Paradigm Shift in Plant Disease Diagnostics: A Journey from Conventional Diagnostics to Nano-diagnostics

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Introduction

Disease diagnosis can be defined as either the identification of a disease or the identification of the agent that causes the disease. The early and rapid identification of a plant pathogen is the key for appropriate management practices to be applied prior for avoiding further spread of the disease or its introduction due to transboundary movement of plant material. The demand for rapid, accurate, sensitive, standard, high throughput and simultaneous detection assays of plant pathogens has risen in the last few decades due to intensive monocropping. Conventional methods relied on study of symptoms and morphological studies. However, it is well known fact that symptoms are not always unique and can be confused with other diseases. Conventional methods are often time-consuming, laborious, and require expert taxonomist. The limitations posed by conventional diagnostics have led to the development of techniques with improved accuracy and reliability. Present era demands fast and sensitive methods for detection and identification of specific fungal pathogens. Accordingly, Plant disease diagnosticians have an array of methodologies that allow much faster, more specific, more sensitive, more accurate and multiple detection of plant fungal pathogens, leaving the need of skilled taxonomist. The present chapter highlights several techniques developed that have revolutionized the field of plant diagnostics.

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Conventional Diagnostics

The conventional way of identification of plant pathogenic fungi involves interpreting visual symptoms of disease or signs like presence of structures: mycelia, sporophore, spores and fructifications of the pathogen (Nezhad 2014). It may be followed by pathogen confirmation using microscopic and cultural techniques. Most of the traditional fungi were identified by taxonomists based on spore morphology and conidiogenesis (Kendrick 1971; Barnett and Hunter 1972; Agrios 2005). The methods related to fungal morphology require extensive knowledge of classical taxonomy. Other limitations include the difficulty of some species to be cultured in vitro, and the inability to accurately quantify the pathogen (Goud and Termorshuizen 2003). Based on symptomatology, cultivar susceptibility and epidemiology *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (FORC) was identified as a different *forma specialis* and was distinguished from *F. oxysporum* f.sp. *cucumerinum* (FOC) (Vakalounakis 1996). The other conventional methods namely: direct inspection of dry seeds, washing test, soaking test, incubation tests, blotter tests, embryo count test and; filter and centrifuge extraction technique were often used for detection of seed borne fungal pathogens (Castro et al. 1994). Visual inspection is relatively easy when symptoms clearly are characteristic of a specific disease. The direct inspection method has been used for detection of seed borne pathogens e.g. Presence of sclerotia of *Sclerotinia sclerotiorum* and *Claviceps purpurea*. Incubation methods such as blotter and agar plate were most popular and frequently used for the detection of a great number of seed-transmitted pathogens (De Tempe and Binnerts 1979; Majumder et al. 2013). Culturing is another traditional method requiring a few days or weeks to detect the presence of a pathogen in a plant. Direct agar plating technique (DAPT) in which acidified potato dextrose agar (APDA having pH=3.5 amended with 25 % strength lactic acid per litre of medium) has been used to detect latent infections in asymptomatic nuts and fruits (Michailides et al. 2005). These methods are cheap and simple but are time consuming, laborious and require skilled labour and cannot diagnose pathogens before the symptoms are observable.

Physical Diagnostics

Electron Microscopy (EM)

EM is one of the most important tool since its first use in Germany in 1939. In electron microscope, a beam of short wave electrons are used instead of visible light passing through conventional light microscopes. It works under high vacuum and focusing is done by electromagnetic/electrostatic lenses (Bos 1983). The specimen to be studied is mounted on a copper grid containing apertures covered with a thin film of plastic (formvar) (Noordam 1973).

Scanning Electron Microscopy (SEM)

Introduction of scanning electron microscopy (SEM) has revolutionized the study of the microscopic world owing to high quality three dimensional images, large magnitude of increase from 10 to 1,000,000 times, rapid processes of image digitalization and acquisition, easiness to prepare and operate samples, as well as affordable costs (Bozzola and Russell 1999). SEM has been potentially used for identification and detection of seed-borne fungi e.g. *Colletotrichum lindemuthianum* in seeds of common bean (*Phaseolus vulgaris* L.), *Colletotrichum truncatum* in maize (*Zea mays* L.) and *Colletotrichum gossypii* var. *cephalosporioides* in cotton (*Gossypium hirsutum* L.) (De Carvalho Alves and Pozza 2012). SEM was also employed effectively for the detection of destructive pathogen *Sphaeropsis sapinea* (anamorph-*Diplodia pinea*) infecting conifers. The pathogen differes in conidial morphology which cannot be recognized by light microscope. However, SEM was able to group 30 isolates of *S. sapinea* into Type-A having smooth conidial surface and Type-B having pits distributed over the conidial surface (Wang et al. 1985).

Cryo-Scanning Electron Microscopy (Cryo -SEM)

Cryo-SEM is used for imaging of samples containing moisture without causing drying artifacts such as extraction, solubilization and shrinkage. This is a rapid method which enables three-dimensional in situ visualization of fungal invasion within roots and is broadly applicable for identification of plant diseases caused by necrotrophic fungi. This method was effectively used to visualize internal infection of wheat (*Triticum aestivum*) roots by the pathogenic fungus *Rhizoctonia solani* AG-8. Cryo-SEM helped in retaining fungal hyphae and root cells in situ in disinteged root tissues, avoiding the distortions that are usually introduced during conventional preparation by chemical fixation, dehydration and embedding (Refshauge et al. 2006). Cryo-SEM has also been used to show that hyphae of *Bipolaris sorokiniana* remain adhered to the wax surfaces of barley leaves by means of an extensive extracellular matrix (Jansson and Akesson 2003).

Serological Diagnostics

Serology is the use of specific antibodies to detect their respective antigens in test samples. Antibodies are composed of immunoglobulin (Ig) proteins produced in the body of the vertebrate in response to the antigens which are usually foreign proteins, complex carbohydrates, polynucleotides or lipopolysaccharides. Each antibody is specific to a particular antigen and binds to it. Antibodies are produced by B lymphocytes and include five classes- IgG, IgM, IgA, IgE and IgD (Hull 2002). The major soluble antibody IgG (Gamma immunoglobulins) is the most commonly used and is Y shaped molecule with two antigen binding sites (Dickinson 2005). Serum

containing antibodies is known as antiserum which is of two types: polyclonal, containing antibodies to all the available epitopes on the antigen, and monoclonal, containing antibodies to single epitope (Hull 2002) Polyclonal antisera was first produced in rabbits as they are most convenient animal, easy to keep, easy to inject with antigens and relatively a straight-forward process to extract blood containing antibodies by slight cut to the ear vein. Later, other animals such as cows, rats, mice and chickens were also used (Singh 2005). Monoclonal antiserum is raised in mice (Harlow and Lane 1988) and is highly specific against a single type of antigen. Both polyclonal and monoclonal antibodies are now available commercially against a wide range of fungal pathogens from companies such as Adgen®, Neogen® Agdia®, Loewe® and Bioreba®. Numerous serological techniques have been developed and are being used for specific detection and identification of plant fungal pathogens as:

Immuno-sorbent Electron Microscopy (ISEM)

This technique was introduced by Derrick (1973) as serologically specific electron microscopy (SSEM) and has been widely used in plant virology (Milne 1972; Milne and Luisoni 1977). Because of its similarity with solid phase immunoassays, the method was known as immunosorbent electron microscopy by Roberts and Harrison (1979). ISEM has been extensively used for detection and in situ characterization of phytoplasma (Musetti and Favali 2004). ISEM combines the specificity of serological assays with the visualization capabilities of the EM. It is an ideal confirmatory test requiring small amount of samples, if the EM facility and specific antisera are available. ISEM 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 (Narayanasamy 2011) e.g. Monoclonal antibodies have been raised against the species-specific eipitopes on the surface of zoospores and cysts *of Phytophthora cinnamomi* to detect six isolates of *P. cinnamomi* and six species of *Pythium* using immunoflorescence (Hardham et al. 1986).

Enzyme Linked Immuno-sorbent Assay (ELISA)

ELISA, a solid phase heterogeneous immunoassay has been proved to be a valuable serological tool in detection of plant fungal pathogens (Casper and Mendgen 1979; Johnson et al. 1982). ELISA is based on the specific recognition capabilities of antibodies. These antibodies are usually derived from the immunization of animals (usually rabbits, mice, chicken or goat) with certain immunogens such as culture filtrates or mycelial compounds. After repeated injections of the immunogen, blood samples are taken and the serum is used either as a whole or it is applied after certain clean-up steps for the ELISA tests. Polyclonal antibodies are mostly used, but often

lead to problem of high background due to reactions with host proteins. However, monoclonal antibodies increasingly available from commercial companies have overcome the problem of background reactions with host proteins. Different monoclonal antibodies are being used to detect specific strains of a pathogen or to detect a group of strains or species. Many variations of ELISA have been developed and include: direct and indirect ELISA procedures. They differ in the way the antigen–antibody complex is detected, but the underlying theory and the final results are the same. Mostly, double antibody sandwich form of ELISA (DAS-ELISA), which is a direct form of ELISA, as described by Clark and Adams (1977) is used for the detection of plant pathogens. In direct ELISA procedures, the antibodies (usually as an IgG fraction of the antiserum) bound to the well surface of the microtitre plate (polystyrene inflexible rigid plates or polyvinyl chloride, flexible plates) capture the fungus. The captured fungus sample is detected by incubation with an antibody-enzyme conjugate followed by addition of color development reagents (substrate or substrate/dye combination). The capturing and detecting antibodies can be from the same or different sources. Since the pathogen is sandwiched between two antibody molecules, this method is called the double antibody sandwich (DAS-ELISA). DAS-ELISA procedure is known to be highly specific and often detects closely related strains (Koenig 1978). There are also several alternative indirect forms of ELISA available for fungus detection. One among them includes: direct antigen coating procedure (DAC-ELISA), the method developed by Mowat (1985) with minor modifications (Hobbs et al. 1987). In this method, plant extracts prepared in a carbonate buffer are applied directly to the wells and antibodies raised in two different animal species are used. This method is by far the simplest of all the forms of ELISA test (Reddy et al. 1988). There are number of examples in which ELISA has been employed for specific detection of plant pathogenic fungi. E.g. ELISA method was used as a tool to detect fungal pathogens *Rhizoctonia solani*, *Pythium* spp. and *Sclerotinia homoeocarpa* in turfgrass (Fidanza and Dernoeden 1995; Shane 1991) and for early detection of karnal bunt pathogen in wheat when the infection levels are very low (Varshney 1999). DAC-ELISA was standardized and used for detection of *Collectotrichum falcatum* causing red rot of sugarcane (Hiremath and Naik 2003). Polyclonal antibodies (IgG K91) were raised to detect a quarantine pathogen of strawberry – *Colletotrichum acutatum* using ELISA (Kratka et al. 2002) and against *Aspergillus oryzae*, the common plant pathogenic fungi found associated with wheat, sorghum and other crops (Kamraj et al. 2012) for use in ELISA.

Dot Immuno-binding Assay (DIBA)

DIBA technique is similar to ELISA in principle except that the plant extracts are spotted on to a nitrocellulose or nylon membrane rather than using a polystyrene plate as the solid support matrix. Unlike in ELISA, where a soluble substrate is used for color development, a precipitating (chromogenic) substrate is used for probing

pathogen. Hydrolysis of chromogenic substrates results in a visible coloured precipitate at the reaction site on the membrane. DIBA procedure was applied for the detection of the resting spores of *Plasmodiophora brassicae*, causing club root disease of crucifers (Orihara and Yamamoto 1998).

Tissue Immuno Blot Assay (TIBA)

TIBA is a variation of DIBA in which a freshly-cut edge of a leaf blade, stem, leaf, tuber, root or an insect is blotted on the membrane, followed by detection with labelled antibodies. This method is simple, does not require elaborate sample preparation or extraction, and even can provide information on the distribution of fungal pathogen in plant tissues. The disadvantages of DIBA and TIBA are possible interference of sap components with the subsequent diagnostic reactions. However, their sensitivity, the relatively short time required to assay large numbers of samples, the need for minimum laboratory facilities for the assay, the ability to store blotted membranes for extended periods, and low costs favor TIBA and DIBA as useful diagnostic techniques. The other advantage is that the samples can be blotted onto the membranes right in the field and such membranes can be carried or shipped by mail for further processing at a central location either within the country or in a different country. A direct tissue immuno blot assay (DTIBA) procedure has been developed to detect *Fusarium* spp. in the transverse sections from stems and crown of tomato and cucumber plants by employing a combination of the MAb (AP19-2) and Fluorescein 5-isothiocyanate (FITC)-conjugated antimouse IgM-sheep IgG (Arie et al. 1995).

Lateral Flow Assay (LFA)

LFA is a one step, fast, simple, versatile based on the serological specificity of polyclonal or monoclonal antibodies. Lateral flow assay also referred to as Dipstick method is equivalent to medical detection systems such as pregnancy kits (Dickinson 2005). The advantages of these devices are that they are simple to use and results are quick, usually in less than 10 min. Lateral Flow Devices (LFDs) typically consist of a porous nitrocellulose membrane bound to a narrow plastic strip on which pathogen-specific antibodies are immobilized in a band partway up the strip. Species-specific antibodies bound to microparticles of latex, colloidal gold, or silica are placed between the band of immobilized antibodies and a sample application pad. The lateral-flow assay kit usually has an inlet for receiving the pathogen infected sap. After the sample fluid is placed in the sample inlet, the sample flows from the sample pad through embedded reagents, in which specific chemical reactions occur by capillary forces. The reaction product continues to flow through the membrane arriving at the capture reagents. The capture reagents are immobilized on the

membrane as a band shape. The captured reaction product generates visually distinguishable color on the bands. Typically two bands are formed on the membrane, one of which is a test band for detecting the sample by its concentration, and the other is the control band for confirming the success of the assay. Sample fluids may continue to flow and can be collected in an absorbent pad. The test kit does not require a permanent dedicated space, high-priced instruments and skillful operators. An LFA test was developed using an IgM monoclonal antibody to detect *Rhizoctonia solani* (sensitivity 3 ng ml^{-1} of antigen) (Thornton et al. 2004).

Polymerase Chain Reaction (PCR) Based Diagnostics

PCR is a popular molecular biology in vitro technique developed in 1983 by Kary Mullis for enzymatically replicating DNA. The technique allows a small amount of DNA molecule to be amplified many times, in an exponential manner by repeated cycles of denaturation, polymerisation and elongation at different temperatures using specific oligonucleotides (primers), deoxyribonucleotide triphosphates (dNTPs) and a thermostable *Taq* DNA polymerase in the adequate buffer (Mullis and Faloona 1987). Oligonucleotides, flanking part of the genome of the pathogen, are extended by a thermostable DNA polymerase to increase the copies of target DNA (Webster et al. 2004). PCR technique is extremely sensitive, fairly inexpensive and requires minimal skill to perform. The presence of a specific DNA band of the expected size indicates the presence of the target pathogen in the sample. In 1993, Mullis was awarded the Nobel prize in Chemistry for his work on PCR (Bartlett and Stirling 2003). PCR is commonly carried out in small reaction tubes (0.2–0.5 ml volumes) in a machine called as thermal cycler. The thermal cycler alternatively heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use of the Peltier's effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current (Rahman et al. 2013). PCR allows the amplification of millions of copies of specific DNA sequences. The amplified DNA fragments are visualized by electrophoresis in agarose gel stained with ethidium bromide (Capote et al. 2012). Several attempts have been made to develop species-specific PCR primers for fungal plant pathogens (Henson and French 1993). Fungal mitochondrial DNA has been widely used as a source of molecular markers for evolution (Bruns et al. 1991), taxonomy (Martin and Kistler 1990) and genetic diversity studies (Forster and Coffey 1993). DNA region mostly targeted for PCR based diagnostic include ribosomal DNA (rDNAs) as it is present in all organisms at high copy number, inter transcribed spacers (ITS) region for developing DNA barcodes to identify the fungal species and β-tubulin genes used extensively for phylogenetics (Sanchez-Ballesteros et al. 2000; Hirsch et al. 2000; Fraaije et al. 2001; White et al. 1990; El-Sheikha and Ray 2014). The intergenic spacer sequence (IGS) primers based PCR have been used to detect and identify *Verticillium dahliae* and *V. alboatrum* (Schena et al. 2004) and to distinguish pathogenic and non-pathogenic

Fusarium oxysporum in tomato (Validov et al. 2011). The ITS region has also been widely used in fungal taxonomy and is known to show variation between species e.g., between *Pythium ultimum* and *P. helicoides* (Kageyama et al. 2007); *Peronospora arborescens* and *P. cristata* (Landa et al. 2007); *Colletotrichum gloeosporioides* and *C. acutatum* (Kim et al. 2008). PCR technology has many applications in plant pathology and several variants of PCR are being used for detection (El-Sheikha and Ray 2014):

Nested PCR

Nested PCR is a modification of standard PCR involving two consecutive PCR runs, in which the first round PCR products are subjected to a second round PCR amplification with more specific primers. Initial primer pair is used to generate PCR products, which may contain products amplified from non-target areas. The products from the first PCR are used as template in a second PCR, using one (hemi-nesting) or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity. In nested PCR mode, two primer pairs are used; one for the outer fragment and other for the inner fragment. These include outer forward primer, outer reverse primer, forward inner primer and reverse inner primer. Usually, the products of the first amplification are transferred to another tube before the nested PCR is carried out using one or two internal primers. Nested PCR requires more detailed knowledge of the sequence of the target and aims to reduce the product contamination due to the amplification of unintended primer binding sites (mispriming). The nested-PCR is an ultrasensitive technique for detection of several plant pathogenic bacteria, fungi and phytoplasma. E.g. Nested PCR primers based on microsatellite regions were designed for *Monilinia fructicola*, the causal agent of brown rot of stone fruits, and *Botryosphaeria dothidea*, the causal agent of panicle and shoot blight of pistachio (Ma et al. 2003). Intra and inter specific variations in *Ustilainoidea virens*, the causal agent of false smut/green smut of rice were utilized for its detection using nested PCR (Young-Li 2004). Similarly, nested PCR primers have been designed for detection of *Gremmeniella abietina*, the causal agent of stem canker and shoot dieback of conifers namely Abies, Picea, Pinus, Larix, Pseudotsuga, and Tsuga based on 18S rDNA sequence variation pattern in (Zeng et al. 2005) and for detection of *Colletotrichum gloeosporioides*, the causal agent of anthracnose in *Camellia oleifera* based on ITS region (Liu et al. 2009). A rapid nested PCR based diagnostic was developed for detection of *Ramularia collo-cygni*, the causal agent of leaf spot of *Hordeum vulgare* based on species specific primers developed from entire nuclear ribosomal internal transcribed spacer and 5.8S rRNA gene (Havis et al. 2006). Nested PCR was successfully developed for detection of *Verticillium dahliae*, the causal agent of verticillium wilt of strawberry (Kuchta et al. 2008). Single nucleotide polymorphism (SNP) in the FOW1 gene in *Fusarium oxysporum* f. sp. *chrysanthemi*, an economically important pathogen of ornamentals namely *Gerbera jamesonii*, *Osteospermum sp*., and *Argyranthemum frutescens* was exploited for nested PCR (Li et al. 2010).

Multiplex PCR

Multiplex PCR allows the simultaneous and sensitive detection of different DNA targets in a single reaction reducing time and cost (Webster et al. 2004; Lopez et al. 2009). Multiplex PCR is useful in plant pathology because different fungi frequently infect a single host and consequently sensitive detection is needed and helps in reducing the number of tests required (James et al. 2006). Different fragments specific to the target fungi are simultaneously amplified and identified based on molecular sizes on agarose gels but care is needed to optimize the conditions so that respective amplicons can be generated efficiently. E.g. To detect and quantify four foliar fungal pathogens in wheat namely *Septoria tritici* (leaf blotch) and *S. nodorum* (leaf and glume blotch), the β-tubulin gene was used as the target region (Fraaije et al. 2001). Multiplex PCR has been effectively utilized for detecting *Phytophthora lateralis* in cedar trees (Winton and Hansen 2001), for determining the mating type of the pathogens *Tapesia yallundae* and *T. acuformis* (Dyer et al. 2001), to differentiate two pathotypes of *Verticilliun albo-atrum* infecting hop (Radisek et al. 2006) and for distinguishing 11 taxons of wood decay fungi infecting hardwood trees (Guglielmo et al. 2007). Multiplex PCR technique was also used for the simultaneous detection and differentiation of powdery mildew fungi: *Podosphaera xanthii* and *Golovinomyces cichoracearum* infecting sunflower (Chen et al. 2008).

Cooperational PCR (Co-PCR)

Co-PCR is a highly sensitive method of amplification, originally developed for detection of plant viruses. This technique involves the use of four primers and reaction process consists of the simultaneous reverse transcription of two different fragments from the same target, one containing the other. Four amplicons are produced by the combination of the two pair of primers, one pair external to other and largest fragment is produced due to the co-operational action of amplicons (Olmos et al. 2002) it can be coupled with dot blot hybridization, making it possible to characterize the nucleotide sequence (Bertolini et al. 2007). E.g. Co-operational PCR coupled with dot blot hybridization was developed for the detection of *Phaeomoniella chlamydospora*, causing petri disease of grapevine. Co-PCR was able to amplify the partial region of the fungal rDNA including the internal transcribed spacer (ITS) region for detection of *P. chlamydospora* and 17 additional grapevine-associated fungi belonging to the genera *Botryosphaeria*, *Cryptovalsa*, *Cylindrocarpon*, *Dematophora*, *Diplodia*, *Dothiorella*, *Eutypa*, *Fomitiporia*, *Lasiodiplodia*, *Neofusicoccum*, *Phaeoacremonium*, *Phomopsis* and *Stereum*, based on the use of primer pairs NSA3/NLC2 (external pair) and NSI1/NLB4 (inner pair). A specific probe (Pch2D) targeting the ITS2 region in the rDNA was further developed for carrying out dot blot for specific detection of *P. chlamydospora* (Martos et al. 2011).

Real-Time PCR

Real-time PCR, also called quantitative PCR (qPCR) is a laboratory technique based on the PCR, used to amplify and quantify a targeted DNA molecule (Paplomatas 2006). Real-Time PCR is based on the principle of PCR except that the progress of the reaction can be monitored on-line by a camera or detector while they accumulate at each reaction cycle, without the need of post-reaction processing such as gel electrophoresis. Target DNA is quantified using a calibration curve that relates threshold cycles to a specific amount of template DNA. Amplicons are detected using several chemistries based on the emission of fluorescence signal produced proportionally during the amplification (Heid et al. 1996; Mackay et al. 2002; Makkouk and Kumari 2006). Four main chemistries are currently used in real time PCR: SYBR Green I (amplicon sequence non-specific) and; TaqMan, Molecular beacons, and Scorpion-PCR method (sequence specific) (Mackay et al. 2002; Thelwell et al. 2000; Schaad and Frederick 2002; Schaad et al. 2003). Amplicon sequence nonspecific methods are based on the use of a dye that emits fluorescent light when intercalated into double stranded DNA while amplicon sequence specific methods are based on the use of oligonucleotide probes labeled with a donor fluorophore and an acceptor dye (quencher) (Schena et al. 2004). All of these methods are based upon the hybridization of fluorescently labelled oligonucleotide probe sequences to a specific region within the target amplicon that is amplified using traditional forward and reverse PCR primers. TaqMan® probes, developed by Applied Biosystems (Foster City, California, USA), consist of single-stranded oligonucleotides that are complementary to one of the target strands (Lopez et al. 2003). Molecular beacons are the simplest hairpin probes and have complementarity nucleotide sequences that are complementary to the target amplicon (Alemu 2014). Scorpion probes covalently couple the stem-loop structure to a PCR primer due to intramolecular hybridization of probe sequence with PCR amplicon (Lopez et al. 2003). A number of plant pathogenic fungi such as *Helminthosporium solani* were detected in soil and in tubers using TaqMan probe based real time PCR (Cullen et al. 2001). Real time PCR was also used for detection of *Rhizoctonia solani* in soil samples (Lees et al. 2002) and to quantify different species of *Fusarium* in wheat kernels using TaqMan chemistry (Waalwijk et al. 2004). Even oomycete plant pathogen and *Phytophthora ramorum*, the cause of sudden oak death disease were detected by Cepheid SmartCycler real time PCR (Nezhad 2014).

Isothermal Nucleic Acid Amplification Based Diagnostics

Isothermal nucleic acid amplification facilitates rapid target amplification through single-temperature incubation, reducing system complexity compared to PCR-based methods. The method differs in terms of complexity (multiple enzymes or primers), sensitivity, and specificity. Isothermal DNA amplification produces longer

DNA fragments with higher yields than the conventional PCR technique and has greater amplification efficiency owing to undisrupted and sustained enzyme activity. Isothermal nucleic acid amplification includes several methods as:

Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA is a novel transcription based isothermal amplification method developed by Compton (1991). NASBA also known as self sustained sequence replication (3SR) and Transcription Mediated Amplification (TMA) is a sensitive transcription-based amplification system (TAS) for the specific replication of nucleic acids in vitro (Guatelli et al. 1990; Gill and Ghaemi 2008). The assay targets rRNA, which is more stable than mRNA (Zhang 2013). The reaction involves two-stage protocol: the initial phase of denaturation and primer annealing at 65 °C, and the cycle phase for target amplification at the predefined temperature of 41 °C (Chang et al. 2012). NASBA requires three enzymes namely *Avian myeloblastosis virus reverse transcriptase* (AMV-RT), *RNase* H and T7 *DNA dependent RNA polymerase* (DdRp) and two primers. The first primer (P1) carrying the binding/promotor sequence is used to initiate the RNA reverse-transcription (RT) reaction, catalyzed by a reverse-transcriptase after which RNA–cDNA hybrid molecules are degraded by *RNase*H. The remaining cDNA is accessible to the second primer (P2) which initiates the synthesis of the complementary strand. A third enzyme, T7 RNA Polymerase, docks the double strand DNA on the sequence at the 5′ end of P1, transcribing many RNA copies of the gene. This process, i.e. the cycle of first strand synthesis, RNA hydrolysis, second strand synthesis and RNA transcription, is repeated indeterminately starting from the newly transcribed RNA. RNA and double stranded cDNA accumulates exponentially and can be detected by EtBr/agarose gel electrophoresis (Fakruddin et al. 2012). This technology was initially applied for detection of a number of plant viruses such as *Apple stem pitting virus* (Klerks et al. 2001), *Plum pox virus* (Olmos et al. 2007), *Potato virus Y*, *Arabis mosaic virus* (ArMV) and the bacteria *Clavibacter michiganensis* subsp. *Sepedonicus* and *R. solanacearum* (Szemes and Schoen 2003). Recently, NASBA combined with real time has also been used for detection of fungi such as *Candida* sp. and *Aspergillus* sp. (Zhao and Perlin 2013).

Loop-Mediated Isothermal Amplification (LAMP)

LAMP assay first described by Notomi et al. (2000) is a novel DNA amplification technique that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. LAMP is based on the principle of autocycling strand displacement DNA synthesis performed by the *Bst* polymerase derived from *Bacillus stearothermophilus* (isolated from hot springs having temperature 70 °C,

with polymerization and 5′–3′ exonuclease activity) for the detection of a specific DNA sequence (Chang et al. 2012). The technique makes use of four specially designed primers, a pair of outer primers and a pair of inner primers, which together recognize six distinct sites flanking the amplified DNA sequence. As inner primers possess hybrid design, amplified DNA structures take on a loop configuration at one or both ends of the elongated strands, which in turn serve as stem-loop structured templates for further displacement DNA synthesis. The final amplified product consists of a mixture of stem loop DNA strands with various stem lengths and structures with multiple loops (De Boer and Lopez 2012). Amplification can be carried out in a simple and inexpensive device like a water bath at temperatures between 60 and 65 °C (Rigano et al. 2014). LAMP products can be directly observed by the naked eye or using a UV transilluminator in the reaction tube by addition of SYBR Green I stain to the reaction tube separately (Tsui et al. 2011). LAMP assay has been employed for the specific detection of *Fusarium graminearum*, the major causative agent of *Fusarium* head blight of small cereals based on the *gaoA* gene (galactose oxidase) of the fungus (Niessen and Vogel 2010) and for detection of *Ganoderma lucidum* associated with the basal stem rot disease of coconut based on primers targeting small subunit ribosomal RNA gene (Sharadraj et al. 2015).

Molecular Inversion Probe (MIP) Assay Based Diagnostics

MIPs were initially used for high-throughput analysis of single nucleotide polymorphisms, DNA methylation, detection of genomic copy number changes and other genotyping applications (Diep et al. 2012; Hardenbol et al. 2003). Now, the methodology is being utilized for the detection of plant pathogens and can detect as little as 2.5 ng of pathogen DNA due to high specificity (Lau et al. 2014). MIPs originally called as Padlock probes (PLPs), are single-stranded DNA molecules containing two regions complementary to the target DNA that flank SNP in question. Each probe contains universal primers' sequences separated by endoribonuclease recognition site and a 20-nt tag sequence. During the assay the probes undergo a unimolecular rearrangement as: circularization due to filling of gaps with nucleotides corresponding to the SNP in four separate allele-specific polymerization (A, C, G and T) and ligation reactions followed by linearization due to mode of enzymatic reaction. As a result they become inverted followed by PCR amplification step. Further processing of the probes depends on specific assay (Absalan and Ronaghi 2007). MIPs have high accuracy due to fidelity of both polymerase and ligase in the gap-fill step, high specificity due to hybridization, polymerization and ligation (Thiyagarajan et al. 2006). A specific assay has been developed based on padlock probes along with microarray having detection limit of 5 pg of pathogen DNA for the detection of economically important plant pathogens including oomycetes (*Phytophthora* spp. and *Pythium* spp.), fungi (*Rhizoctonia* spp., *Fusarium* spp. and *Verticillium* spp.) and a nematode (*Meloidogyne* spp.) (Szemes et al. 2005). Two padlock probes have been designed to target species-specific single nucleotide

polymorphisms (SNPs) located at the inter-generic spacer two region and large subunit of the rRNA respectively, to discriminate the two fungal species, *Grosmannia clavigera* and *Leptographium longiclavatum*, intimately associated with the mountain pine beetles (*Dendroctonus ponderosae*) in western Canada (Tsui et al. 2010). MIP technology was used as a diagnostic tool to screen the plant pathogens, *Fusarium oxysporum* f.sp. *conglutinans*, *Fusarium oxysporum* f.sp. *lycopersici* and *Botrytis cinerea* (Lau et al. 2014).

Hybridization Based Diagnostics

Fluorescent In Situ Hybridization (FISH)

FISH is a powerful method for in situ detection of pathogens which combines microscopic observation of pathogen along with the specificity of hybridization and is dependent on the hybridization of DNA probes to species-specific regions (Wullings et al. 1998; Volkhard et al. 2000). FISH probes often target sequences of ribosomal RNA or mitochondrial genes as they are abundant in sequence databases and exist in multiple copies in each cell (Tsui et al. 2011). The major step of FISH involves the preparation of biological samples and labeling (incorporation of a fluorescent label/marker e.g. carboindocyanine dye) of a nucleic acid sequence to form a probe. The probe is hybridized to the DNA or RNA in biological materials to form a double-stranded molecule under controlled experimental conditions followed by detection of hybridization (Amann et al. 1995). The first FISH probe targeting a living fungus was designed and used for detection of *Aureobasidium pullulans* on the phylloplane of apple seedlings (Li et al. 1996).

Array Based Diagnostics

Arrays both, microarrays and macroarrays, hold promise for quick and accurate detection and identification of plant pathogens due to multiplex capabilities of the system (Saikia and Kadoo 2010). Array refers to reverse dot blot assays in which assorted DNA probes are bound to a fixed matrix (e.g. nylon membrane or microscope slides for microarrays) in a highly regular pattern (De Boer and Lopez 2012). Macro arrays are generally membrane-based with spot sizes greater than 300 μm while microarrays are high-density arrays with spot sizes smaller than 150 μm. The macroarray technology is now commercially available in four European countries under the name DNA Multiscan (http://www.dnamultiscan.com) for the test of plant pathogens (Tsui et al. 2011).

A typical microarray slide can contain up to 30,000 spots (Webster et al. 2004). ssDNA probes are irreversibly fixed as an array of discrete spots to a surface of glass, membrane or polymer. Each probe is complementary to a specific DNA sequence (genes, ITS, ribosomal DNA) and hybridization with the labeled

complementary sequence provides a signal that can be detected and analyzed. Arrays printed with probes corresponding to a large number of fungal pathogens can be utilized to simultaneously detect all the pathogens within the tissue of an infected host. The steps include extraction of nucleic acid, RT-PCR and labeling with a fluorescent probe such as fluorescin, Cy3 or Cy5. The labeled target molecule is denatured and allowed to hybridize with the arrayed probes. Specific patterns of fluorescence in the form of spots are detected by a microarray reader which allows the identification of specific gene sequences found only in the pathogen of interest (Schmitt and Henderson 2005). DNA array technology was developed for rapid and efficient detection of tomato vascular wilt pathogens *Fusarium oxysporum* f.sp. *lycopersici*, *Verticillium albo-atrum*, and *V. dahliae*. The array successfully detected the tomato wilt pathogens from complex substrates like soil, plant tissues, and irrigation water as well as samples collected from tomato growers (Lievens et al. 2003). Tambong et al. (2006) developed macroarray for simultaneous detection of most of the known *Pythium* species. Recently, *Magnaporthe grisea* array was developed and is commercially available from Agilent Technologies having genome-wide coverage of *Magnaporthe grisea* and inclusion of relevant rice genes in a single microarray with 60-mer oligo probe length (http://www.agilent.com/).

Sequencing Based Diagnostics

Routine sequencing is likely to play an increasingly important role in species identification. PCR amplicons can often be sequenced relatively inexpensively and rapidly. Genetic databases available on the internet such as GenBank allow rapid comparison of one's sample sequence to extensive and growing libraries of sequences (Vincelli and Tisserat 2008). With the advancement in the field of sequencing, full genome sequencing of plant pathogens is possible at lower rate and offers a means for pathogen detection.

Next-Generation Sequencing (NGS)

NGS techniques also referred to as second-generation sequencing (SGS) emerged in 2005 using commercial Solexa sequencing technology. In this technique, sequencing reaction is detected on amplified clonal DNA templates by emulsion or solid phase PCR methods (Nezhad 2014). It involves isolation of total DNA or RNA from diseased plant, elimination of host nucleic acid, enrichment of pathogen DNA, and exploitation of different NGS technologies (Adams et al. 2009; Studholme et al. 2011). Three platforms: Roche/454 FLX, the Illumina/Solexa genome analyzer and the applied biosystems SOLID™ system were widely used and recently, two more parallel platforms came into existence: Helicos Heliscope™ and Pacific Biosciences SMRT instruments (Mardis 2008). Nunes et al. (2011) applied 454 sequencing

technology to elucidate and characterize the small RNA transcriptome (15–40 nt) of mycelia and appressoria of *Magnaporthe oryzae*. A number of both known and unknown plant pathogenic fungi have been detected using NGS e.g. *Pyrenophora teres* f. sp. *teres* and *Phytophthora infestans* in sweet potato (Zhou and Holliday 2012; Neves et al. 2013). The draft genome of the soil borne *Pyrenochaeta lycopersici* causing corky root rot (CRR) disease in tomato and affecting other solanaceous species including pepper, eggplant and tobacco, as well as other cultivated crops such as melon, cucumber, spinach and safflower was characterized based on paired-end Illumina reads is highly effective in reconstructing contigs containing almost full length genes (Aragona et al. 2014).

Third Generation Sequencing (TGS)

More recent single molecule sequencing technologies are known as third-generation sequencing (TGS). TGS also referred as single molecule sequencing (SMS) uses single DNA molecules for sequence reactions without the need for DNA template amplification. TGS has been used in plant genomics and pathogen detection (Pan et al. 2008; Rounsle et al. 2009). TGS is superior to SGS as it simplifies the sample preparation, increases the detection accuracy by eliminating PCR-caused errors, and generates longer sequence reads by better throughput platforms. Oxford Nanopore technology and recently IBM's plan of silicon-based nanopores are the recent devices developed for third generation DNA sequencing (Kircher and Kelso 2010).

Biosensor Based Diagnostics

Immunosensors are those biosensors in which the recognition element is antibody and offers direct label free pathogen detection. It is a device comprising of an antigen or antibody species coupled to a single transducer which detects the binding of the complementary species (Priyanka et al. 2013). Different types such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM) and cantilever-based sensors are currently the most promising (Skottrup et al. 2008).

Quartz Crystal Microbalance (QCM) Immune-Sensors

In this novel technique of plant pathogen detection, a quartz crystal disk is coated with pathogen-specific antibodies. Voltage is applied across the disk, making the disk warp slightly via a piezoelectric effect (Webster et al. 2004). Adsorption of pathogen to the crystal surface changes its resonance oscillation frequency in a

concentration dependent manner. It is both qualitative and quantitative method. The QCM term broadly includes bulk acoustic wave (BAW), quartz crystal resonance sensors (QCRS) and thickness shear mode (TSM). The difference between BAW, QCRS and TSM acoustic sensors, is their mode of wave propagation (Cooper and Singleton 2007).

Surface Plasmon Resonance (SPR) Sensors

SPR uses surface plasmons, which are electromagnetic waves that can be excited by light at gold sensor interfaces. As incoming light interacts with the gold interface at angles larger than the critical angle, the reflected light displays a characteristic decrease, the so-called SPR minimum, due to resonant energy transfer from the incoming photons to surface plasmons. SPR sensors have been shown to be rapid, label-free, and selective tools for the detection (Skottrup et al. 2008). Surface plasmon resonance (SPR) was first used for detection of fungal spores (urediniospores) of *Puccinia striiformis* f.sp. *tritici* (Pst). The approach involved the use of a mouse monoclonal antibody (Pst mAb8) and a SPR sensor for label-free detection of spores (Skottrup et al. 2007). SPR sensor based on DNA hybridization was also used for the detection of *Fusarium culmorum*, a fungal pathogen of wheat (Zezza et al. 2006; Pascale et al. 2013).

Cantilever-Based Sensors

The use of a cantilever as a sensor dates back to 1943 when Norton proposed a hydrogen gas sensor based on a cantilever and was initially used in atomic force microscopy (AFM) for surface characterization (Datar et al. 2009). An AFM measures the forces between the tip of a cantilever and the sample surface using the tip deflection (contact mode AFM) or changes in the resonance frequency of a vibrating cantilever (dynamic mode AFM). Cantilever technology has been used for biosensing applications using antibodies (Waggoner and Craighead 2007). Cantilevers can be operated in either (a) static mode, which measures cantilever bending upon analyte binding or (b) dynamic mode, which measures resonance frequency changes when analytes binds the surface (similarly to QCM sensors). Cantilever sensors have been applied to the detection of relatively small analytes such as nucleic acids and disease proteins (Waggoner and Craighead 2007). Cantilever sensors use have been demonstrated for detection of fungus *Aspergillus niger* at 10^3 cfu/ml using resonance changes (Nugaeva et al. 2007). A specific micromechanical cantilever array system has been used for detection of *Saccharomyces cerevisiae* (Banik and Sharma 2011).

Spectroscopic and Imaging Technique Based Diagnostics

A number of spectroscopic and imaging techniques are being used for forecasting the occurrence of disease and detection of pathogen such as fluorescence spectroscopy where the fluorescence from the object of interest is measured after excitation with a beam of light (usually ultraviolet spectra), visible and infrared spectroscopy whereby visible and infrared rays are used for a rapid, non-destructive, and cost-effective method for the detection of plant diseases (Sankaran et al. 2010). Hyperspectral imaging has also found application in precision agriculture whereby the spectral reflectance is acquired for a range of wavelengths in the electromagnetic spectra profiling (Okamoto et al. 2009). However, these techniques require trained person having the knowledge of softwares related to image data analysis and requirement of high efficiency computers. Imaging spectroscopy has been used to scan wheat kernels for head blight disease through machine vision techniques (Delwiche and Kim 2000) while spectral and fluorescence data has been employed to monitor winter wheat yellow rust (Moshou et al. 2005).

Volatile Organic Compounds (VOCs) Based Diagnostics

Plants emit many low molecular weight biomolecules in gaseous phase called as volatile organic compounds (VOCs) from their surfaces into their immediate surroundings that serve essential functions (Baldwin et al. 2006). VOC profiling is an emerging innovative avenue and has potential applications in disease diagnosis. The emitted VOC profiles of healthy plants are significantly different than those infected ones (Martinelli et al. 2014). The electronic nose (e-nose) is a platform for VOCs profiling.

Electronic Nose

In recent years, the development of innovative devices such as electronic nose (e-nose) based on different electronic aroma detection (EAD) principles and mechanisms has been investigated and implemented for diverse disciplines within the plant sciences by many researchers (Wilson et al. 2004). The electronic nose is often referred to as an intelligent device, able to mimic the human olfaction functions and may be used for detection, recognition and classification of volatile compounds and odours. This type of electronic olfactory system was introduced in 1982 by Dodd and Persaud from the Warwick Olfaction Research Group, UK (Troy Nagle et al. 1998). A complete electronic-nose system typically consists of several integrated and/or interfaced components including a multisensor array (composed

of several gas sensors with broad sensitivity and cross-reactivity or partially-overlapping selectivity), a data-processing and analysis unit such as an artificial neural network (ANN), software having digital pattern-recognition algorithms, and often aroma reference-library databases containing stored files with digital fingerprints of specific aroma reference (signature) patterns (Wilson 2013). An electronic nose incorporating artificial intelligence to detect basal stem rot (BSR) disease caused by *Ganoderma boninense* fungus affecting oil palm plantations in South East Asia was developed based on three types of odour samples for both healthy and infected oil palm trees, namely odour of the air surrounding the tree, odour of bored tree trunk and odour of soil surrounding the base of the tree trunks (Markom et al. 2009). A Cyranose® 320 was developed to detect postharvest fungal diseases namely gray mold caused by *Botrytis cinerea*, anthracnose caused by *Colletotrichum gloeosporioides* and fruit rot caused by *Alternaria* spp. in blueberries (Li et al. 2009). Electronic Noses (ENs) have also been used to analyse the Volatile Organic Compounds (VOCs) of both healthy and infected powdery mildew infected tomato (*Solanum lycopersicum*) crops (Ghaffari et al. 2010). In order to detect contamination of wheat by *Fusarium* species, an electronic nose based on an array of metalloporphyrin coated quartz microbalances was developed for detection of *Fusarium* species, *F. cerealis*, *F. graminearum*, *F. culmorum* and *F. redolens* based on release of toxic metabolites, especially the mycotoxin deoxynivalenol (DON, Vomitoxin) and zearalenone (ZEA) (Eifler et al. 2011).

Nano-diagnostics

Nano diagnostics is the use of nano-biotechnology to diagnose plant diseases.

Quantum Dots (QDs)

QDs are nanometer scale semiconductor nanoparticles that fluoresce when stimulated by an excitation light source and are defined as particles with physical dimensions smaller than the exciton Bohr radius (Jamieson et al. 2007). QDs are ultrasensitive nanosensor based on fluorescence resonance energy transfer (FRET) can detect very low concentration of DNA and do not require separation of unhybridized DNA (Khiyami et al. 2014). QDs are linked to specific DNA probes to capture target DNA. The target DNA strand binds to a fluorescent-dye (fluorophore) labeled reporter strand and thus forming FRET donor-acceptor assembly. Unbound DNA strand produce no fluorescence but on binding of even small amount of target DNA (50 copies) may produce very strong FRET signal (Chun-Yang Zhang et al. 2005). QD specific antibody sensor was developed for rapid detection of *Polymyxa betae*, an obligate parasite of sugarbeet roots and vector of *Beet necrotic yellow vein virus* (BNYVV), the causal agent of rhizomania (Safarpour et al. 2012).

Nanoprobes

Fluorescent silica nanoprobes have potential for rapid diagnosis of plant diseases. Fluorescent rubpy doped silica nanoparticles (FSNP) at 50 ± 4.2 nm conjugated with the secondary antibody of goat anti-rabbit IgG (using microemulsion method) has been used for successful detection of a bacterial plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria*, the causal agent of bacterial spot disease in solanaceous plants (Yao et al. 2009). In future, nanoprobes can be utilized for detection of other plant pathogens also.

Portable Devices and Kits

On site diagnosis of plant pathogens require portable devices and such a portable system in the form of PCR termed as Palm PCR was developed by Ahram Biosystems Company in Korea in which DNA can be amplified in less than 25 min. The portable system presents a highly functional and user-friendly way to perform different types of PCR tests for both beginners and experienced researchers.

Lab on a Chip

A Lab on a chip is a new micro technique which possess several advantages such as portability, low reagent consumption, short reaction times and on site diagnosis. A large number of samples can be processed directly in the field itself (Figeys and Pinto 2000; Kricka 2001). The first lab-on-a-chip system in the field of plant pathology was developed for rapid diagnosis of *Phytophthora* species (Julich et al. 2011). A portable real-time microchip PCR system was developed for detection of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) strains. The system included fluorescence detector and a battery-powered microcontroller unit for PCR. The entire system was 2,561,668 cm^3 in size and weighs under 850 g (Koo et al. 2013).

Lab in a Box

Lab in a box also termed as nanodiagnostic kits refers to a briefcase sized kit that can be carried to the field to search for pathogens. Nanodiagnostic kit equipment can easily and quickly detect potential serious plant pathogens, allowing experts to help farmers in prevention of disease epidemics from breaking out.

Phytophthora *Test Kits*

The Alert test kit for *Phytophthora* has been used to detect all common *Phytophthora* species. Pathogen detection can be accomplished when as little as 0.5 % of a plant's roots are infected (http://danrcs.ucdavis.edu.)

Conclusion

Plant pathogenic fungi are becoming more widespread globally due to modern high input monocropping based agriculture and easy transboundary movement. In order to manage plant pathogens and restrict their movement as well as secondary spread in new geographical areas, early and timely detection is pre-requisite. Thus, plant disease diagnosis and detection of plant pathogen are critical and integral part of successful disease management and serve as the first and crucial line of defense. Once the pathogen is identified, appropriate control measures can be employed. In the past, detection of fungal pathogens involved time consuming biological indexing for days/weeks at a certain temperature on the appropriate medium or grow out tests for seed borne fungi. These processes are extremely cumbersome and cannot be adopted for routine diagnosis of large number of samples. The constraints posed by these traditional biological indexing methods led to profound advancement in the development of affordable and simple new improved methods which served as powerful tools for detection and identification of phytopathogenic fungi. New innovative detection technologies have been formulated and demonstrated that are accurate, cost effective, portable, rapid, robust, sensitive, and high throughput for routine plant disease diagnosis. Several techniques have been developed which have an edge over the traditional methods of plant pathogen diagnosis; these include physical diagnostic tools (EM; SEM etc.), serological techniques (DIBA; ELISA etc.), molecular techniques (PCR), lateral flow assays, hybridization based assays, nano-based kits, electronic nose etc. are gaining momentum and have potential applications. The era of Next/Third Generation Sequencing, in which the entire DNA or RNA sequences of organisms can be traced, has provided an ocean full of diagnostic techniques involving the complementation of bioinformatics approaches for authentic identification *vis-a-vis* characterization of plant pathogenic fungi. On site molecular diagnostics is in its infancy but is surely evolving faster and will become a boon due to user friendliness. Despite availability of array of frontier tools and techniques for plant pathogen detection in the era of biotechnology, conventional methods can't be completely ignored in some instances. The best approach for both disease diagnosis and detection of plant pathogenic fungi demands blend of diverse range of conventional and advanced unconventional methods. With this continuous evolving spectrum of advanced techniques, the major challenge in the future for phytopathologists will be to choose a particular modus operandi among array of these techniques for specific detection of pathogen.

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