  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0.2 g  $\text{KH}_2\text{PO}_4$ , 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  $1 \times 10^8$ :  $5 \times 10^7$  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  $\mu$ l/well.
- (vi) Add 110  $\mu$ l 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  $\mu$ l of the medium and replacing with 100  $\mu$ l 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  $\text{Na}_2\text{HPO}_4$ , 0.25 g  $\text{KH}_2\text{PO}_4$ , 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  $\mu$ l/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<sub>2</sub>SO<sub>4</sub> (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), Triton 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 bicinchoninic 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<sub>2</sub> 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*-nitrophenyl 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; overlay 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 ( $6 \times 10^7$  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  $5.4 \times 10^7$  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  $-20^{\circ}\text{C}$ ; homogenize the samples separately in distilled water for 5 min; filter as done earlier (step A i above) and adjust the spore concentration to  $1 \times 10^6$  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  $\mu\text{l}$  of each sample to two wells of microtiter plates and incubate overnight at  $4^{\circ}\text{C}$ .
- (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  $\mu\text{g}/\text{ml}$  of anti-resting spore IgG; incubate at  $37^{\circ}\text{C}$  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  $37^{\circ}\text{C}$  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  $37^{\circ}\text{C}$  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  $\mu\text{l}$  onto a 40  $\text{cm}^2$  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  $\mu\text{g}/\text{ml}$  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  $\text{MgCl}_2$ , 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  $\mu\text{m}$ , 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  $\beta$ -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  $\mu$ m 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 immunoprecipitate 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<sup>6</sup> 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  $\mu$ l 2  $\times$  CTAB buffer (2  $\times$  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  $\mu$ l TE buffer (TE = 10 mM Tris, pH 7.6; 1 mM EDTA).
- (iv) Denature DNA at 95°C for 4 min; transfer 20  $\mu$ l 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  $\beta$ -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 isoamylalcohol (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<sup>2</sup> 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; resuspend 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 magnetic 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/microcentrifuge 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 pimarinin, 100 ppm ampicillin, 25 ppm rifampicin and 25 ppm pentachloronitrobenzene (PCNB) into each of the 24-deepwell (DW) Uniplate microtiter 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  $-80^{\circ}\text{C}$  for at least 1 h.
- (iv) Lyophilize the samples for a period of 48 h; use the Labconco stoppering tray drying systems (STDS) (Labconco Corp., USA) with incubation chamber at  $0^{\circ}\text{C}$  for 24 h, followed by 24 h with incubation at  $23^{\circ}\text{C}$ .
- (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  $180^{\circ}$ , after bashing 1 min and bash again for an additional minute.

#### B. Extraction of DNA (Adaptation 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  $\mu\text{l}$  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  $65^{\circ}\text{C}$  chamber for 20 min.
- (iv) Centrifuge the plates at 4,600 g for 2 min; gently remove the capmat; add 150  $\mu\text{l}$  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  $-20^{\circ}\text{C}$  for 30 min to overnight and centrifuge the plates at 4,600 g for 30 min.
- (vi) Transfer 400  $\mu\text{l}$  of the supernatant to a new 2 ml DW plate containing 600  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  of 95% ethanol; centrifuge at 4,600 g for 5 min and incubate the spin column plate at  $65^{\circ}\text{C}$  for 5 min to dry the membrane.
- (x) Add 200  $\mu\text{l}$  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%  $\beta$ -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/iso-amyl 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/iso-amyl 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  $\mu$ g); 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/ $\mu$ l.

### 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  $\mu$ l) (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  $\mu$ l 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  $\mu$ l reaction mixture with DNA Taq polymerase (Promega, Madison, USA) and purify genomic DNA from pathogen isolates.
- (iii) Perform PCR for plant samples in a 20  $\mu$ l 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  $\mu$ g/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  $\mu$ l 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  $\mu$ l of phenol/chloroform; centrifuge at 12,000  $\times$  g for 10 min; separate the upper phase; mix with 500  $\mu$ l of chloroform and centrifuge at 12,000  $\times$  g for 4 min.
- (iv) Transfer the aqueous phase into a new 1.5 ml Eppendorf tube; add 50  $\mu$ l of 3 M sodium acetate and 500  $\mu$ l of isopropanol and centrifuge at 12,000  $\times$  g for 20 min.
- (v) Wash the DNA pellet with 500  $\mu$ l of 70% ethanol and centrifuge at 12,000  $\times$  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  $\mu$ g /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  $\mu$ M primers, 1 U *Taq* polymerase (BioBasic), 1  $\times$  PCR buffer with 1.5 mM  $MgCl_2$  and 10  $\mu$ g of template DNA.
- (iii) Add sterile distilled water to have a final volume of 25  $\mu$ l.
- (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  $\mu$ l) by gel electrophoresis on a horizontal 2% agarose gel and stain the bands with ethidium bromide (0.5  $\mu$ g/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 Tris-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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 Tween-20) 25 mM MgCl<sub>2</sub>, 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)<sub>5</sub> (5'-CAGCAGCAGCAGCAG-3') or (GACAC)<sub>3</sub> (5'-GACACGACACGACAC-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)<sub>5</sub>) or 48°C (for (GACAC)<sub>3</sub>) 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<sup>2</sup>–10<sup>5</sup> 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<sub>280</sub> nm and A<sub>260</sub> 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<sup>2+</sup>, 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  $\mu$ l containing 13.4  $\mu$ l sterile dd water, 25  $\mu$ l TaqMan Universal master mix (Applied Biosystems), 0.3  $\mu$ l of each forward and reverse primer (300 nM), 10  $\mu$ l TaqMan probe (200 nM) and 10  $\mu$ l 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  $\mu$ l of lysate (supernatant) to fresh 2-ml tubes containing 200  $\mu$ l chloroform; mix by vortexing and centrifuge for 5 min at 13,000 g.
- (iii) Transfer 500  $\mu$ l of aqueous layer to clean 2-ml tubes containing 500  $\mu$ l isopropanol and 50  $\mu$ l 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  $\mu$ l 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  $-20^{\circ}\text{C}$ , 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  $\mu$ l SYBR® Green Mix, 0.375  $\mu$ l ROX, passive reference (diluted 1:50), 300 nM primers and 10  $\mu$ l diluted DNA extract made up to 25  $\mu$ l using molecular grade water; carry out SYBR® green assays in duplicate with generic cycling conditions:  $95^{\circ}\text{C}$  for 10 min and 40 cycles of  $60^{\circ}\text{C}$  for 1 min and  $95^{\circ}\text{C}$  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 System (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  $\mu\text{l}^{-1}$  AmpliTaq Gold, 0.2 mM dNTPs and 5.5 mM  $\text{MgCl}_2$ .
  - (v) Use all sets of primers at 300 nM and probes at 100 nM; add 1  $\mu\text{l}$  DNA extract, giving a final volume of 25  $\mu\text{l}$ /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  $\mu\text{l}$  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  $\mu\text{l}$  volumes with 10–15 ng of template DNA, 100  $\mu\text{M}$  of each dNTP, 2.5  $\mu\text{l}$  of 10 × buffer (200 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton® × 100), 2 mM  $\text{Mg Cl}_2$ , 0.2  $\mu\text{M}$  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  $\beta$ -D-1-thiogalactopyranoside) and X-Gal (5-Bromo-4-chloro-3-indolyl  $\beta$ -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  $\times$  g for 10 min and at 130,000  $\times$  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 Freund'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 biotinylation of IgG, dialyze 1 ml of purified IgG overnight at 7°C against coupling buffer (containing 10 g NaCl, 10 g NaHCO<sub>3</sub> 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 disinfested 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 β-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  $12,000 \times g$  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  $5 \times 10 \times 2$  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 MgCl<sub>2</sub>, 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 *ScrF* 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<sub>2</sub>), 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- $\text{Na}_2$ , 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  $\mu\text{l}$  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  $\mu\text{l}$  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 and 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  $\mu\text{l}$  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  $\times$  PCR buffer (10 $\times$ : 500 mM KCl, 15 mM  $\text{MgCl}_2$ , 100 mM Tris-HCl, pH 9.0 at 25°C) and 1 Triton X-100, 1  $\mu\text{l}$  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<sub>2</sub>.
- (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).

## References

- Abad ZG, Abad JA, Coffey MD, Oudemans PV, Man in't Veld WA, de Gruyter H, Cunningham J, Louws FJ (2008) *Phytophthora bischeria* sp. nov., a new species identified in isolates from the Rosaceous raspberry, rose and strawberry in three continents. *Mycologia* 100: 99–110.
- Abdullah I, Koerber M, Stachewicz H, Winter S (2005) The 18S rDNA of *Synchytrium endobioticum* and its utility in microarrays for simultaneous detection of fungal and viral pathogens. *Appl Microbiol Biotechnol* 68: 368–375.
- Abe H, Baba T, Takukuwa T (1969) Serological reaction of root rot pathogen (*Rhizoctonia solani*) of sugar beets. *Ann Phytopathol Soc Jpn* 35: 374.
- Adair S, Kim S, Breuil C (2002) A molecular approach for early monitoring of decay basidiomycetes in wood chips. *FEMS Microbiol Lett* 211: 117–122.
- Adams GC Jr., Butler EE (1979) Serological relationships among anastomosis groups of *Rhizoctonia solani*. *Phytopathology* 69: 629–633.
- Afouda L, Wolf G, Wydra K (2009) Development of a sensitive serological method for specific detection of latent infection of *Macrophomina phaseolina* in cowpea. *J Phytopathol* 157: 15–23.
- Agarwal VK, Sinclair TB (1996) *Principles of Seed Pathology*, 2<sup>nd</sup> edition, CRC Press, Boca Raton, FL, USA.
- Aggarwal A, Sharma D, Anuradha, Prakash V, Mehrotra RS (2001) Electrophoresis pattern of mycelial protein – a tool for differentiation of *Phytophthora* species from *Chukrasia tabularis*. *Ind Phytopathol* 54: 424–428.
- Agrios GN (2005) *Plant Pathology*, 5th edition, Elsevier-Academic Press, Amsterdam.
- Ahmed KM, Ravinder Reddy Ch (1993) A pictorial guide to the identification of seedborne fungi from sorghum, pearl millet, finger millet, chickpea, pigeonpea and groundnut. Information Bull No. 34, Internat Crops Res Inst for Semi-Arid Tropics (ICRISAT), Patancheru, India.
- Alaei H, Baeyen S, Maes M, Höfte M, Heungens K (2009) Molecular detection of *Puccinia horiana* in *Chrysanthemum × morifolium* through conventional and real-time PCR. *J Microbiol Meth* 76: 136–145.
- Al-Samarrai TH, Schmid J (2000) A simple method for extraction of fungal genomic DNA. *Appl Microbiol Lett* 30: 53–56.
- Andrade O, Muñoz G, Galdames R, Durán P, Honorato R (2004) Characterization, *in vitro* culture and molecular analysis of *Thecaphora solani*, the causal agent of potato smut. *Phytopathology* 94: 875–882.

- Anil Kumar, Singh A, Garg GK (1998) Development of seed immunoblot binding assay for the detection of Karnal bunt (*Tilletia indica*) of wheat. *J Plant Biochem Biotechnol* 7: 119–120.
- Appel R, Alder N, Habermeyer J (2001) A method for the artificial inoculation of potato tubers with *Phytophthora infestans* and polymerase chain reaction of latently infected sprouts and stems. *J Phytopathol* 149: 297.
- Arie T, Hayashi Y, Yoneyama K, Nagatani A, Furuya M, Yamaguchi I (1995) Detection of *Fusarium* spp. in plants with monoclonal antibody. *Ann Phytopathol Soc Jpn* 61: 311–317.
- Arie T, Gouthu S, Shimagaki S, Kamakura J, Kimura M, Inoue M, Takio K, Ozaki A, Yoneyama K, Yamaguchi I (1998) Immunological detection of endopolygalacturonase secretion by *Fusarium oxysporum* in plant tissue and sequencing of its encoding gene. *Ann Phytopathol Soc Jpn* 64: 7–15.
- Armengol J, Vicent A, Torné CL, García-Figueres G, García-Jiménez T (2001) Fungi associated with esca and grapevine declines in Spain: a three-year survey. *Phytopathol Mediterr* 40: 325–329.
- Arco A, Raposo R (2007) PCR-based strategy to detect and identify species of *Phaeoacremonium* causing grapevine disease. *Appl Environ Microbiol* 73: 2911–2918.
- Arzanlou M, Abeln ECA, Kema GHJ, Waalwijk C, Carlier J, de Vries I, Guzman M, Crous P (2007) Molecular diagnostics for the Sigatoka disease complex of Banana. *Phytopathology* 97: 1112–1118.
- Atassi MZ, Lee C (1978) The precise and entire antigenic structure of native lysozyme. *Biochem J* 171: 429–434.
- Attallah ZK, Stevenson WR (2006) A methodology to detect and quantify five pathogens causing decay using real-time quantitative polymerase chain reaction. *Phytopathology* 96: 1037–1045.
- Attallah ZK, Bae J, Jansky SH, Rouse DI, Stevenson WR (2007) Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. *Phytopathology* 97: 865–872.
- Babadoost M, Chen W, Bratsch AD, Eastman CE (2004) *Verticillium longisporum* and *Fusarium solani*: two new species in the complex of internal discoloration of horseradish. *Plant Pathol* 53: 669–676.
- Balesdent MH, Jednyczka M, Jain L, Mendes-Pereira E, Betrandy J, Rouxnel (1998) Conidia as a substrate for internal transcribed spacer based PCR identification of components of *Leptosphaeria maculans*-species complex. *Phytopathology* 88: 1210–1217.
- Banks JN, Cox SJ (1992) The solid phase attachment of fungal hyphae in an ELISA to screen for antifungal antibodies. *Mycopathologia* 120: 79–85.
- Banks JN, Cox SJ, Clarke JH, Shamsi RH, Northway BJ (1992) Towards the immunological detection of field and storage fungi. In: Samson RA, Hocking AD, Ritt JJ, King AD (ed), *Modern Methods in Food Mycology*, Elsevier, Holland, pp. 247–252.
- Barnes CW, Szabo LJ (2007) Detection and identification of four common rust pathogens of cereals and grasses using real-time polymerase chain reaction. *Phytopathology* 97: 717–727.
- Barnes CW, Szabo LJ (2008) A rapid method for detecting and quantifying bacterial DNA in rust fungal DNA samples. *Phytopathology* 98: 115–119.
- Bary T, Collieran G, Glennon M, Duncan LK, Gannon F (1991) The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Meth Appl* 1: 51–56.
- Bates JA, Taylor EJA (2001) Scorpion ARMS primers for SNP real-time PCR detection and quantification of *Pyrenophora teres*. *Mol Plant Pathol* 2: 275–280.
- Baysal-Gurel F, Lewis Ivey ML, Dorrance A, Frederick R, Czarnecki J, Boeh M, Miller SA (2008) An immunofluorescence assay to detect urediniospores of *Phakopsora pachyrhizi*. *Plant Dis* 92: 1387–1393.
- Bearehell SJ, Fraaije BA, Shaw MM, Fitt BD (2005) Wheat archive links long-term fungal pathogen population dynamics to air population. *Proc. Natl Acad Sci USA* 102: 5438–5442.
- Beck JJ, Ligon JM (1995) Polymerase chain reaction assays for the detection of *Stagonospora nodorum* and *Septoria tritici* in wheat. *Phytopathology* 85: 319–324.
- Beck JJ, Ligon JM, Etienne L, Binder A (1996) Detection of crop fungal pathogens by polymerase chain reaction technology. *BCPC Symp Proc No. 65, Diagnostics in Crop Production*, pp. 111–118.

- Beever RE, Parkes SL (2003) Use of nitrate non-utilizing (Nit) mutants to determine vegetative compatibility in *Botryotinia fuckeliana* (*Botrytis cinerea*). *Eur J Plant Pathol* 109: 607–613.
- Beever RE, Weeds PL (2004) Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. In: Elad Y, Williamson P, Tudzinski P, Delen N (ed) *Botrytis: Biology, Pathology and Control*, Kluwer Academic, Dordrecht, pp. 29–52.
- Bell KS, Roberts J, Verrall S, Cullen DW, Williams NA, Harrison JG, Toth IK, Cooke DEL, Duncan JM, Claxton JR (1999) Detection and quantification of *Spongospora subterranea* f.sp. *subterranea* in soils and on tubers using specific primers. *Eur J Plant Pathol* 105: 905–915.
- Bellaire L de, Chillet M, Mourichon S (2000) Elaboration of an early quantification of quiescent infections of *Colletotrichum musae* on bananas. *Plant Dis* 84: 128–133.
- Benson DM (1991) Detection of *Phytophthora cinnamomi* in azalea with commercial serological assay kits. *Plant Dis* 75: 478–482.
- Benson DM (1992) Detection by enzyme-linked immunosorbent assay of *Rhizoctonia* species in poinsettia cuttings. *Plant Dis* 76: 578–581.
- Bermingham S, Dewey FM, Fisher PJ, Maltby L (2001) Use of a monoclonal antibody immunoassay for the detection and quantification of *Helicium lugdunensis* colonizing alder leaves and roots. *Microbial Ecol* 42: 506–512.
- Bilodeau GJ, Lévesque CA, de Cock AWAM, Duchaine C, Brière S, Uribe P, Martin FM, Hamelin RC (2007) Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR and molecular beacons. *Phytopathology* 97: 632–642.
- Bindslev L, Oliver RP, Johansen B (2002) *In situ* PCR for detection and identification of fungal species. *Mycol Res* 106: 277–279.
- Bluhm BH, Cousin MA, Woloshuk CP (2004) Multiplex real-time PCR detection of fumonisin-producing and trichothecene-producing groups of *Fusarium* species. *J Food Protect* 67: 536–543.
- Böhm J, Hahn A, Schubert R, Bahnweg G, Adler N, Nechwatal J, Oehmann R, Oßwald W (1999) Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. *J Phytopathol* 147: 404–416.
- Bolwerk A, Lagopodi A, Lugtenberg BJJ, Bolemborg GV (2005) Visualization of interactions between a pathogenic and beneficial *Fusarium* strain during biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* 18: 710–721.
- Bom M, Boland GJ (2000) Evaluation of polyclonal antibody-based immunoassays for detection of *Sclerotinia sclerotiorum* on canola petals and prediction of stem rot. *Canad J Microbiol* 46: 723–729.
- Bonants PJM, van Gent-Pelzer PEM, Hagenaarde Weerdt M (2000) Characterization and detection of *Phytophthora fragariae* in plant, water and soil by molecular methods. *Bull OEPP* 30: 525–531.
- Bonants PJM, van Gent-Pelzer PEM, Hooftman R, Cooke DEL, Guy DC, Duncan JM (2004) A combination of baiting and different PCR formats, including measurement of real-time quantitative fluorescence for the detection of *Phytophthora fragariae* in strawberry plants. *Eur J Plant Pathol* 110: 698–702.
- Bonde MR, Peterson GL, Dowler WM, May B (1984) Isozyme analysis to differentiate species of *Peronosclerospora* causing downy mildews for maize. *Phytopathology* 74: 1278.
- Bonde MR, Peterson GL, Dowler WM, May B (1985) Comparison of *Tilletia indica* isolates from India and Mexico by isozyme analysis. *Phytopathology* 75: 1309.
- Bonde MR, Peterson GL, Matsumoto TT (1989) The use of isozymes to identify teliospores of *Tilletia indica*. *Phytopathology* 79: 596–599.
- Bonde MR, Micales JA, Peterson GL (1993) The use of isozyme analysis for identification of plant pathogenic fungi. *Plant Dis* 77: 961–968.
- Børja I, Solheim H, Hietala AM, Fossdal CG (2006) Etiology and real-time polymerase chain reaction-based detection of *Gremmeniella*- and *Phomopsis*-associated disease in Norway spruce seedlings. *Phytopathology* 96: 1305–1314.

- Börjesson T, Johnson L (1998) Detection of common bunt (*Tilletia caries*) infestation in wheat with an electronic nose and human panel. *J Plant Dis Protect* 105: 306–313.
- Bosland PW, Williams PH (1987) An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism vegetative compatibility and geographic origin. *Canad J Bot* 65: 2067–2073.
- Bossi R, Dewey FM (1992) Development of a monoclonal antibody-based immunodetection assay for *Botrytis cinerea*. *Plant Pathol* 41: 472–482.
- Bounou S, Jabaji-Hare SH, Hogue R, Charest PM (1999) Polymerase chain reaction-based assay for specific detection of *Rhizoctonia solani* AG-3 isolates. *Mycol Res* 103: 1–8.
- Bouterige S, Robert R, Bouchara JP, Marot-Leblond A, Molinero V, Senet JM (2000) Production and characterization of two monoclonal antibodies specific for *Plasmopara halstedii*. *Appl Environ Microbiol* 66: 3277–3282.
- Boyle B, Hamelin RC, Séguin A (2005) *In vivo* monitoring of obligate biotrophic pathogen growth by kinetic-PCR. *Appl Environ Microbiol* 71: 1546–1552.
- Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
- Braun H, Levivier S, Eber F, Renard M, Chevre AM (1997) Electrophoretic analysis of natural populations of *Leptosphaeria maculans* directly from leaf lesions. *Plant Pathol* 46: 147–154.
- Brill LM, McClary RD, Sinclair JD (1994) Analysis of two ELISA formats and antigen preparations using polyclonal antibodies to *Phomopsis longicolla*. *Phytopathology* 84: 173–179.
- Brown AE, Muthumeenakshi S, Sreenivasaprasad S, Mills RR, Swinburne TR (1993) A PCR primer specific to *Cylindrocarpon heteronema* for detection of the pathogen in apple wood. *FEMS Microbiol Lett* 108: 117–120.
- Bruns TD, White JJ, Taylor JW (1991) Fungal molecular systematics. *Ann Rev Ecol Syst* 22: 525–564.
- Bulman SR, Marshall JW (1988) Detection of *Spongospora subterranea* in potato tuber lesions using the polymerase chain reaction (PCR). *Plant Pathol* 47: 759–766.
- Burdon JJ, Luig NH, Marshall DR (1983) Isozyme uniformity and virulence variation in *Puccinia graminis* f.sp. *tritici* in Australia. *Austr J Biol Sci* 36: 403.
- Burdon JJ, Roelfs AP, Brown AHD (1986) The genetic basis of isozyme variation in the wheat stem rust fungus (*Puccinia graminis tritici*). *Canad J Genet Cytol* 28: 171.
- Bussaban B, Lumyong S, Lumyong P, Seelanan T, Park DC, McKenzie EHC, Hyde KD (2005) Molecular and morphological characterization of *Pyricularia* and allied genera. *Mycologia* 97: 1002–1011.
- Cadle-Davidson L (2008) Monitoring pathogenesis of natural *Botrytis cinerea* infections in developing grape berries. *Amer J Enol Vitic* 59: 367–395.
- Cahill DM, Hardham AR (1994) A dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* in soils. *Phytopathology* 84: 1284–1292.
- Caiazzo R, Tarantino P, Porrone G, Lahoz E (2006) Detection and early diagnosis of *Peronospora tabacina* Adam in tobacco plant with systemic infection. *J Phytopathol* 154: 432–435.
- Call DR (2005) Challenges and opportunities for pathogen detection using DNA microarrays. *Critical Rev Microbiol* 31: 91–99.
- Camele I, Marcone C, Cristinzio G (2005) Detection and identification of *Phytophthora* species in southern Italy by RFLP and sequence analysis of PCR-amplified nuclear ribosomal DNA. *Eur J Plant Pathol* 113: 1–14.
- Campanile G, Schena L, Luisi N (2008) Real-time PCR identification and detection of *Fuscoporia torulosa* in *Quercus ilex*. *Plant Pathol* 57: 76–83.
- Cao T, Tewari J, Strelkov E (2007) Molecular detection of *Plasmodiophora brassicae*, causal agent of clubroot of crucifers in plant and soil. *Plant Dis* 91: 80–87.
- Carzaniga R, Fiocca D, Bowyer P, O'Connell RJ (2002) Localization of melanin in conidia of *Alternaria alternata* using phage antibodies. *Mol Plant-Microbe Interact* 15: 216–224.
- Causin R, Scopel C, Grendene A, Montechio L (2005) An improved method for the detection of *Phytophthora cactorum* (L.C.) Schröeter in infected plant tissues using SCAR markers. *J Plant Pathol* 87: 25–35.

- Celi FS, Zenilman ME, Shuldiner AR (1993) A rapid and versatile method to synthesize initial standards for competitive PCR. *Nucleic Acids Res* 21: 1047.
- Chakraborty V, Basu P, Das R, Saha A, Chakraborty BN (1996) Evaluation of antiserum raised against *Pestalotiopsis theae* for the detection of grey blight of tea by ELISA. *Folia Microbiol* 41: 413–418.
- Chandelier A, Ivors K, Garbelotto M, Zini J, Laurent F, Cavelier M (2006) Validation of a real-time PCR method for the detection of *Phytophthora ramorum*. *Bull OEPP/EPPO* 36: 409–414.
- Chang GH, Yu RC (1997) Rapid immunoassay of fungal mycelia in rice and corn. *J Chinese Agric Chem Soc* 35: 533–539.
- Chee HY, Kim WG, Cho WD (1998) Detection of *Plasmodiophora brassicae* by using polymerase chain reaction. *Kor J Plant Pathol* 14: 589–593.
- Chen W, Gray LE, Grau CR (1996) Molecular differentiation of fungi associated with brown stem rot and detection of *Phialophora gregata* in resistant and susceptible soybean cultivars. *Phytopathology* 86: 1140–1148.
- Chen LC, Chen TZ, Chen HL, Yeh H (1998) Establishment of molecular markers for detection and diagnosis of *Botrytis cinerea* and *B. elliptica*. *Plant Pathol Bull* 7: 177–188.
- Chen Y-Y, Conner RL, Gillard CL, Boland GJ, Babcock C, Chang K-F, Hwang SF, Balasubramanian PM (2007) A specific and sensitive method for the detection of *Colletotrichum lindemuthianum* in dry bean tissue. *Plant Dis* 91: 1271–1276.
- Chen R-S, Chu C, Cheng C-W, Chen W-Y, Tsay J-G (2008) Differentiation of two powdery mildews of sunflower (*Helianthus annuus*) by a PCR-mediated method based on ITS sequences. *Eur J Plant Pathol* 121: 1–8.
- Chilvers MI, duToit LJ, Peever TL (2007) A real-time quantitative PCR assay for *Botrytis* spp. that cause neck rot of onion. *Plant Dis* 91: 599–608.
- Chu PWG, Waterhouse PM, Martin RR, Gerlach WL (1989) New approaches to the detection of microbial plant pathogens. *Biotechnol Genet Eng Rev* 7: 45–111.
- Cilliers AJ, Swart AJ, Wingfield MJ (1994) Selective medium for isolating *Lasidiplodia theobromae*. *Plant Dis* 78: 1052–1055.
- Cipriani MG, Schena L, Sialer MMF, Gallitelli D (2000) Characterization and cloning of a molecular probe for diagnosis of *Verticillium* spp. *Atti Gionrate fitopatologiche* 2: 551–558.
- Clark MF, Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. *J Gen Virol* 34: 475–483.
- Clear RM, Patrick SK (1992) A simple medium to aid the identification of *Fusarium moniliforme*, *F. proliferatum* and *F. subglutinans*. *J Food Protect* 55: 120–122.
- Coff C, Poupara P, Xiao Q, Garner A, Lund V (1998) Sequence of a plastocyanin cDNA from wheat and the use of the gene product to determine serological tissue degradation after infection with *Pseudocercospora herpotrichoides*. *J Phytopathol* 146: 11–17.
- Colas V, Lacourt I, Ricci P, Vanlerberghe-Masulli F, Venard P, Poupet A, Panabieres F (1998) Diversity of virulence in *Phytophthora parasitica* in tobacco as reflected by nuclear RFLPs. *Phytopathology* 88: 205–212.
- Cooke DEL, Schena L, Cacciola SO (2007) Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. *J Plant Pathol* 89: 145–160.
- Coolong TW, Walcott RR, Randle WM (2008) Quantitative real-time polymerase chain reaction assay for *Botrytis aclada* in onion bulb tissues. *HortScience* 43: 408–413.
- Cooper B, Eckert D, Andon NL, Yates JR (2003) Investigative proteomics: Identification of an unknown virus from infected plants using mass spectrometry. *J Amer Soc Mass Spectro* 14: 736–741.
- Correll JC, Puhalla JE, Schneider RW (1986a) Identification of *Fusarium oxysporum* f.sp. *apii* on the basis of colony size, virulence and vegetative compatibility. *Phytopathology* 76: 396–400.
- Correll JC, Puhalla JE, Schneider RW (1986b) Vegetative compatibility of groups among nonpathogenic root colonizing strains of *Fusarium oxysporum*. *Canad J Bot* 64: 2358–2361.
- Correll JC, Klittich, CJR, Leslie JF (1987) Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77: 1640–1646.

- Côté MJ, Tardif MC, Meldrum AJ (2004) Identification of *Monilinia fructigena*, *M. fructicola*, *M. laxa* and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant Dis* 88: 1219–1225.
- Coutts RHA, Covelli L, Di Serio F, Citir A, Açikgöz S, Hernández C, Ragozzino A, Flores R (2004) Cherry chlorotic rusty spot and Amasya cherry disease are associated with a complex pattern of mycoviral-like double stranded RNAs. II Characterization of a new species in the genus *Partitivirus*. *J Gen Virol* 85: 3349–3353.
- Cruz P, Buttner MP (2008) Development and evaluation of a real-time quantitative PCR assay for *Aspergillus flavus*. *Mycologia* 100: 683–690.
- Cullen DW, Lees AK, Toth IK, Duncan JM (2002) Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. *Plant Pathol* 51: 1365–1369.
- Cullen DW, Toth IK, Pitkin Y, Boonham N, Walsh K, Barker I, Lees AK (2005) Use of quantitative molecular diagnostic assays to investigate Fusarium dry rot in potato stocks and soil. *Phytopathology* 95: 1462–1471.
- Daayf F, Nicole M, Geiger JP (1995) Differentiation of *Verticillium dahliae* populations on the basis of vegetative compatibility and pathogenicity on cotton. *Eur J Plant Pathol* 101: 69–79.
- Degola F, Berni E, Dall'Asta C, Spotti E, Marchelli R, Ferrero I, Restivo FM (2007) A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. *J Appl Microbiol* 103: 409–417.
- Delcan J, Melgarejo P (2002) Mating behaviour and vegetative compatibility in Spanish populations of *Botryotinia fuckeliana*. *Eur J Plant Pathol* 108: 391–400.
- Delfosse P, Reddy AS, Legreve A, Thirumala Devi K, Abdurahaman MD, Maraite H, Reddy DVR (2000) Serological methods for detection of *Polymyxa graminis*, an obligate root parasite and vector of plant viruses. *Phytopathology* 90: 537–545.
- Demontis MA, Cacciola SO, Orru M, Balmas V, Chessa V, Maserti BE, Mascia L, Raudino F, di San Lio GM, Migheli Q (2008) Development of real-time PCR systems based on SYBR® Green I and TaqMan® technologies for specific quantitative detection of *Phoma tracheiphila* in infected citrus. *Eur J Plant Pathol* 120: 339–351.
- Derrick KS (1972) Immuno-specific grids for electron microscopy of plant viruses. *Phytopathology* 62: 753.
- Derrick KS (1973) Quantitative assay for plant viruses using serologically specific electron microscopy. *Virology* 56: 652–653.
- Dewey FM (1998) Use of monoclonal antibodies to study plant invading fungi particularly *Botrytis cinerea* and *Septoria nodorum*. *Beit Zucht Bundes Kultur* 8: 45–47.
- Dewey FM, Brasier CM (1988) Development of ELISA for *Ophiostoma ulmi* using antigen-coated wells. *Plant Pathol* 37: 28–35.
- Dewey FM, MacDonald M, Philipps S (1989a) Development of monoclonal antibody ELISA, dot-blot and dipstick immunoassays for *Humicola lanuginosa* in rice. *J Gen Microbiol* 135: 361–374.
- Dewey FM, Munday CJ, Brasier CM (1989b) Monoclonal antibodies to specific components of the Dutch elm disease pathogen *Ophiostoma ulmi*. *Plant Pathol* 38: 9–20.
- Dewey FM, MacDonald M, Philipps S, Priestley R (1990) Development of monoclonal antibody ELISA and dipstick immunoassays for *Penicillium islandicum* in rice grains. *J Gen Microbiol* 136: 753–760.
- Dharam Singh, Maheshwari VK (2001) Influence of stack burn disease of paddy on seed health status. *Seed Res* 29: 205–209.
- Dhingra OD, Muchovej JJ (1980) Twin stem abnormality disease of soybean seedlings caused by *Sclerotium* sp. *Plant Dis* 64: 176.
- Dhingra OD, Sediya C, Carraro IM, Ries MS (1978) Behavior of four soybean cultivars to seed infecting fungi in delayed harvest. *Fitopatol Brasil* 3: 277.
- Dobrowolski MP, O'Brien PA (1993) Use of RAPD-PCR to isolate a species-specific DNA probe for *Phytophthora cinnamomi*. *FEMS Microbiol Lett* 113: 43–47.
- Dobrowolski MP, Tommerup IC, Chearer BL, O'Brien PA (2003) Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. *Phytopathology* 93: 695–704.



- Doster MA, Michailides TJ (1998) Production of bright greenish yellow fluorescence in figs infected by *Aspergillus* species in California orchards. *Plant Dis* 82: 669–673.
- Dowell FE, Boratynski TN, Ykema RE, Dowdy AK, Staten RT (2002) Use of optical sorting to detect wheat kernels infected with *Tilletia indica*. *Plant Dis* 86: 1011–1013.
- Drenth A, Wagals G, Smith B, Sendall B, O'Dwyer C, Irvine G, Irwin JAG (2006) Development of a DNA-based method for the detection and identification of *Phytophthora* species. *Austr Plant Pathol* 35: 147–159.
- Duffy BK, Weller DM (1994) A semiselective and diagnostic medium for *Gauemannomyces graminis* var. *tritici*. *Phytopathology* 84: 1407–1415.
- Duncan JM (1980) A technique for detecting red stele (*Phytophthora fragariae*) infection in strawberry stocks before planting. *Plant Dis* 77: 517–520.
- Duncan JM (1990) *Phytophthora* species attacking strawberry and raspberry. *EPPO Bull* 20: 107–115.
- Duncan JM, Kennedy DM, Chard J, Ali A, Rankin PA (1993) Control of *Phytophthora fragariae* on strawberry and raspberry in Scotland by bait tests. In: Ebbels D (ed), *Plant Health and the European Single Market*, BCPC Symp, pp. 301–305.
- Dupont J, Laloui W, Magnin S, Larignon P, Roquebert MF (2000) *Phaeoacremonium viticola*, a new species associated with Esca disease of grapevine in France. *Mycologia* 92: 499–504.
- Dushnicky LG, Ballance GM, Summer MJ, MacGregor AW (1998) Detection of infection and host responses in susceptible and resistant wheat cultivars to a toxin-producing isolate of *Pyrenophora tritici-repens*. *Canad J Plant Pathol* 20: 19–27.
- Dyer RB, Kendra DF, Brown DW (2006) Real-time PCR assay to quantify *Fusarium graminearum* wild-type and recombinant mutant DNA in plant material. *J Microbiol Meth* 67: 534–542.
- Edwards SG, Seddon B (2001) Selective media for the specific isolation and enumeration of *Botrytis cinerea* conidia. *Lett Appl Microbiol* 32: 63–66.
- Edwards SG, Pirgozliev SR, Hare MC, Jenkinson P (2001) Quantification of trichothecene-producing *Fusarium* species in harvested grains by competitive PCR to determine efficacies of fungicides against *Fusarium* head blight of winter wheat. *Appl Environ Microbiol* 67: 1575–1580.
- Edwards J, Constable F, Wiechel T, Salib S (2007) Comparison of the molecular tests-single PCR, nested PCR and quantitative PCR (SYBR® Green and TaqMan®)- for detection of *Phaeoaniella chlamydospora* during grapevine nursery propagation. *Phytopathol Mediterr* 46: 58–72.
- Eibel P, Wolf GA, Koch AE (2005a) Detection of *Tilletia caries*, causal agent of common bunt of wheat by ELISA and PCR. *J Phytopathol* 153: 297–306.
- Eibel P, Wolf GA, Koch AE (2005b) Development and evaluation of an enzyme-linked immunosorbent assay (ELISA) for detection of loose smut of barley (*Ustilago nuda*). *Eur J Plant Pathol* 111: 113–124.
- Ekefan EJ, Simons SA, Nwankiti AO, Peters JC (2000) Semi-selective medium for isolation of *Colletotrichum gloeosporioides* from soil. *Experi Agric* 35:313–321.
- Elliot ML, DesJardin EA, Henson JM (1993) Use of a polymerase chain reaction assay to aid in identification of *Gauemannomyces graminis* var. *graminis* from different grass hosts. *Phytopathology* 83: 414–418.
- Elwakil MA, Ghoneem KM (2002) An improved method of seed health testing for detecting the lurked seedborne fungi of fenugreek. *Pak J Plant Pathol* 1: 11–13.
- Elwakil MA, El-Sherif EM, El-Metwally MA (2007) An innovative method for detecting slow-growing seedborne fungi of peanut. *Plant Pathol J* 6: 306–311.
- Errampalli S, Saunders J, Cullen DW (2001) A PCR-based method for detection of potato pathogen, *Helminthosporium solani* in silver scurf-infected tuber tissue and soils. *J Microbiol Meth* 44: 59–68.
- Erwin DC, Ribeiro OK (1996) *Phytophthora* diseases worldwide. The Amer Phytopathol Soc Press St. Paul, MN, USA.
- Eun AJC, Wong S-M (2000) Molecular beacons: a new approach to plant virus detection. *Phytopathology* 90: 269–275.

- Eynck C, Koopmann B, Grunewaldt-Stoecker G, Karlovsky P, von Tiedemann A (2007) Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. *Eur J Plant Pathol* 118: 259–274.
- Fahleson J, Lagercrantz U, Hu Q, Steventon LA, Dixelium C (2003) Estimation of genetic variation among *Verticillium* isolates using AFLP analysis. *Eur J Plant Pathol* 109: 361–371.
- Feodorova RN (1987) New and improved methods of detecting smuts in wheat and barley seeds. *Biul Inst Hodowli i Aklim Ros'lin* 201: 253–256.
- Ferraris L, Cardinale F, Valentino D, Roggero P, Tamietti G (2004) Immunological discrimination of *Phytophthora cinnamomi* from other *Phytophthora* pathogenic on chestnut. *J Phytopathol* 152: 193–199.
- Förster H, Adaskaveg JE (2000) Early brown rot infections in sweet cherry fruit are detected by *Monilinia*-specific DNA primers. *Phytopathology* 90: 171–178.
- Fouly HM, Wilkinson HT (2000) Detection of *Gaeumannomyces graminis* varieties using polymerase chain reaction with variety-specific primers. *Plant Dis* 84: 947–951.
- Fountaine JM, Shaw MW, Napier B, Ward E and Fraaije BA (2007) Application of real-time and multiplex polymerase chain reaction assays to study leaf blotch epidemics in barley. *Phytopathology* 97: 297–303.
- Fourie PH, Halleen F (2000) Investigation on the occurrence of *Phaeomoniella chlamydospora* in canes of rootstock mother vines. *Austr Plant Pathol* 31: 425–426.
- Fraaije BA, Lovell DJ, Rohel EA, Hollomon DW (1999) Rapid detection and diagnosis of *Septoria tritici* epidemics in wheat using a polymerase chain reaction/PicoGreen assay. *J Appl Bacteriol* 86: 701–708.
- Francis SA, Roden BC, Adams MJ, Weiland J, Michael A (2007) Comparison of ITS sequences from UK and North American sugar beet powdery mildews and the designation of *Erysiphe betae*. *Mycol Res* 111: 204–212.
- Fraser DE, Shoemaker PB, Ristaino JB (1999) Characterization of isolates of *Phytophthora infestans* from tomato and potato in North Carolina from 1993 to 1995. *Plant Dis* 83: 633–638.
- Frederick RD, Snyder KE, Tooley PW, Berthier-Schaad Y, Peterson GI, Bonde MR, Schaad NW, Knorr DA (2000) Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. *Phytopathology* 90: 951–960.
- Fredlund E, Gidlund A, Olsen M, Borjesson T, Spliid NHH, Simonsson M (2008) Method of evaluation of *Fusarium* DNA extraction from mycelia and wheat for downstream real-time PCR quantification and correlation to mycotoxin levels. *J Microbiol Meth* 73: 33–40.
- Freeman S, Maimon M, Pinkas Y (1999) Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. *Phytopathology* 89: 456–461.
- French-Monar RD, Jones JB, Roberts PD (2006) Characterization of *Phytophthora capsici* associated with roots of weeds on Florida vegetable farms. *Plant Dis* 90: 345–350.
- Fu G, Huang SL, Wei JG, Yuan GQ, Ren JG, Yan WH, Cen ZL (2007) First record of *Jatropha podagrica* gummosis caused by *Botryodiplodia theobromae* in China. *Austr Plant Dis Notes* 2: 75–76.
- Fulton CE, Brown AE (1997) Use of SSU rDNA group I intron to distinguish *Monilinia fructicola* from *M. laxa* and *M. fructigena*. *FEMS Microbiol Lett* 157: 307–312.
- Gabor Bk, O'Gara ET, Philip BA, Horan DP, Hardham AR (1993) Specificities of monoclonal antibodies to *Phytophthora cinnamomi* in two rapid diagnosis assays. *Plant Dis* 77: 1189–1197.
- Ganley RJ, Bradshaw RE (2000) Rapid identification of polymorphic microsatellite loci in a forest pathogen, *Dothistroma pini* using anchored PCR. *Mycol Res* 105: 1075–1078.
- Gao X, Jackson TA, Lambert KN, Li S, Harman GL, Niblack TL (2004) Detection and quantification of *Fusarium solani* f.sp. *glycines* in soybean roots with real-time quantitative polymerase chain reaction. *Plant Dis* 88: 1372–1380.
- Garcia Pedrajas MD, Bainbridge BW, Heale JB, Perez Artés E, Jimenez Diaz Rm (1999) A simple PCR-based method for the detection of the chickpea wilt pathogen *Fusarium oxysporum* f.sp. *ciceris* in artificial and natural soils. *Eur J Plant Pathol* 105: 251–259.
- Garraway MO, Evans R (1984) *Fungal Nutrition and Physiology*. John Wiley & Sons, New York.

- Garrido C, Carbù M, Fernández-Acero FJ, Boonham N, Colyer A, Cantoral JM, Budge G (2009) Development of protocols for detection of *Colletotrichum acutatum* and monitoring of strawberry anthracnose using real-time PCR. *Plant Pathol* 58: 43–51.
- Garzón CD, Geiser DM, Moorman GW (2005) Diagnosis and population analysis of *Pythium* species using AFLP fingerprinting. *Plant Dis* 89: 81–89.
- Gayoso C, de Ilárduya OM, Pomar F, de Cáceres FM (2007) Assessment of real-time PCR as a method for determining the presence of *Verticillium dahliae* in different Solanaceae cultivars. *Eur J Plant Pathol* 118: 199–209.
- Gindrat D, Pezet R (1994) Paraquat, a tool for rapid detection of latent fungal infections and endophytic fungi. *J Phytopathol* 14: 86–98.
- Giraud T, Fortini D, Levis C, Larmarque C, Leroux P, LoBuglio K, Brygoo Y (1999) Two sibling species of the *Botrytis cinerea* complex, *transposa* and *vacuina* are found in sympatry on numerous host plants. *Phytopathology* 89: 967–973.
- Gleason ML, Ghabrial SA, Ferriss RS (1987) Serological detection of *Phomopsis longicola* in soybean seeds. *Phytopathology* 77: 371–375.
- Glen M, Smith AH, Langrell SRH, Mohammed CL (2007) Development of nested polymerase chain reaction detection of *Mycosphaerella* spp. and its application to the study of leaf disease in Eucalyptus plantations. *Phytopathology* 97: 132–144.
- González E, Sutton TB, Correll JC (2006) Clarification of the etiology of Glomerella leaf spot and bitter rot of apple caused by *Colletotrichum* spp. based on morphology and genetic, molecular and pathogenicity tests. *Phytopathology* 96: 982–992.
- González-Jaén MT, Mirete S, Patiño B, López-Errasquín E, Vázquez C (2004) Genetic markers for the analysis of variability and for production of specific diagnostic sequences in fumonisin-producing strains of *Fusarium verticillioides*. *Eur J Plant Pathol* 110: 525–532.
- Goodwin PH, Kirkpatrick BC, Duniway JM (1989) Cloned probes for identification of *Phytophthora parasitica*. *Phytopathology* 79: 716–721.
- Goodwin PH, English JT, Neber DA, Duniway JM, Kirkpatrick BC (1990) Detection of *Phytophthora parasitica* from soil and host tissue with a species-specific DNA probe. *Phytopathology* 80: 277.
- Goodwin SB, Schneider RE, Fry WE (1995) Use of cellulose acetate electrophoresis for rapid identification of allozyme genotypes of *Phytophthora infestans*. *Plant Dis* 79: 1181–1185.
- Gough KC, Li Y, Vaigan TJ, Williams AJ, Cockburn W, Whitlam GC (1999) Selection of phage antibodies to surface epitopes of *Phytophthora infestans*. *J Immunol Meth* 228: 97–108.
- Griffin DW, Kellog CA, Peak KK, Shinn EA (2002) A rapid and efficient assay for extracting DNA from fungi. *Lett Appl Microbiol* 34: 210–214.
- Grote D, Olmos A, Kofoet A, Tuset JJ, Bertolini E, Cambra M (2000) Detection of *Phytophthora nicotianae* by PCR. *Bull OEPP* 30: 539–541.
- Grote D, Olmos A, Kofoet A, Tuset JJ, Bertolini E, Cambra M (2002) Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested PCR. *Eur J Plant Pathol* 108: 197–207.
- Gubis J, Hudcovicová M, Klčová L, Červená V, Bojnanská K, Kraic J (2004) Detection of leaf blotches- causal agents in barley leaves and grains. *Czech J Genet Plant Breed* 40: 111–117.
- Guglielmo F, Bergemann SE, Gonthier P, Nicolotti G, Garbelotto M (2007) A multiplex PCR-based method for the detection and early identification of wood rotting fungi in standing trees. *J Appl Microbiol* 103: 1490–1507.
- Guillemete T, Iacomí-Vasilescu B, USAMV (2004) Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. *Plant Dis* 88: 490–496.
- Gutierrez WA, Shew HD (1998) Identification and quantification of ascospores as the primary inoculum for collar rot of greenhouse-produced tobacco seedlings. *Plant Dis* 82: 485–490.
- Guo J-R, Schnieder F, Beyer M, Verreet J-A (2005) Rapid detection of *Mycosphaerella graminicola* in wheat using reverse transcription-PCR assay. *J Phytopathol* 153: 674–679.
- Gutierrez LJ, Wang Y, Lutton E, McSpadden Gardner BB (2006) Distribution and fungicide sensitivity of fungal pathogens causing anthracnose-like lesions in tomatoes grown in Ohio. *Plant Dis* 90: 397–403.

- Gwinn KD, Collins-Shepard MH, Reddick BB (1991) Tissue print-immunoblot, an accurate method for the detection of *Acremonium coenophialum* in tall fescue. *Phytopathology* 81: 747–748.
- Hahn F (2002) Fungal spore detection on tomatoes using spectral fourier signatures. *Biosystem Eng* 81: 249–259.
- Hamer J, Farrel L, Orbach M, Valent A, Chumley F (1989) Host species specific conservation of a family of repeated DNA sequences in the genome of a fungal pathogen. *Proc. Nat Acad Sci USA* 86: 9981–9985.
- Hampson MC (1993) History, biology and control of potato wart disease in Canada. *Canad J Plant Pathol* 15: 223–224.
- Han SS, Ra DS, Nelson RJ (1995) Relationship between DNA fingerprints and virulence of *Pyricularia grisea* from rice and new hosts in Korea. *Internat Rice Res Notes* 20(1): 26–27.
- Hardham AR, Suzuki E, Perkin JL (1986) Monoclonal antibodies to isolate- species- and genus-specific components on the surface of zoospores and cysts of the genus *Phytophthora cinnamomi*. *Canad J Bot* 64: 311–321.
- Hardham AR, Gubler F, Duniec J, Elliott J (1991) A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. *J Microscopy* 162: 305–318.
- Harmon PF, Dunkle LD, Latin R (2003) A rapid PCR-based method for the detection of *Magnaporthe oryzae* from infected perennial ryegrass. *Plant Dis* 87: 1072–1076.
- Harrison JG, Barker H, Lowe R, Rees EA (1990) Estimation of amounts of *Phytophthora infestans* mycelium in leaf tissue by enzyme-linked immunosorbent assay. *Plant Pathol* 39: 274–277.
- Harrison JG, Rees EA, Barker H, Lowe R (1993) Detection of spore balls of *Spongopora subterranea* on potato tubers by enzyme-linked immunosorbent assay. *Plant Pathol* 42: 181–186.
- Harvey HP, Ophel-Keller K (1996) Quantification of *Gaeumannomyces graminis* var. *tritici* in infected roots and arid soil using slot-blot hybridization. *Mycol Res* 100: 962–970.
- Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995) *Ainsworth & Bisby's Dictionary of the Fungi*, CAB Internat, Oxon, UK.
- Hayden KJ, Rizzoso D, Tse J, Garbelotto M (2004) Detection and quantification of phytophthora ramorum from California forests using a real-time polymerase chain reaction assay. *Phytopathology* 94: 1075–1083.
- Hayden K, Ivors K, Wilkinson C, Garbelotto M (2006) TaqMan chemistry for *Phytophthora ramorum* detection and quantification with comparison of diagnostic methods. *Phytopathology* 96: 846–854.
- Henriquez JL, Sugar D, Spotts RA (2004) Etiology of bull's eye rot of pear caused by *Neofabraea* spp. in Oregon, Washington and California. *Plant Dis* 88: 1134–1138.
- Henson JM, French R (1993) The polymerase chain reaction and plant disease diagnosis. *Ann Rev Phytopathol* 31: 81–109.
- Henson JM, Goins T, Grey W, Mathre DE, Elliott ML (1993) Use of polymerase chain reaction to detect *Gaeumannomyces graminis* DNA in plants grown in artificially and naturally infested soil. *Phytopathology* 83: 283–297.
- Hermansen A, Herrero M-L, Gausla E, Razzaghian J, Naerstad R (2007) *Pythium* species associated with cavity spot on carrots in Norway. *Ann Appl Biol* 150: 115–121.
- Hewett PD (1977) Pretreatment in seed health testing: hypochlorite in the 2,4-D-blotter for *Leptosphaeria maculans* (*Phoma lingam*). *Seed Sci Tech* 5: 599.
- Hogg AC, Johnston RH, Dyer AT (2007) Applying real-time quantitative PCR to Fusarium crown rot of wheat. *Plant Dis* 91: 1021–1028.
- Holtz BA, Karu AF, Weinhold AR (1994) Enzyme-linked immunosorbent assay for detection of *Thielaviopsis basicola*. *Phytopathology* 84: 977–984.
- Hood ME, Shew HD (1996) Applications of KOH-aniline blue fluorescence in the study of plant-fungal interactions. *Phytopathology* 86: 704–708.
- Hsieh SPY, Huang RZ, Wang TC (1996) Application of tannic acid in qualitative and quantitative growth assay of *Rhizoctonia* spp. *Plant Pathology Bull* 5: 100–106.
- Hu X, Nazar RN, Robb J (1993) Quantification of *Verticillium* biomass in wilt disease development. *Physiol Mol Plant Pathol* 42: 23–36.

- Hu CJ, Li YR, Wei YW, Huang SL (2008) A PCR-based method to detect *Sclerotium hydrophilum* in infected rice leaf sheaths. *Austr Plant Pathol* 37: 40–42.
- Hughes KJD, Inman AJ, Beales PA, Cook RIA, Fulton CE, McReynolds ADK (1998) PCR-based detection of *Phytophthora fragariae* in raspberry and strawberry roots. *Brighton Crop Protect Conf Pest Dis* 2: 687–692.
- Hughes KJD, Giltrap PM, Barton VC, Hobden E, Tomilson JA, Barber P (2006) On-site real-time PCR detection of *Phytophthora ramorum* causing dieback of *Parrotia persica* in the UK. *Plant Pathology* 55: 813.
- Hussain S, Lees AK, Duncan JM, Cooke DEL (2005) Development of a species-specific and sensitive detection assay for *Phytophthora infestans* and its application for monitoring of inoculum in tubers and soil. *Plant Pathol* 54: 373–382.
- Hyakumachi M, Priyatmojo A, Kubota M, Fukui H (2005) New anastomosis groups AG-T and AG-U of binucleate *Rhizoctonia* spp. causing root and stem rot of cut-flower and miniature roses. *Phytopathology* 95: 784–792.
- Hyun JW, Peres NA, Yi S-Y, Timmer LW, Kim KS, Kwon H-M, Lim H-C (2007) Development of PCR assays for the identification of species and pathotypes of *Elsinoe* causing scab on citrus. *Plant Dis* 91: 865–870.
- Iacomi-Vasilescu B, Blancard D, Guénard M, Molinero-Demilly V, Laurent E, Simoneau P (2002) Development of a PCR-based diagnostic assay for detecting pathogenic *Alternaria* species in cruciferous seeds. *Seed Sci Technol* 30: 87–95.
- Infantino A, Pucci A (2005) A PCR-based assay for the detection and identification of *Pyrenochaeta lycopersici*. *Eur J Plant Pathol* 112: 337–437.
- Ingle CA, Kushner SR (1996) Development of an *in vitro* mRNA decay system for *Escherichia coli*: poly(A) polymerase I is necessary to trigger degradation. *Proc Natl Acad Sci USA* 93: 12926–12931.
- International Seed Testing Association (ISTA) (1994) ISTA Handbook on Seed Health Testing, Section 2, Working Sheets. ISTA, Zurich, Switzerland.
- International Seed Testing Association (ISTA) (1996) International rules for seed testing. *Proc. Internat Seed Testing Assoc* 31: 1.
- Ioos R, Iancu G (2008) European collaborative studies for the validation of PCR-based detection tests targeting regulated fungi and oomycetes. *EPPO Bull* 38: 198–204.
- Ivors KL, Tse J, Garbelotto M (2002) TaqMan PCR for detection of *Phytophthora* DNA in environmental plant samples. *Proc Sudden Oak Death, Sci Symp, Monterey, CA, USA*, p. 56.
- Ivors KL, Garbelotto M, Vries IDE, Ruyter-Spira C, Hekkert B Te, Rosenzweig N, Bonants P (2006) Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forests and European nursery populations. *Mol Ecol* 15: 1493–1505.
- Jackson EW, Avant JB, Overturf KE, Bonman JM (2006) A quantitative assay of *Puccinia coronata* f.sp. *avenae* DNA in *Avena sativa*. *Plant Dis* 90: 692–636.
- Jacobson DJ, Gordon TR (1990) Further investigations of vegetative compatibility within *Fusarium oxysporum* f.sp. *melonis*. *Canad J Bot* 68: 1245–1248.
- Jamaux I, Spire D (1994) Development of a polyclonal antibody-based immunoassay for the early detection of *Sclerotinia sclerotiorum* in rapeseed petals. *Plant Pathol* 43: 847–852.
- Jamaux I, Spire D (1999) Comparison of responses of ascospores and mycelium by ELISA with anti-mycelium and anti-ascospore antisera for the development of a method to detect *Sclerotinia sclerotiorum* on petals of oilseed rape. *Ann Appl Biol* 134: 171–179.
- Jayasinghe CK, Fernando THPS (1998) Growth at different temperatures and on fungicide-amended media: two characteristics to distinguish *Colletotrichum* species pathogenic to rubber. *Mycopathologia* 143: 93–95.
- Jerne NK (1960) Immunological speculations. *Annu Rev Microbiol* 14: 341–358.
- Joaquin RR, Rowe RC (1990) Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. *Phytopathology* 80: 1160–1166.
- Joaquin RR, Rowe RC (1991) Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plants. *Phytopathology* 81: 552–558.



- Johansen DA (1940) Plant Microtechnique. McGraw-Hill Book Co Inc., New York.
- Johnson RD, Johnson L, Kohmoto K, Otani H, Lane CR, Kodama M (2000) A polymerase chain reaction-based method to specifically detect *Alternaria alternata* apple pathotype (*A. mali*), the causal agent of Alternaria blotch of apple. *Phytopathology* 90: 973–976.
- Judelson HS, Messenger-Routh B (1996) Quantitation of *Phytophthora cinnamomi* in avocado roots using a species-specific DNA probe. *Phytopathology* 86: 763–768.
- Justesen AF, Hansen HJ, Pinnschmidt HO (2008) Quantification of *Pyrenophora graminea* in barley seed using real-time PCR. *Eur J Plant Pathol* 122: 253–263.
- Jyan M-H, Huang L-C, Ann P-J, Liou R-F (2002) Rapid detection of *Phytophthora infestans* by PCR. *Plant Pathol Bull* 11: 45–56.
- Kageyama K, Kobayashi M, Tomita M, Kubota N, Suga H, Hyakumachi M (2002) Production and evaluation of monoclonal antibodies for the detection of *Pythium sulcatum* in soil. *J Phytopathol* 150: 97–104.
- Kaminski JE, Demoeen PH, O'Neill NR, Wetzel II HC (2005) A PCR-based method for the detection of *Ophiopogon agrostis* in creeping bentgrass. *Plant Dis* 89: 980–985.
- Karajeh MR (2006) Seed transmission of *Verticillium dahliae* in olive as detected by a highly sensitive nested PCR-based assay. *Phytopathol Mediterr* 45: 15–23.
- Karolewski Z, Fitt BDL, Latunde-Dada AO, Foster SJ, Todd AD, Downes K, Evans N (2006) Visual and PCR assessment of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) cultivars. *Plant Pathol* 55: 387–400.
- Karpovich-Tate N, Spanu P, Dewey FM (1998) Use of monoclonal antibodies to determine biomass of *Cladosporium fulvum* in infected tomato leaves. *Molec Plant-Pathogen Interact* 11: 710–716.
- Karthikeyan M, Radhika K, Bhaskaran R, Mathiyazhagan S, Samiyappan R, Velazhahan R (2006) Rapid detection of *Ganoderma* disease of coconut and assessment of inhibition effect of various control measures by immunoassay and PCR. *Plant Protect Sci* 42: 49–57.
- Kaufmann PJ, Weidemann GJ (1996) Isozyme analysis of *Colletotrichum gloeosporioides* from five host genera. *Plant Dis* 80: 1289–1293.
- Kawaradani M, Kusakari S, Morita S, Tanaka Y (1994) The enzyme activities in eggplant infected with soilborne diseases and application to diagnosis for diseases. *Ann. Phytopathol Soc Jpn* 60: 507–613.
- Kawaradani M, Kusakari S, Kimura M, Takizawa H, Nishihashi H (1998) A new method for measuring  $\beta$ 1,3-glucanase activity using p-nitrophenyl- $\beta$ -D laminaritetraside as a substrate to diagnose Verticillium wilt of egg plant. *Ann Phytopathol Soc Jpn* 64: 489–493.
- Kawasaki ES, Chehab FF (1994) Analysis of gene sequences by hybridization of PCR-amplified DNA to covalently bound oligonucleotides probes. The reverse dot-blot method. *Methods Mol Biol* 28: 225–236.
- Keer JT, Birce L (2003) Molecular methods for assessment of bacterial viability. *J Microbiol Meth* 53: 175–183.
- Keiper FJ, Hayden MJ, Wallwork H (2006) Development of sequence tagged microsatellites for the barley scald pathogen *Rhynchosporium secalis*. *Mol Ecol Notes* 6: 543–546.
- Keiper FJ, Capio E, Grcic M, Wallwork H (2007) Development of sequence tagged microsatellites for the barley net blotch pathogen *Pyrenophora teres*. *Mol Ecol Notes* 7: 664–666.
- Keiper FJ, Grcic M, Capio E, Wallwork H (2008) Diagnostic microsatellite markers for the barley net blotch pathogens *Pyrenophora teres* f.sp. *maculata* and *Pyrenophora teres* f. *teres*. *Austr Plant Pathol* 37: 428–430.
- Kellens JTC, Peumans WJ (1991) Biochemical and serological comparison of lectins from different anastomosis groups of *Rhizoctonia solani*. *Mycol Res* 95: 1235–1241.
- Kendall JJ, Hollomon DW, Sellely A (1998) Immunodiagnosis as an aid to timing of fungicide sprays for the control of *Mycosphaerella graminicola* on winter wheat in the UK. *Brighton Crop Protect Conf* 2: 701–706.
- Kennedy R, Wakeham AJ, Cullington JE (1999) Production and immunodetection of ascospores of *Mycosphaerella brassicola*: Ringspot of vegetable crucifers. *Plant Pathol* 48: 297–307.



- Kessel GJT, de Haas BH, Lombaers-van der Plas CH, Meijer EMJ, Dewey FM, Goudriaan J, van der Werf W, Köhl J (1999) Quantification of mycelium of *Botrytis* spp. and the antagonist *Ulocladium atrum* in necrotic leaf tissue of cyclamen and lily by fluorescence microscopy and image analysis. *Phytopathology* 89: 868–876.
- Khaldeeva EV, Medyantseva EP, Glushko NI, Budnikov GK (2001) Amperometric immuno-enzyme sensor for evaluating the degree of infection of vegetable crops by phytopathogenic fungi. *Agrokhimiya* 5: 81–86.
- Khanzada AK, Mathur SB (1988) Influence of extraction rate and concentration of stain on loose smut infection of wheat seed. *Pak J Agric Res* 9: 218–222.
- Khanzada AK, Rennie WJ, Mathur SB, Neergaard P (1980) Evaluation of two routine embryo test procedures for assessing the incidence of loose smut infection in seed samples of wheat (*Triticum aestivum*). *Seed Sci Technol* 8: 363.
- Kim HJ, Lee YS (2001) Development of an *in planta* molecular marker for the detection of Chinese cabbage (*Brassica campestris pekinensis*) club root pathogen *Plasmodiophora brassicae*. *J Microbiol* 39: 56–61.
- Kiss L, Takamatsu S, Cunnington JH (2005) Molecular identification of *Oidium neolycopersici* as the causal agent of the recent tomato powdery mildew epidemics in North America. *Plant Dis* 89: 491–496.
- Kitagawa T, Sakamoto Y, Furumi K, Ogura H (1989) Novel enzyme immunoassays for specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* and for general detection of various *Fusarium* species. *Phytopathology* 79: 162–165.
- Klassen GR, Blacerzak M, de Cock AWAM (1996) 5S ribosomal RNA gene spacers as species-specific probes for eight species of *Pythium*. *Phytopathology* 86: 581–587.
- Klemsdal SS, Herrero ML, Wanner LA, Lund G, Hermansen A (2008) PCR-based identification of *Pythium* spp. causing cavity spot in carrots and sensitive detection in soil samples. *Plant Pathol* 57: 877–886.
- Klich MA, Mullaney EJ (1987) DNA restriction enzyme fragment polymorphism as a tool for rapid differentiation of *Aspergillus flavus* from *Aspergillus oryzae*. *Exp Mycol* 11: 170–175.
- Knoll S, Vogel RF, Niessen L (2002) Identification of *Fusarium graminearum* in cereal samples by DNA Detection Test Strips™. *Lett Appl Microbiol* 34: 144–148.
- Ko SS, Kunimoto RK, Ko WH (2001) A simple technique for purifying fungal cultures contaminated with bacteria and mites. *J Phytopathol* 149: 509–510.
- Koch G, Kohler W (1990) Isozyme variation and genetic distances of *Erysiphe graminis* DC. *formae speciales*. *J Phytopathol* 129: 89.
- Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predetermined specificity. *Nature (Lond)* 256: 495–497.
- Konstantinova P, Bonants PJM, Genter-Pelzer MPE van, Zouwen P van der, Bulk R van den (2002) Development of specific primers for detection and identification of *Alternaria* spp. in carrot material by PCR and comparison with blotter and plating assays. *Mycol Res* 106: 23–33.
- Korolev N, Elad Y, Katan T (2008) Vegetative compatibility grouping in *Botrytis cinerea* using sulphate non-utilizing mutants. *Eur J Plant Pathol* 122: 369–383.
- Kox LFF, van Brouwershaven IR, van de Vossen BTLH, van den Beld HE, Bonants PJM, de Gruyter J (2007) Diagnostic values and utility of immunological, morphological and molecular methods for *in planta* detection of *Phytophthora ramorum*. *Phytopathology* 97: 1119–1129.
- Kozlakidis Z, Covelli L, DiSerio F, Citir A, Açıkgöz S, Hernández C, Ragozzino A, Flores R, Coutts RHA (2006) Molecular characterization of the largest mycoviral-like double-stranded RNAs associated with Amasya cherry disease, a disease of presumed fungal etiology. *J Gen Virol* 87: 3113–3117.
- Kozlakidis Z, Citir A, Açıkgöz S, Coutts RHA (2007) Development of a reverse transcription-polymerase chain reaction (RT-PCR) assay for the detection of Amasya cherry disease. *Plant Pathol* 56: 1032–1035.
- Kraft JM, Boge WL (1994) Development of an antiserum to quantify *Aphanomyces euteiches* in resistant pea lines. *Plant Dis* 78: 179–183.

- Krátká J, Pekárova-Kyněrová B, Kudlíková I, Slováček J, Zemánková M (2002) Utilization of immunochemical methods for detection of *Colletotrichum* spp. in strawberry. *Plant Protect Sci* 38: 55–63.
- Kristensen R, Gauthier G, Berdal KG, Hamels S, Remacle J, Host-Jenson A (2007) DNA microarray to detect and identify trichothecene- and moniliformin-producing *Fusarium* species. *J Appl Microbiol* 102: 1060–1070.
- Kroon LPNM, Verstappen ECP, Kox LFF, Flier WG, Bonants PJM (2004) A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*. *Phytopathology* 94: 613–620.
- Kushalappa AC, Lui LH (2002) Volatile fingerprinting (SPME-GC-FID) to detect and discriminate diseases of potato tubers. *Plant Dis* 86: 131–137.
- Kutilek V, Lee R, Kitto GB (2001) Development of immunochemical techniques for detecting Karnal bunt in wheat. *Food Agric Immunol* 13: 103–114.
- Láday M, Szécsi Á (2001) Distinct electrophoretic isozyme profiles of *Fusarium graminearum* and closely related species. *System Appl Microbiol* 24: 67–75.
- Láday M, Szécsi Á (2002) Identification of *Fusarium* species by isozyme analysis. *Acta Microbiol Immunol Hung* 49: 321–330.
- Lamour K, Finley L (2006) A strategy for recovering high quality genomic DNA from a large number of *Phytophthora* isolates. *Mycologia* 98: 514–517.
- Langerak CJ, van den Bulk RW, Franken AAJM (1996) Indexing seeds for pathogens. *Adv Bot Res* 23: 171–215.
- Langrell SRH, Barbara dJ (2001) Magnetic capture hybridization for improved PCR detection of *Nectria galligena* from lignified apple extracts. *Plant Molec Biol re* 19: 5–11.
- Lardner R, Stummer BE, Sosnowski MR, Scott ES (2005) Molecular identification and detection of *Eutypa lata* in grapevine. *Mycol Res* 109: 799–808.
- Larkin RP, Ristaino JB, Campbell CL (1995) Detection and quantification of *Phytophthora capsici*. *Phytopathology* 85: 1057–1063.
- Larsen JE, Hollingsworth CR, Flor J, Dornbusch MR, Simpson NL, Samac DA (2007) Distribution of *Phoma sclerotoides* on alfalfa and winter wheat crops in the North Central United States. *Plant Dis* 91: 551–558.
- Leach JE, White FE (1991) Molecular probes for disease diagnosis and monitoring. In: Khush GS, Toenniessen GH (ed) *Rice Biotechnology*, CAB Internat UK and Internat Rice Res Inst, Philippines pp. 281–307.
- Lecomte P, Péros JP, Blancard D, Bastien N, Délye C (2000) PCR assays that identify the grapevine die-back fungus *Eutypa lata*. *Appl Environ Microbiol* 66: 4475–4480.
- Lee HK, Tewari JP (2001) A PCR-based assay to detect *Rhynchosporium secalis* in barley seed. *Plant Dis* 85: 220–225.
- Lee HK, Tewari JP, Turkington TK (2001) Symptomless infection of barley seed by *Rhynchosporium secalis*. *Canad J Plant Pathol* 23: 315–317.
- Lee HK, Tewari JP, Turkington TK (2002) Quantification of seedborne infection by *Rhynchosporium secalis* in barley using competitive PCR. *Plant Pathol* 51: 217–224.
- Lees AK, van de Graaf P, Wale S (2008) The identification and detection of *Spongopora subterranea* and factors affecting infection and disease. *Amer J Potato Res* 85: 247–252.
- Lees AK, Sullivan L, Cullen DW (2009) A quantitative polymerase chain reaction assay for the detection of *Polyscytalum pustulans*, the cause of skin spot disease of potato. *J. Phytopathol* 157: 154–158.
- Leisova L, Kucera L, Minarikova V, Ovesna J (2005) AFLP-based PCR markers that differentiate spot and net forms of *Pyrenophora teres*. *Plant Pathol* 54: 66–73.
- Leisova L, Minarikova V, Kucera L, Ovesna J (2006) Quantification of *Pyrenophora teres* in infected barley leaves using real-time PCR. *J Microbiol Meth* 67: 446–455.
- Leslie JP (1993) Fungal vegetative compatibility. *Annu Rev Phytopathol* 31: 127–150.
- Leung H, Williams PH (1986) Enzyme polymorphism and genetic differentiation among geographic isolates of the rice blast fungus. *Phytopathology* 76: 778.
- Lévesque CA, Vrain TC, De Boer SM (1994) Development of a species-specific probe for *Pythium ultimum* using amplified ribosomal DNA. *Phytopathology* 84: 474–478.

- Lévesque CA, Harlton CE, de Cock AWAM (1998) Identification of some oomycetes by reverse dot-blot hybridization. *Phytopathology* 88: 213–222.
- Levy M, Romano J, Marchetti MA, Hamer JE (1991) DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. *Plant Cell* 3: 95–102.
- Levy L, Lee IM, Hadidi A (1994) Simple and rapid preparation of infected plant tissue extracts for PCR amplification of virus, viroid and MLO nucleic acids. *J Virol Meth* 49: 295–304.
- Lewis Ivey ML, Nava-Diaz C, Miller SA (2004) Identification and management of *Colletotrichum acutatum* on immature bell pepper. *Plant Dis* 88: 1198–1204.
- Licciardello G, Grasso FM, Bella P, Cirvilleri G, Grimaldi V, Catara V (2006) Identification and detection of *Phoma tracheiphilla*, causal agent of citrus mal secco disease by real-time polymerase chain reaction. *Plant Dis* 90: 1523–1530.
- Lievens B, Thomma BPHJ (2005) Recent developments in pathogen detection arrays: implications for fungal plant pathogens and use in practice. *Phytopathology* 95: 1374–1380.
- Lievens B, Brouwer M, Vanachter ACRC, Lévesque CA, Cammue BPA, Thomma BPHJ (2003) Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogen. *FEMS Microbiol Lett* 223: 113–122.
- Lievens B, Hansen IRM, Vanachter ACRC, Cammue BPA, Thomma BPHJ (2004) Root and foot rot on tomato caused by *Phytophthora infestans* detected in Belgium. *Plant Dis* 88: 86.
- Lievens B, Claes L, Vanachter ACRC, Bruno PA, Cammue BPA, Thomma BPHJ (2006) Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis. *FEMS Microbiol Lett* 255: 129–139.
- Lievens B, Claes L, Vakalounakis DJ, Vanachter ACRC, Thomma BPHJ (2007) A robust identification and detection assay to discriminate the cucumber pathogens *Fusarium oxysporum* f.sp. *cucumerinum* and f.sp. *radicis-cucumerinum*. *Environ Microbiol* 9: 2145–2161.
- Liew ECY, Maclean DJ, Irwin JAG (1998) Specific PCR-based detection of *Phytophthora medicaginis* using the intergenic spacer region of the ribosomal DNA. *Mycol Res* 102: 73–80.
- Lima CS, Pfenning LH, Costa SS, Campos MA, Leslie JF (2008) A new *Fusarium* lineage within the *Gibberella fujikuroi* species complex is the main causal agent of mango malformation disease in Brazil. *Plant Pathol* : Doi: 10.1111.j.1365-3059.2008-01946.x
- Lima CS, Monteiro JHA, Crespo NC, Costa SS, Leslie JF, Pfenning LH (2009) VCG and AFLP analyses identify the same groups in the causal agents of mango malformation in Brazil. *Eur J Plant Pathol* 123: 17–26.
- Lin Y-H, Chang J-Y, Liu E-T, Chao C-P, Huang J-W, Chang PL (2009) Development of a molecular marker for specific detection of *Fusarium oxysporum* f.sp. *cubense* race 4. *Eur J Plant Pathol* 123: 353–365.
- Lind V (1990) Isolation of antigens for serological identification of *Pseudocercospora herpotrichoides* (Fron)Deighton. *J Plant Dis Plant Protect* 97: 490–501.
- Liu Z, Nickrent DL, Sinclair JB (1990) Genetic relationships among isolates of *Rhizoctonia solani* anastomosis group-2 based on isozyme analysis. *Canad J Plant Pathol* 12: 376.
- LoBuglio KF, Pfister DH (2008) A *Glomerella* species phylogenetically related to *Colletotrichum acutatum* on Norway maple in Massachusetts. *Mycologia* 100: 710–715
- Lovic BR, Martyn RD, Miller ME (1995) Sequence analysis of the ITS regions of rDNA in *Monosporascus* spp. to evaluate its potential for PCR-mediated detection. *Phytopathology* 85: 655–661.
- Lu TH, Groth JV (1987) Isozyme detection and variation in *Uromyces appendiculatus*. *Canad J Bot* 66: 885.
- Lübeck M, Poulsen H (2001) UP-PCR cross blot hybridization as a tool for identification of anastomosis groups in the *Rhizoctonia solani* complex. *FEMS Microbiol Lett* 201: 83–89.
- Lyons NF, White JG (1992) Detection of *Pythium violae* and *Pythium sulcatum* in carrots with cavity spots using competition ELISA. *Ann Appl Biol* 120: 235–244.
- Ma, Luo Y, Michailides TJ (2003) Nested PCR assay for detection of *Monilinia fructicola* in stone fruit orchards and *Botryosphaeria dothidea* from pistachios in California. *J Phytopathol* 151: 312–322.

- MacNish GC, O'Brien PA (2005) RAPD-PCR used to confirm that four pectic isozyme (zymograms) groups within the Australian *Rhizoctonia solani* AG-8 populations are true intraspecific groups. *Austr Plant Pathol* 34: 245–250.
- MacNish GC, Sweetingham MW (1993) Evidence of stability of pectic zymogram groups within *Rhizoctonia solani* AG-8. *Mycol Res* 97: 1056–1058.
- MacNish GC, Carling DE, Brainard KA (1997) Relationship of microscopic and macroscopic vegetative reaction in *Rhizoctonia solani* and the occurrence of vegetatively compatible populations (VCPs) in AG-8. *Mycol Res* 101: 61–68.
- Magan N, Evans P (2000) Volatiles as an indicator of fungal activity and differentiation between species and the potential use of electronic nose technology for early detection of grain spoilage. *J Stored Prod Res* 36: 319–340.
- Maguire JD, Gabrielson RL (1983) Testing techniques for *Phoma lingam*. *Seed Sci Tech* 11: 599–605.
- Mahuku GS, Platt (Bud) HW, Maxwell P (1999) Comparison of polymerase chain reaction-based method with plating on media to detect and identify *Verticillium* wilt pathogen of potato. *Canad J Plant Pathol* 21: 125–131.
- Mangan A (1983) The use of plain water agar for detection of *Phoma betae* on beet seeds. *Seed Sci Technol* 6: 925–926.
- Marakakis EA, Tjamos SE, Antoniou PP, Paplomatas EJ, Tjamos EC (2009) Symptom development, pathogen isolation and real-time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes. *Eur J Plant Pathol* 124: 603–611.
- Marasas WFO, Ploetz RC, Wingfield MJ, Wingfield BD, Steenkamp ET (2006) Mango malformation disease and the associated *Fusarium* species. *Phytopathology* 96: 667–672.
- Markovic VL, Stummer BE, Hill AS (2002) Immunodetection and characterization of antigens expressed by *Uncinula necator*. *J Phytopathol* 150: 667–673.
- Martin B (1987) Rapid tentative identification of *Rhizoctonia* spp. associated with diseased turf grasses. *Plant Dis* 71: 47–49.
- Martin FN (1991) Selection of DNA probes useful for isolate identification of two *Pythium* spp. *Phytopathology* 81: 742.
- Martin MT, Cobos R (2007) Identification of fungi associated with grapevine decline in Castilla y León (Spain). *Phytopathol Mediterr* 46: 18–25.
- Martin FN, Tooley PW (2004) Identification of *Phytophthora* isolates to species level using restriction fragment length polymorphism analysis of a polymerase chain reaction-amplified region of mitochondrial DNA. *Phytopathology* 94: 983–991.
- Martin FN, Tooley PW, Blomquist C (2004) Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California and two additional species commonly recovered from diseased plant material. *Phytopathology* 94: 621–631.
- Martin FN, Coffey MD, Zeller K, Hamelin RC, Tooley P, Garbelotto M, Hughes KJD, Kubisiak T, Bilodeau GJ, Levy L, Blomquist C, Berger PH (2009) Evaluation of molecular markers for *Phytophthora ramorum* detection and identification: Testing for specificity using standardized library of isolates. *Phytopathology* 99: 390–403.
- Martinez-Culebras PV, Querol A, Suarez-Fernandez MB, Garcia-Lopez MD, Barrio E (2003) Phylogenetic relationships among *Colletotrichum* pathogens of strawberry and design PCR primers for their identification. *J Phytopathol* 151: 135–143.
- Mathur S, Ukhede R (2002) Development of a dot-blot technique for rapid identification of *Botrytis cinerea*, the causal organism of gray mold in greenhouse tomatoes. *J Horti Sci Biotechnol* 77: 604–608.
- Matsuda Y, Sameshima T, Moriura N, Inoue K, Nonomura T, Kakutani K, Nishimura H, Kusakari S, Tamamatsu S, Toyoda H (2005) Identification of individual powdery mildew fungi infecting leaves and direct detection of gene expression of single conidium by polymerase chain reaction. *Phytopathology* 95: 1137–1143.
- Matsumoto M, Matusyama N (1998) Trials of identification of *Rhizoctonia solani* AG 1-1A, the causal agent of rice sheath rot disease using specifically primed PCR analysis in diseased plant tissues. *Bull Inst Trop Agric Kyushu Univ* 21: 27–32.
- Matthew JS, Brooker JD (1991) The isolation and characterization of polyclonal and monoclonal antibodies to anastomosis group 8 of *Rhizoctonia solani*. *Plant Pathol* 40: 67–97.

- Maude RB (1996) Seedborne Diseases and Their Control - Principles and Practice. CAB Internat, Wallingford, Oxon, UK.
- May KJ, Ristaino JB (2004) Identity of the mtDNA haplotype(s) of *Phytophthora infestans* in historical specimens from Irish Potato Famine. *Mycol Res* 108: 471–479.
- McDonald JG, Wong E, Kristjansson GT, White GP (1999) Direct amplification by PCR of DNA from ungerminated teliospores of *Tilletia* species. *Canad J Plant Pathol* 21: 78–80.
- McDonald JG, Wong E, White GP (2000) Differentiation of *Tilletia* spp. by rep-PCR genomic printing. *Plant Dis* 84: 1121–1125.
- McMaugh SJ, Lyon BR (2003) Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. *Biotechniques* 34: 982–986.
- Mehl HL, Epstein L (2007) Identification of *Fusarium solani* f.sp. *cucurbitae* race 1 and race 2 with PCR and production of disease-free pumpkin seeds. *Plant Dis* 91: 1288–1292.
- Mehl HL, Epstein L (2008) Sewage and community shower drains are environmental reservoirs of *Fusarium solani* species complex group 1, a human and plant pathogen. *Environ Microbiol* 10: 219–227.
- Meijerink G (1997) The International Seed Health Initiative. In: Hutchins D, Reeves JC (ed), *Seed Health Testing*, CAB International, Oxon, UK, pp. 87–94.
- Meng J, Wang Y (2010) Rapid detection of *Phytophthora nicotianae* in infected tobacco tissues and soil samples based on its *Ypt1* gene. *J Phytopathol* 158: 1–7.
- Mertely JC, Legard DE (2004) Detection, isolation and pathogenicity of *Colletotrichum* spp. from strawberry petioles. *Plant Dis* 88: 407–412.
- Mew T, Bride J, Hibino H, Bonman J, Merca S (1988) Rice pathogens of quarantine importance. *Proc Interat Workshop on Rice Seed Health*, Internat Rice Research Inst Los Banos, Philippines, pp. 101–115.
- Meyer UM, Spotts RA (2000) Detection and quantification of *Botrytis cinerea* by ELISA in pear stems during cold storage. *Plant Dis* 84: 1099–1103.
- Meyer L, Sanders GM, Jacobs R, Korsten L (2006) A one-day sensitive method to detect and distinguish between the citrus black spot pathogen *Guignardia citricarpa* and the endophyte *Guignardia mangiferae*. *Plant Dis* 90: 97–101.
- Micales JA, Bonde MR (1995) Isozymes: Methods and applications. In: Singh RP and Singh US (ed) *Molecular Methods in Plant Pathology*, CRC/Lewis Publishers, Boca Ratan, USA
- Miller SA, Bhat RG, Scmitthener (1994) Detection of *Phytophthora capsici* in peper and cucurbit crops in Ohio with two commercial immunoassay kits. *Plant Dis* 78: 1042–1046.
- Mills PR, Sreenivasaprasad J, Brown AE (1992) Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. *FEMS Microbiol Lett* 98: 137–143.
- Minerdi D, Moretti M, Li Y, Gaggero L, Garibaldi A, Gullino ML (2008) Conventional PCR and real-time quantitative PCR detection of *Phytophthora cryptogea* on *Gerbera jamesonii*. *Eur J Plant Pathol* 122: 227–237.
- Mirzaei S, Goltapeh EM, Shams-Bakhsh M, Safaie N (2008) Identification of *Botrytis* spp. on plants grown in Iran. *J Phytopathol* 156: 21–28.
- Montes-Borrego M, Muñoz Ledesma FJ, Jiménez-Díaz, Land BB (2009) A nested polymerase reaction protocol for detection and population biology studies of *Peronospora arborescens*, the downy mildew pathogen of opium poppy, using herbarium specimens and asymptomatic fresh plant tissues. *Phytopathology* 99: 73–81.
- Morrice S, Ragazzi A, Kasuga T, Mitchelson KR (1998) Detection of *Fusarium oxysporum* f.sp. *vasinfectum* in cotton tissue by polymerase chain reaction. *Plant Pathol* 47: 486–494.
- Mostert L, Groenewald JZ, Summerbell RC, Robert V, Sutton DA, Padhye AA, Crous PW (2005) Species of *Phaeoacremonium* associated with infection in humans and environmental reservoirs in infected woody plants. *J Clin Microbiol* 43: 1752–1767.
- Moukhamedov R, Hu X, Nazar RN, Robb J (1994) Use of polymerase chain reaction amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. *Phytopathology* 84: 256–259.
- Mulé G, Suca A, Stea G, Moretti A (2004) A species-specific PCR based on the calmodulin partial gene for identification of *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans*. *Eur J Plant Pathol* 110: 495–502.
- Mullis KB (1990) The unusual origin of the polymerase chain reaction. *Sci Amer* April 56.



- Mullis KB, Faloona FA (1987) Specific synthesis of DNA *in vitro* via polymerase catalyzed chain reaction. *Meth Enzymol* 155: 335–350.
- Mumford R, Boonham N, Tomlinson J, Barker I (2006) Advances in molecular phytodiagnostics – new solutions for old problems. *Eur J Plant Pathol* 116: 1–19.
- Nakamura M, Suprapta DN, Iwai H (2008) Differentiation of pathogenic and nonpathogenic isolates of *Geotrichum candidum* sensu Suprapta on citrus fruit based on PCR-RFLP analysis of rDNA, ITS and PCR using specific primers designed in polygalacturonase genes. *Mycoscience* 49: 155–158.
- Nannapaneni R, Gergerich RC, Lee FN (2000) Technology for rapid detection, identification and quantification of rice blast fungus *Pyricularia grisea*. *Ark Agric Exp Sta Res Ser No* 476: 480–485.
- Narayanasamy P (2001) *Plant Pathogen Detection and Disease Diagnosis*, Second edition, Marcel Dekker Inc., New York.
- Narayanasamy P (2005) *Immunology in Plant Health and Its Impact on Food Safety*, The Haworth Press, New York.
- Nanayakkara UN, Mathuresh Singh, Al Mugharabi KI, Peters RD (2009) Detection of *Phytophthora erythroseptica* in above-ground potato tissues, progeny tubers, stolons and crop debris using PCR techniques. *Amer J Potato Res*: DOI 10.1007/s12230-009-9077-x
- Nazar RN, Hu X, Schmidt J, Gulham D, Robb J (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. *Physiol Mol Plant Pathol* 39: 1–11.
- Newton AC (1991) Isozyme analysis in isolates of some facultative phytopathogenic fungi. *J Phytopathol* 131: 199.
- Newton AC, Caten CE, Johnson R (1985) Variations for isozymes and double-stranded RNA among isolates of *Puccinia striiformis* and two other cereal rusts. *Plant Pathol* 34: 235.
- Nicholson P, Simpson DR, Wilson AH, Chandler E, Thomsett M (2004) Detection and differentiation of trichothecene- and enniatin-producing *Fusarium* species on small grain cereals. *Eur J Plant Pathol* 110: 503–514.
- Nicolaisen M, Justesen AF, Thrane U, Skoube P, Holmström K (2005) An oligonucleotide microarray for the identification and differentiation of trichothecene producing and nonproducing *Fusarium* species occurring on cereal grains. *J Microbiol Meth* 62: 57–69.
- Nicolaisen M, Suproniene S, Nielsen LK, Lazzaro I, Spliid NH, Justesen AF (2009) Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *J Microbiol Meth* 76: 234–240.
- Niepold F, Schöber-Butin G (1995) Application of PCR technique to detect *Phytophthora infestans* in potato tubers and leaves. *Microbiol Res* 150: 379–385.
- Niessen L, Vogel RF (1997) Specific identification of *Fusarium graminearum* by PCR with *gaoA* targeted primers. *Syst Appl Microbiol* 20: 111–123.
- Northover J, Cerkaskas RF (1994) Detection and significance of symptomless latent infection of *Monilinia fructicola* in plums. *Canad J Plant Pathol* 16: 30–36.
- O'Brien PA (2008) PCR primers for specific detection of *Phytophthora cinnamomi*. *Austr Plant Pathol* 37: 69–71.
- O'Gorman DT, Sholberg PL, Stokes SC, Ginns J (2008) DNA sequence analysis of herbarium specimens facilitates the revival of *Botrytis mali*, a postharvest pathogen of apple. *Mycologia* 100: 227–235.
- Ogoshi A, Oniki M, Araki T, Ui T (1983) Anastomosis groups of binucleate *Rhizoctonia* spp. in Japan and North America and their perfect stages. *Trans Mycol Soc Jpn* 24: 79–87.
- Okoli CAN, Carder JH, Barbara DJ (1994) Restriction fragment length polymorphisms (RFLPs) and the relationships of some host-adopted isolates of *Verticillium dahliae*. *Plant Pathol* 43: 33–40.
- Okubara PA, Schroeder KL, Paulitz TC (2008) Identification and quantification of *Rhizoctonia solani* and *R. oryzae* using real-time polymerase chain reaction. *Phytopathology* 98: 837–847.
- Old KM, Morgan GF, Bell JC (1984) Isozyme variability among isolates of *Phytophthora cinnamomi* from Australia and Papua New Guinea. *Canad J Bot* 62: 2016.
- Oliver RP, Farman ML, Jones JDG, Harmon-Kosack KE (1993) Use of fungal transformants expressing  $\beta$ -glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. *Molec Plant-Microbe Interact* 6: 521–525.



- Olsson CHB, Heiberg N (1997) Sensitivity of the ELISA test to detect *Phytophthora fragariae* var. *rubi* in raspberry roots. J Phytopathol 145: 285–288.
- Omer MA, Johnson DA, Douhan LI, Hamm PB, Rowe RC (2008) Detection, quantification and vegetative compatibility of *Verticillium dahliae* in potato and mint production soils in the Columbia basin of Oregon and Washington. Plant Dis 92: 1127–1131.
- Orihara S, Yamamoto T (1998) Detection of resting spores of *Plasmodiophora brassicae* from soil and plant tissues by enzyme immunoassay. Ann Phytopathol Soc Jpn 64: 569–573.
- Otero AJ, Sarracent J, Hernández H, Sánchez M, Muirragui D, Villamar M, Moreta D, Jiménez MI, Pérez L, Maribona RH (2007) Monoclonal antibody-based TAS-ELISA for quantitative detection of *Mycosphaerella fijiensis*. J Phytopathol 155: 713–719.
- Oudemans P, Coffey MD (1991a) Isozyme comparison within and among world wide sources of three morphologically distinct *Phytophthora*. Mycol Res 95: 19–30.
- Oudemans P, Coffey MD (1991b) A revised systematics of twelve papillate *Phytophthora* species based on isozyme analysis. Mycol Res 95: 1025–1046.
- Overton BE, Stewart EL, Qu X, Wenner NG, Christ BJ (2004) Qualitative real-time PCR-SYBR® Green detection of Petri disease fungi. Phytopathol Mediterr 43: 403–410.
- Paavananen-Huhtala S, Hyvönen J, Bulat SA, Yli-Mattilia T (1999) RAPD-PCR, isozyme, rDNA, RFLP and rDNA sequence analysis in identification of Finnish *Fusarium oxysporum* isolates. Mycol Res 103: 625–634.
- Padliya ND, Garrett WM, Campbell KB, Tabb DL, Cooper B (2007) Tandem mass spectrometry for the detection of plant pathogenic fungi and the effects of database composition on protein inferences. Proteomics 7: 3932–3942.
- Padmanabhan R, Mohanraj D, Alexander KC, Jothi R (1995) Early and rapid detection of sugarcane smut by histological/immunological methods. In: Detection of Plant Pathogens and Their Management (ed) Verma JP, Varma A, Dinesh Kumar, Angkor Publishers, New Delhi, India, pp. 344–356.
- Pan SQ, Ye XS, Kuć J (1991) A technique for detection of chitinase  $\beta$ -1,3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocusing. Phytopathology 81: 970–974.
- Panabieres F, Marais A, Trentin F, Bonnet P, Ricci P (1989) Repetitive DNA polymorphism analysis as a tool for identifying *Phytophthora* species. Phytopathology 79: 1105–1109.
- Pasquali M, Dematheis F, Gilardi G, Gullino ML, Garibaldi A (2005) Vegetative compatibility groups of *Fusarium oxysporum* f.sp. *lactucae* from lettuce. Plant Dis 89: 237–240.
- Patiño B, Mirete S, González-Jaen MT, Mulé G, Rodríguez T, Vázquez C (2004) PCR detection assay of fumonisin producing *Fusarium verticillioides* strains. J Food Protect 67: 1278–1283.
- Patzak J (2003) PCR detection of hop fungal pathogens. Proc Internat Hop Growers Conv. Dobrna-Zalec, Slovenia, pp. 12–16.
- Paulitz TC, Schroeder KL (2005) A new method for the quantification of *Rhizoctonia solani* and *R. oryzae* from soil. Plant Dis 89: 767–772.
- Pekárová B, Krátka J, Slovák J (2001) Utilization of immunochemical methods to detect *Phytophthora fragariae* in strawberry plants. Plant Protect Sci 37: 57–65.
- Peraza-Echeverría L, Rodríguez-García CM, Zapata-Salazar DM (2008) A rapid, effective method for profuse *in vitro* conidial production of *Mycosphaerella fijiensis*. Austr Plant Pathol 37: 460–463.
- Peres NA, Harakava R, Adaskaveg JE, Timmer LW (2007) Comparison of molecular procedures for detection and identification of *Guignardia citricarpa* and *G. mangiferae*. Plant Dis 91: 525–531.
- Pérez-Hernández O, Nam MH, Gleason ML, Kim HG (2008) Development of a nested polymerase chain reaction assay for detection of *Colletotrichum acutatum* on symptomless strawberry leaves. Plant Dis 92: 1665–1671.
- Peters RD, Platt (Bud) HW, Hall R (1999) Use of allozyme markers to determine genotype to *Phytophthora infestans* in Canada. Canad J Plant Pathol 21: 144–153.
- Peterson GL, Bonde MR, Phillips JG (2000) Size-selective sieving for detecting teliospores of *Tilletia indica* in wheat seed samples. Plant Dis 84: 999–1007.
- Pethybridge SJ, Hay F, Jones S (2006) Seedborne infection of pyrethrum by *Phoma ligulicola*. Plant Dis 90: 891–897.

- Pettitt TR, Wakeham AJ, Wainwright MF, White JG (2002) Comparison of serological, culture, and bait methods for detection of *Pythium* and *Phytophthora* zoospores in water. *Plant Pathol* 51: 720–727.
- Pianzola MJ, Moscatelli M, Vero S (2004) Characterization of *Penicillium* isolates associated with blue mold in apple in Uruguay. *Plant Dis* 88: 23–28.
- Plantiño-Álvarez B, Rodríguez-Cámara MC, Rodríguez Fernandez T, González-Jaen MT, Vázquez Estévez C (1999) Immunodetection of an exo-polygalacturonase in tomato plants infected with *Fusarium oxysporum* f.sp. *radicis-lycopersici*. *Bol Sandad Vegetal Plagas* 25: 529–536.
- Plyler TR, Simone GW, Fernandez D, Kistler HB (1999) Rapid detection of the *Fusarium oxysporum* lineage containing the Canary Island date palm wilt pathogen. *Phytopathology* 89: 407–413.
- Polashock JJ, Vaiciunas J, Oudemans PV (2005) Identification of a new *Phytophthora* species causing root and runner rot of cranberry in New Jersey. *Phytopathology* 95: 1237–1243.
- Polevaya Y, Alkalai-Tuvia S, Copel A, Fallik E (2002) Early detection of gray mold development in tomato after harvest. *Postharvest Biol Technol* 25: 221–225.
- Prasad MNN, Bhat SS, Charith Raj AP, Janardhana GR (2006) Molecular detection of *Phomopsis azadirachtae*, the causative agent of dieback disease of neem by polymerase chain reaction. *Curr Sci* 91: 158–159.
- Priestley RA, Dewey FM (1993) Development of a monoclonal antibody immunoassay for the eye spot pathogen *Pseudocercospora herpotrichoides*. *Plant Pathol* 42: 403–412.
- Prospiero S, Black JA, Winton LM (2004) Isolation and characterization of microsatellite markers in *Phytophthora ramorum*, the causal agent of sudden oak death. *Molec Ecol Notes* 4: 672–624.
- Pryor BM, Davis RM, Gilbertson RL (1994) Detection and eradication of *Alternaria radicina* in carrot seed. *Plant Dis* 78: 452–456.
- Puhalla JE (1979) Classification of isolates of *Verticillium dahliae* based on heterokaryon incompatibility. *Phytopathology* 69: 1186–1189.
- Puhalla JE (1985) Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Canad J Bot* 63: 1305–1308.
- Puhalla JE, Hummel M (1983) Vegetative incompatibility groups within *Verticillium dahliae*. *Phytopathology* 73: 1305–1308.
- Qi YX, Zhang X, Pu JJ, Xie YX, Zhang HQ, Huang SL, Li SL, Zhang H (2009) Nested PCR assay for detection of *Corynespora* leaf fall disease caused by *Corynespora cassicola*. *Austr Plant Pathol* 38: 141–148.
- Qu X, Kavanagh JA, Egan D, Christ BJ (2006) Detection and quantification of *Spongopora subterranea* f.sp. *subterranea* by PCR in host tissue and naturally infested soils. *Amer J Potato Res* 83: 21–30.
- Rachdawong S, Cramer CL, Grabau EA, Stromberg VK, Lacy GH, Strombeg EL (2002) *Gauemannomyces graminis* vars. *avenae*, *graminis* and *tritici* identified using PCR amplification of avinacinase-like genes. *Plant Dis* 86: 652–660.
- Radisšek S, Jakše J, Javornik B (2004) Development of pathotype-specific SCAR markers for detection of *Verticillium albo-atrum* isolates from hop. *Plant Dis* 88: 1115–1122.
- Rafin C, Nodet P, Tirilly R (1994) Immunoenzymatic staining procedure for *Pythium* species with filamentous noninflated sporangia in soilless cultures. *Mycol Res* 98: 535–541.
- Rajeswari S, Palaniswami A, Rajappan K (1997) A chemodiagnostic method for the detection of symptomless latent infection of *Colletotrichum musae* in banana fruits. *Plant Dis Res* 12: 50–52.
- Rasmussen OF, Wulff BS (1991) Detection of *Pseudomonas syringae* pv. *pisi* using PCR. *Proc 4<sup>th</sup> Internat Working Group*, Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 367–376.
- Rey MEC (1984) Immunofluorescence and protein A- gold technique in Lowicryl K4 M embedded tissue. *J Microscope* 136: 373–381.
- Ridgway HJ, Steyaert JM, Pottinger BM, Carpenter M, Nicol D, Stewart A (2005) Development of an isolate-specific marker for tracking *Phaeoconiella chlamydospora* infection in grapevines. *Mycologia* 97: 1093–1101.

- Rigotti S, Gindro K, Richter H, Viret O (2002) Characterization of molecular markers for specific and sensitive detection of *Botrytis cinerea* Pers.: Fr. in strawberry (*Fragaria x ananassa* Duch.) using PCR. FEMS Microbiol Lett 209: 169–174.
- Rigotti S, Viret O, Gindro K (2006) Two new primers highly specific for the detection of *Botrytis cinerea* Pers.: Fr. Phytopathol Mediterr 45: 253–260.
- Ristaino JB, Johnson A, Blanco-Meneses M, Liu B (2007) Identification of the tobacco blue mold pathogen *Peronospora tabacina* by polymerase chain reaction. Plant Dis 91: 685–691.
- Rittenburg JH, Petersen FP, Grothaus GD, Miller SA (1988) Development of a rapid, field-usable immunoassay format for detection and quantification of *Pythium*, *Rhizoctonia* and *Sclerotinia* spp. in plant tissues. Phytopathology 78: 156.
- Roberts RG (2005) *Alternaria yaliinficiens* sp. nov. on Ya Li pear fruit: from interception to identification. Plant Dis 89: 134–145.
- Roberts IM, Harrison BD (1979) Detection of potato leafroll and potato mop top viruses by immunosorbent electron microscopy. Ann Appl Biol 93: 289–297.
- Robold AV, Hardham AR (1998) Production of species-specific antibodies that react with surface components on zoospores and cysts of *Phytophthora nicotianae*. Canad J Microbiol 44: 1161–1170.
- Roebroeck EJA, Groen NPA, Mes JJ (1990) Detection of latent *Fusarium oxysporum* infection in gladiolus corms. Acta Horti 266: 468–476.
- Rollo F, Amici A, Foesi F, di Silvestro L (1987) Construction and characterization of a cloned probe for the detection of *Phoma tracheiphila* in plant tissues. Appl Microbiol Biotechnol 26: 352.
- Rollo F, Salvi R, Torchia P (1990) Highly sensitive and fast detection of *Phoma tracheiphila* by polymerase chain reaction. Appl Microbiol Biotechnol 32: 572–576.
- Rolshausen PE, Trouillas FP, Gubler WD (2004) Identification of *Eutypa lata* by PCR-RFLP. Plant Dis 88: 925–929.
- RueyShyang C, JwuGuh T, YuFen H, Chiou RYY (2002) Polymerase chain reaction-mediated characterization of molds belonging to the *Aspergillus flavus* group and detection of *Aspergillus parasiticus* in peanut kernels by multiplex polymerase chain reaction. J Food Protect 65: 840–844.
- Ruiz E, Ruffner HP (2002) Immunodetection of Botrytis-specific invertase in infected grapes. J. Phytopathol 150: 76–85.
- Saiki RK, Walsh PS, Levenson CH, Ehrlich HA (1989) Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc. Nat Acad Sci USA 86: 6230–6234.
- Salazar O, Julian MC, Rubio V (2000) Primers based on specific rDNA-ITS sequences for PCR detection of *Rhizoctonia solani*, *R. solani* AG2 subgroups and ecological types and binucleate *Rhizoctonia*. Mycol Res 104: 281–285.
- Salinas J, Schots A (1994) Monoclonal antibodies-based immunofluorescence test for detection of conidia of *Botrytis cinerea* on cut flowers. Phytopathology 84: 351–356.
- Sarlin T, Yli-Mattila T, Jestoi M, Rizzo A, Paavanen-Huhtala S, Harikara A (2006) Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt. Eur J Plant Pathol 114: 371–380.
- Sauer KM, Hulbert SH, Tisserat NA (1993) Identification of *Ophiostoma herpotricha* by cloned DNA probes. Phytopathology 83: 97–102.
- Savage SD, Sall MA (1981) Radioimmunosorbent assay for *Botrytis cinerea*. Phytopathology 71: 411–415.
- Sayler RJ, Yang Y (2007) Detection and quantification of *Rhizoctonia solani* AG-1 IA, the rice sheath blight pathogen in rice using real-time PCR. Plant Dis 91: 1663–1668.
- Says-Lesage V, Meliala C, Nicolas P, Roeckel-Drevet P, de Labrouhe TD, Archambault D, Billand F (2001) Molecular test to show the presence of mildew (*Plasmopara halstedii*) in sunflower seeds. OCL-Oléagineux, Corps Gras, Lipides 8: 258–260.
- Schaad NW, Frederick RD, Shaw J, Schneider WL, Hickson R, Petrillo MD, Luster DG (2003) Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. Annu Rev Phytopathol 41: 305–324.
- Schena L, Nigro F, Ippolito A (2002) Identification and detection of *Rosellinia necatrix* by conventional and real-time Scorpion-PCR. Eur J Plant Pathol 108: 355–366.

- Schena L, Hughes KJ, Cooke DEL (2006) Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. *Mol Plant Pathol* 7: 365–379.
- Schena L, Duncan JM, Cooke DEL (2008) Development and application of a PCR-based “molecular tool box” for the identification of *Phytophthora* species damaging forests and natural ecosystems. *Plant Pathol* 57: 64–75.
- Schenk S (1998) Evaluation of a PCR amplification method for detection of systemic smut infection in sugarcane. *SugarCane* No.6: 2–5.
- Schenk PM, Kazan K, Manners JM, Anderson JP, Simpson RS, Wilson IW, Somerville SC, Maclean DJ (2003) Systemic gene expression in *Arabidopsis* during an incompatible interaction with *Alternaria brassicola*. *Plant Physiol* 132: 999–1010.
- Schlenzig A, Habermeyer J, Zinkernagel V (1999) Serological detection of latent infection with *Phytophthora infestans* in potato stems. *J Plant Protect Plant Dis* 106: 221–230.
- Schlenzig A, Cooke DEL, Chard JM (2005) Comparison of a baiting method and PCR for the detection of *Phytophthora fragariae* var. *rubi* in certified raspberry stocks. *EPPO Bull* 35: 87–91.
- Schnerr H, Niessen L, Vogel R (2001) Real-time detection of the *tri5* gene in *Fusarium* species by Light Cycler™-PCR using SYBR® Green I for continuous fluorescence monitoring. *Internat J Food Microbiol* 71: 53–61.
- Schroeder KL, Okubara PA, Tambong JT, Lévesque CA, Paulitz TC (2006) Identification and quantification of pathogenic *Pythium* spp. from soils in eastern Washington using real-time polymerase chain reaction. 96: 637–647.
- Schulze S, Bahnweg G (1998) Critical review of identification techniques for *Armillaria* spp. and *Heterobasidion annosum* root and butt rot diseases. *J Phytopathol* 146: 61–72.
- Schweigkofler W, O'Donnell K, Garbelotto M (2004) Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker from two California sites by using a real-time PCR approach combined with a simple spore trapping method. *Appl Environ Microbiol* 70: 3512–3520.
- Scott Jr DL, Clark CW, Tooley PW, Carras MM, Maas JL (2002) The use of biomagnetic separation to recover DNA suitable for PCR from *Claviceps* species. *Lett Appl Microbiol* 31: 95–99.
- Serdani M, Crous PW, Holz G, Petrini O (1998) Endophytic fungi associated with core rot of apples in South Africa with specific reference to *Alternaria* species. *Sydowia* 50: 257–271.
- Serdani M, Kang JC, Andersen B, Crous PW (2002) Characterization of *Alternaria* species-groups associated with core rot of apples in South Africa. *Mycol Res* 106: 561–569.
- Sheridan GEC, Masters CI, Shallcross JA, Mackey BM (1998) Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Appl Environ Microbiol* 64: 1313–1318.
- Shi L, Hu W, Su Z, Lu X, Tong W (2003) Microarrays: Technologies and applications. *Appl Mycol Biotechnol Ser Vol 3*, Elsevier Sci BV, Amsterdam, pp. 271–293.
- Silva-Mann R, Vieira MGGC, Machado JC, Filho JRB, Salgado KCC, Stevens MR (2005) AFLP markers differentiate isolates of *Colletotrichum gossypii* from *C. gossypii* var. *cephalosporioides*. *Fitopatol Brasileira* 30: 169–172.
- Silvar C, Díaz J, Merino F (2005a) Real-time polymerase chain reaction quantification of *Phytophthora capsici* in different pepper genotypes. *Phytopathology* 95: 1423–1429.
- Silvar C, Duncan JM, Cooke DEL, Williams NA, Díaz J, Merino F. (2005b) Development of specific PCR primers for identification and detection of *Phytophthora capsici*. *Eur J Plant Pathol* 112 : 43–52.
- Sinha OK, Singh Kishan, Mishra R (1982) Stain technique for detection of smut hyphae in nodal buds of sugarcane. *Plant Dis* 66: 932–933.
- Sippell DN, Hall R (1995) Glucose phosphate isomerase polymorphism distinguish weakly virulent from highly virulent strains of *Leptosphaeria maculans*. *Canad J Plant Pathol* 17: 1–6.
- Sissons JGP, Oldstone MBA (1980) Antibody-mediated destruction of virus-infected cells. *Adv Immunol* 29: 209–260.

- Skottrup P, Frokiaer H, Hearty S, O'Kennedy R, Hejgaard J, Nicolaisen M, Justesen AF (2007a) Monoclonal antibodies for the detection of *Puccinia striiformis* urediniospores. *Mycol Res* 111: 332–338.
- Skottrup P, Nicolaisen M, Justesen AF (2007b) Rapid determination of *Phytophthora infestans* sporangia using a surface plasmon resonance immunosensor. *J Microbiol Meth* 68: 507–515.
- Smith DR, Stanosz GR (2006) A species-specific PCR assay for detection of *Diplodia pinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. *Plant Dis* 90: 307–313.
- Smith PK, Krohn RI, Hermanson GT (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76–85.
- Sneh N, Burpee L, Ogoshi A (1991) Identification of *Rhizoctonia* species. The Amer Phytopathol Soc St. Paul MN, USA.
- Söchting HP, Verreet JA (2004) Effects of different cultivation systems (soil management, nitrogen fertilization) on the epidemics of fungal diseases in oilseed rape (*Brassica napus* L. var. *napus*). *J Plant Dis Protect* 111: 1–29.
- Somai BM, Keinath AP, Dean RA (2002) Development of PCR-ELISA detection and differentiation of *Didymella bryoniae* from related *Phoma* species. *Plant Dis* 86: 710–716.
- Stace-Smith R, Bowler DJ, McKenzie DJ, Ellis P (1993) Monoclonal antibodies differentiate the weekly virulent from highly virulent strain of *Leptosphaeria maculans*, the organism causing black leg of canola. *Canad J Bot* 15: 127–133.
- Stackebrandt E, Liesack W, Witt D (1992) Ribosomal RNA and rDNA sequence analyses. *Gene* 115: 225–260.
- Strandberg JO (2002) A selective medium for the detection of *Alternaria dauci* and *Alternaria radicina*. *Phytoparasitica* 30: 269–284.
- Sundaram S, Plascencia S, Banttari EE (1991) Enzyme-linked immunosorbent assay for detection of *Verticillium* spp. using antisera produced to *V. dahliae* from potato. *Phytopathology* 81: 1485–1489.
- Svircev AM, Gardiner RB, McKeen WE, Day AW, Smith RJ (1986) Detection by protein A-gold of antigens of *Botrytis cinerea* in cytoplasm of infected *Vicia faba*. *Phytopathology* 76: 622–626.
- Szemes M, Bonants P, de Weerd M, Baner J, Landegren U, Schoen CD (2005) Diagnostic application of padlock probes-multiplex detection of plant pathogens using universal micro-arrays. *Nucleic Acids Res* 33(8)- doi 10.1093/nar.gni069
- Takamatsu S, Kano Y (2001) PCR primers useful for nucleotide sequencing of rDNA of the powdery mildew fungi. *Mycoscience* 42: 135–139.
- Takamatsu S, Nakano M, Yokota H, Kunoh H (1998) Detection of *Rhizoctonia solani* AG-2-2-IV, the causal agent of large patch of Zoysia grass, using plasmid DNA as probe. *Ann Phytopathol Soc Jpn* 64: 451–457.
- Takenaka S (1992) Use of immunological methods with antiribosome serums to detect snow mold fungi in wheat plants. *Phytopathology* 82: 896–901.
- Takenaka S, Kawasaki S (1994) Characterization of alanine-rich hydroxyproline, containing cell wall proteins and their application for identifying *Pythium* species. *Physiol Molec Plant Pathol* 45: 249–261.
- Tambong JT, de Cock AWAM, Tinker NA, Lévesque CA (2006) Oligonucleotide array for identification and detection of *Pythium* species. *Appl Environ Microbiol* 72: 2691–2706.
- Tan MK, Ghalayini A, Sharma I, Yi J, Shivas R, Priest M, Wright D (2009) A one-tube fluorescent assay for the quarantine detection and identification of *Tilletia indica* and other grass bunts in wheat. *Austr Plant Pathol* 38: 101–109.
- Tanaka A, Kitabayashi H, Tani T, Ogoshi A (1994) A pathogen causing patch so-called “elephant footprint” on zoysia grasses. *Ann Phytopathol Soc Jpn* 60: 344.
- Tempel A (1959) Serological investigations in *Fusarium oxysporum*. *Meded No.* 138 Lanbouwhogeschool, Wageningen 59: 1–60.



- Tenzer I, delgi Ivanisovic S, Morgante M, Gessler C (1999) Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. *Phytopathology* 89: 748–753.
- Thelwell N, Millington S, Solinas A, Booth J, Brown T (2000) Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res* 28: 3752–3761.
- Themann K, Werres S, Diener HA, Lüttmann R (2002) Comparison of different methods to detect *Phytophthora* spp. in recycling water from nurseries. *J Plant Pathol* 84: 41–50.
- Thornton CR, O'Neill TM, Hilton G, Gilligan CA (1999) Detection and recovery of *Rhizoctonia solani* in naturally infested glasshouse soils using a combined baiting double monoclonal antibody ELISA. *Plant Pathol* 48: 627–634.
- Timmer LW, Menge JA, Zitko SE, Pond E, Miller SA, Johnson EL (1993) Comparison of ELISA techniques and standard isolation methods for *Phytophthora* detection in citrus orchards in Florida and California. *Plant Dis* 77: 791–796.
- Tisserat NA, Hulbert SH, Nus A (1991) Identification of *Leptosphaeria korrae* by cloned DNA probes. *Phytopathology* 81: 917–921.
- Tisserat NA, Hulbert SH, Sauer KM (1994) Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiostoma korrae* and *O. herpotricha*. *Phytopathology* 84: 478–482.
- Toit LJ, Derie ML, Hernandez-Perez P (2005) Verticillium wilt in spinach production. *Plant Dis* 89: 4–11.
- Tomioka K, Sato T (2001) Restriction landmark genomic scanning (RLGs) in fungi. *Mycoscience* 42: 295–299.
- Tomlinson JA, Boonham N, Hughes KJO, Griffin RL, Barker I (2005) On-site DNA extraction and real-time PCR for detection of *Phytophthora ramorum* in the field. *Appl Environ Microbiol* 71: 6702–6710.
- Tooley PW, Martin FN, Carras MM, Frederick RD (2006) Real-time fluorescent polymerase chain reaction detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. *Phytopathology* 96: 336–345.
- Tooley PW, Fry WE, Villareal-Gonzalez MJ (1985) Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. *J Hered* 76: 431.
- Tooley PW, Carras MM, Lambert DH (1998) Application of a PCR-based test for detection of potato late blight and pink rot in tubers. *Amer J Potato Res* 75: 187–194.
- Triki MA, Pirou S, El-Mahjoub B, Baudry A (2001) Leak syndrome of potato in Tunisia caused by *Pythium aphanidermatum* and *P. ultimum*. *Potato Res* 44: 221–231.
- Trout CL, Ristaino JB, Madritch M, Wangsomboonde T (1997) Rapid detection of *Phytophthora infestans* in late blight-infected potato and tomato using PCR. *Plant Dis* 81: 1042–1048.
- Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol* 14: 303–308.
- Úrbes-Torres JR, Leavitt GM, Guerrero JC, Guevara J, Gubler WD (2008) Identification and pathogenicity of *Lasiodiplodia theobromae* and *Diplodia seriata*, the causal agents of Bot canker disease of grapevines in Mexico. *Plant Dis* 92: 519–529.
- Urena-Padilla AR, Mac Kenzie SJ, Bowen BW, Legard DE (2002) Etiology and population genetics of *Colletotrichum* spp. causing crown and fruit rot of strawberry. *Phytopathology* 92: 1245–1252.
- Vakalounakis DJ (1996) Root and stem rot of cucumber caused by *Fusarium oxysporum* f.sp. *radicis-cucumerinum* f.sp. nov. *Plant Dis* 80: 313–316.
- Vakalounakis DJ, Fragkiadakis GA (1999) Genetic diversity of *Fusarium oxysporum* isolates from cucumber: differentiation by pathogenicity, vegetative compatibility and RAPD fingerprinting. *Phytopathology* 89: 161–168.
- Vallad GE, Bhat RG, Koike ST, Ryder EJ, Subbarao KV (2005) Weed-borne reservoirs and seed transmission of *Verticillium dahliae* in lettuce. *Plant Dis* 89: 317–324.
- Valseesia G, Gobbin D, Patocchi A, Vecchione A, Pertot I, Gessler C (2005) Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. *Phytopathology* 95: 672–678.



- van de Graaf P, Lees AK, Cullen DW, Duncan JM (2003) Detection and quantification of *Spongospora subterranea* in soil, water and plant tissue samples using real-time PCR. *Eur J Plant Pathol* 109: 589–597.
- van de Graaf P, Lees AK, Wale SJ, Duncan JM (2005) Effect of soil inoculum level and environmental factors on potato powdery scab caused by *Spongospora subterranea*. *Plant Pathol* 54: 22–28.
- van Doorn R, Szemes M, Bonants P, Kowalchuk GA, Salles JF, Ortenberg E, Schoen CD (2007) Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on Open Arrays™. *BMC Genomics* 8: 276 doi: 10.1186/1471-2164-8-276
- van Gent-Pelzer MPE, Krijger M, Bonants PJM (2010) Improved real-time PCR assay for detection of the quarantine potato pathogen *Synchytrium endobioticum* in zonal centrifuge extracts from soil and in plants. *Eur J Plant Pathol* 126: 129–133.
- Van Regenmortel MHV (1982) *Serology and Immunochemistry of Plant Viruses*, Academic Press, New York.
- Vandemark GJ, Ariss JJ (2007) Examining interaction between legumes and *Aphanomyces euteichus* with real-time PCR. *Austr Plant Pathol* 36: 102–108.
- Vandemark GJ, Barker BM (2003) Quantifying *Phytophthora medicaginis* in susceptible and resistant alfalfa with real-time fluorescent assay. *J Phytopathol* 151: 577–583.
- Velichetti RK, Lamison C, Brill LM, Sinclair JB (1993) Immunodetection of *Phomopsis* species in asymptomatic plants. *Plant Dis* 77: 70–73.
- Verreest JA, Klink H, Hoffmann GM (2000) Regional monitoring for disease prediction and optimization of plant protection measures: the IPM wheat model. *Plant Dis* 84: 816–826.
- Viswanathan R, Samiyappan R, Padmanaban P (1998) Specific detection of *Colletotrichum falcatum* in sugarcane by serological techniques. *SugarCane* No.6: 18–3.
- Vöhringer G, Sander G (2001) Comparison of antibodies in chicken egg yolk (IgY) and rabbit (IgG) for quantitative strain detection of *Colletotrichum falcatum* and *Fusarium subglutinans*. *J Plant Dis Protect* 108: 39–48.
- Waalwijk C, Heide R, van der deVries I, Lee T, van der Schoen C, Coainville GC, Häuser-Hahn I, Kastelein P, Köhl J, Demarquet T, Kema GH (2004) Quantitative detection of *Fusarium* species in wheat using TaqMan. *Eur J Plant Pathol* 110: 481–494.
- Wahlström K, Karlsson JO, Holdenrieder O, Stenlid J (1991) Pectinolytic activity and isoenzymes in European *Armillaria* species. *Canad J Bot* 69: 2732–2739.
- Walcott RR, Gitaitis RD, Langston DB (2004) Detection of *Botrytis aclada* in onion seed using magnetic capture hybridization and the polymerase chain reaction. *Seed Sci Technol* 32: 425–438.
- Wang PH, White JG (1996) Development of a species-specific primer for *Pythium violae*. *Proc BCPC Symp* 65: 205–210.
- Wang YC, Yu RC (1998) Detection of toxigenic *Aspergillus* spp. in rice and corn by ELISA. *J Chinese Agric Chem Soc* 36: 512–520.
- Wang CG, Blanchette RA, Jackson WA, Palmer MA (1985) Differences in conidial morphology among isolates of *Sphaeropsis sapinea*. *Plant Disease* 69: 838–841.
- Wang PH, Lo HS, Yeh Y (2001) Identification of *F. oxysporum cucumerinum* and *F. oxysporum luffae* by RAPD-generated DNA probes. *Lett Appl Microbiol* 33: 397–401.
- Wang PH, Chung CY, Lin YS, Yeh Y (2003a) Use of polymerase chain reaction to detect the soft rot pathogen, *Pythium myriotylum* in infected ginger rhizomes. *Lett Appl Microbiol* 36: 116–120.
- Wang PH, Wang YT, White JG (2003b) Species-specific PCR primers for *Pythium* developed from ribosomal ITS1 region. *Lett Appl Microbiol* 37: 127–132.
- Wang Y, Zhang W, Wang Y, Zheng X (2006) Rapid and sensitive detection of *Phytophthora sojae* in soil and infected soybeans by species-specific polymerase chain reaction assays. *Phytopathology* 96: 1315–1321.
- Wang X, Zheng W, Buchenauer H, Zhao J, Han Q, Huang L, Kang Z (2008) The development of a PCR-based method for detecting *Puccinia striiformis* latent infections in wheat leaves. *Eur J Plant Pathol* 120: 241–247.
- Wangsomboondee T, Ristaino JB (2002) Optimization of sample size and rDNA extraction methods to improve PCR detection of different propagules of *Phytophthora infestans*. *Plant Dis* 86: 247–253.

- Ward E, Gray RM (1992) Generation of a ribosomal DNA probed by PCR and its use in identification of fungi within the *Gauemannomyces-Phialophora* complex. *Plant Pathol* 41: 730–736.
- Ward CM, Wilkinson AP, Bramham S, Lee HA, Chan WHS, Butcher GW, Hutchings A, Morgan MRA (1990) Production and characterization of polyclonal and monoclonal antibodies against aflatoxin B1 oxime-BSA in an enzyme-linked immunosorbent assay. *Mycotoxin Res* 6: 73–83.
- Ward LI, Beales PA, Barnes AV, Lane CR (2004) A real-time PCR assay based method for routine diagnosis of *Spongospora subterranea* on potato tubers. *J Phytopathol* 152: 633–638.
- Watson WT, Kenerley CM, Appel DM (2000) Visual and infra-red assessment of root colonization of apple trees by *Phymatotrichopsis omnivora*. *Plant Dis* 84: 539–543.
- Webster J, Weber R (2007) *Introduction to Fungi*, Third edition, Cambridge University Press, Cambridge, UK.
- Weeds PL, Beever RE, Long PL (1998) New genetic markers for *Botrytis cinerea* (*Botryotinia fuckeliana*). *Mycol Res* 102: 791–800.
- Weier HU, Gray JW (1988) A programmable system to perform polymerase chain reaction. *DNA* 7: 441–447.
- Whisson DL, Herdina A, Francis L (1995) Detection of *Rhizoctonia solani* AG-8 in soil using a specific DNA probe. *Mycol Res* 99: 1299–1302.
- Whiteman SA, Jaspers MV, Stewart A, Ridgway JJ (2002) Detection of *Phaeoconiella chlamydospora* in soil using species-specific PCR. *New Zealand Plant Protoc* 55: 139–145.
- Whiteman SA, Jaspers MV, Stewart A, Ridgway JJ (2004) *Phaeoconiella chlamydospora* detection in the grapevine propagation process by species-specific PCR. *Phytopathol Mediterr* 43: 156.
- Wiechel TJ, Salib S, Edwards J (2005) Real-time PCR detection and quantification of *Phaeoconiella chlamydospora* during grapevine propagation in the nursery. 15<sup>th</sup> Austr Plant Pathol Bienn Conf, Geelong, Australia.
- Wijekoon CP, Goodwin PH, Hsiang T (2008) Quantifying fungal infection of plant leaves by digital image analysis using Scion Image software. *J Microbiol Meth* 74: 94–101.
- Willits DA, Sherwood JE (1999) Polymerase chain reaction detection of *Ustilago hordei* in leaves of susceptible and resistant barley varieties. *Phytopathology* 89: 212–217.
- Wiwart M, Korona A (1998) Application of a color image analysis of kernels in evaluation of the infection of triticale grown in different cultivation system. *Plant Breed Sci* 42: 69–79.
- Woong NK, Hae KC, Haiseong H (1996) Studies on the pear abnormal leaf spot disease. 5. Selection of indicator plants. *Kor J Plant Pathol* 12: 214–216.
- Wu WS, Chen TW (1999) Development of a new selective medium for detecting *Alternaria bras-sicola* in cruciferous seeds. *Seed Sci Technol* 27: 397–409.
- Xiao CL, Rogers JD (2004) A postharvest fruit rot in d'Anjou pears caused by *Sphaeropsis pyriputrescens* sp. nov. *Plant Dis* 88: 114–118.
- Xiaojie W, Chunlei T, Jimlong C, Buchenauer H, Jie Z, Qingmei H, Lili H, Zhensheng K (2009) Detection of *Puccinia striiformis* in latently infected wheat leaves by nested polymerase chain reaction. *J Phytopathol* DOI 10.1111/j.1439-0434.2008.01521.x
- Xu ML, Melchinger AE, Lübberstedt T (1999) Species-specific detection of the maize pathogens *Sporisorium reiliana* and *Ustilago maydis* by dot-blot hybridization and PCR-based assays. *Plant Dis* 83: 390–395.
- Xu X-M, Parry DW, Nicholson P, Thomsett MA, Simpson D, Edwards SG, Cooke BM, Doohan FM, Monaghan S, Moretti A, Tocco G, Mule G, Hornok L, Béki E, Tatnell J, Ritieni A (2008) Within-field variability of Fusarium head blight pathogens and their associated mycotoxins. *Eur J Plant Pathol* 120: 21–34.
- Xue P, Goodwing PH, Annis SL (1992) Pathotype identification of *Leptosphaeria maculans* with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. 141: 179–188.
- Yan L, Zhang C, Ding L, Ma Z (2008) Development of a real-time PCR assay for the detection of *Cladosporium fulvum* in tomato leaves. *J Appl Microbiol* 104: 1417–1424.
- Yao CL, Frederiksen RA, Magill CW (1990) Seed transmission of sorghum downy mildew: detection by DNA hybridization. *Seed Sci Technol* 18: 201–207.

- Yao CL, Magill CW, Frederiksen RA (1991) Use of an A-T rich DNA clone for identification and detection of *Peronospora sorghi*. Appl Environ Microbiol 57: 2027.
- Yates IE, Hiatt KL, Kapczynski DR, Smart W, Glenn AE, Hinton DM, Bacon CW, Meinersmann R, Liu S, Jaworski AJ (1999) GUS transformation of the maize endophyte *Fusarium moniliforme*. Mycol Res 103: 129–136.
- Yin Y, Ding L, Liu X, Yang J, Ma Z (2009) Detection of *Sclerotinia sclerotiorum* in planta by a real-time PCR assay. J Phytopathol 157: 465–469.
- Yoshida S, Tsukiboshi T, Shinohara H, Koitabashi M, Tsushima S (2007) Occurrence and development of *Colletotrichum acutatum* on symptomless blueberry bushes. Plant Pathology 56: 871–877.
- Yu FH, Chiu FS (1998) Analysis of fumonisins and *Alternaria alternata* toxin by liquid chromatography-enzyme-linked immunosorbent assay. J AOAC Internat 81: 749–756.
- Yuan Q, Nian S, Yin Y, Li M, Cai J, Wang Z (2009) Development of a PCR-based diagnostic tool specific to wheat dwarf bunt caused by *Tilletia controversa*. Eur J Plant Pathol 124: 585–594.
- Yuen GY, Craig ML, Avila F (1993) Detection of *Pythium ultimum* with a species-specific monoclonal antibody. Plant Dis 77: 692–698.
- Yuen GY, Xia JQ, Sutula CL (1998) A sensitive ELISA for *Pythium ultimum* using polyclonal and species-specific monoclonal antibodies. Plant Dis 82: 1029–1032.
- Zelinger E, Hawves CR, Gurr SJ, Dewey FM (2004) An immunochemical and ultra-structural study of the extracellular matrix produced by germinating spores of *Stagonospora nodorum* on natural and artificial surfaces. Physiol Mol Plant Pathol 65: 123–135.
- Zhang AW, Hartman GL, Riccinoi L, Chen WD, Ma RZ, Pedersen WL (1997) Using PCR to distinguish *Diaporthe phaseolorum* and *Phomopsis longicolla* from other soybean fungal pathogens and to detect them in soybean tissues. Plant Dis 81: 1143–1149.
- Zhang AW, Hartman GL, Curio-Penny B, Pedersen WL, Becker KB (1999) Molecular detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* from soybean seeds. Phytopathology 89: 796–804.
- Zhang Z, Zhang J, Wang Y, Zheng X (2005) Molecular detection of *Fusarium oxysporum* f.sp. *niveum* and *Mycosphaerella melonis* in infected plant tissues and soil. FEMS Microbiol Lett 249: 39–47.
- Zhang N, Geiser DM, Smart CD (2007) Macroarray detection of solanaceous plant pathogens in the *Fusarium solani* species complex. Plant Dis 91: 1612–1620.
- Zhao J, Wang XJ, Chen CQ, Huang LL, Kang ZS (2007) A PCR-based assay for detection of *Puccinia striiformis* f.sp. *tritici* in wheat. Plant Dis 91: 1669–1674.
- Zheng FC, Ward E (1998) Variation within and between *Phytophthora* species for rubber and citrus trees in China by polymerase chain reaction using RAPDs. J Phytopathol 146: 103–109.
- Zink AR, Reischl U, Wolf H, Nerlich AG (2002) Molecular analysis of ancient microbial infections. FEMS Microbiol Lett 213: 141–147.
- Zur G, Shimoni E, Hallerman E, Kashi Y (2002) Detection of *Alternaria* fungal contamination in cereal grains by a polymerase chain reaction-based assay. J Food Protect 65: 1433–1440.

Microbial Plant Pathogens-Detection and Disease

Diagnosis:

Fungal Pathogens, Vol.1

Narayanasamy, P.

2011, XXII, 291 p., Hardcover

ISBN: 978-90-481-9734-7