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Diagnostic Techniques of Soil Borne Plant Diseases: Recent Advances and Next Generation Evolutionary Trends

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ABSTRACT: All about 80000 diseases have been recorded in plants throughout the world, of them majority are associated with soil-borne diseases. Early, speedy and reliable detection of plant pathogens is prerequisite to optimize suitable and accurate management strategy. Traditionally, the most prevalent techniques used to identify plant pathogens relied upon culture-based morphological approaches; these methods were laborious, time-consuming. Molecular detection strategies could solve these limitations with improved accuracy and reliability. The DNA and protein based pathogen detection techniques such as DNA fingerprinting, biochemical assays, isothermal amplification techniques and serology are gaining importance in rapid soil borne pathogen detection due to their high degree of specificity to distinguish closely related organisms at different taxonomic levels. Here, we review the various molecular tools used for detection of several soil-borne plant pathogens and its implementation in agriculture.

Keywords: soil-borne; plant pathogen; disease diagnosis; next generation; advance techniques

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INTRODUCTION

In 2015, the hunger statistic of World Food Program (WFP) revealed that 815 million people in the world, on average one in nine is chronic hunger and still go to bed each night on an empty stomach and simultaneously the WFP opened an information that 3.1 million children dies under the age of 5 every year due to malnutrition. The huge loss in agricultural products, estimated value of $1500 billion^{-year}, due to occurrence of crop diseases is one of the key concerns behind those vital issues, chronic hunger and child-malnutrition which are the big hurdle in the way of world food security (Agrios, 2005). In 2015, the global community adopted 'Zero hunger' mission to make the world hunger-free by 2030. But no single organization can achieve 'Zero Hunger' if it works alone (www.zerohungerchallange.org). The agriculture sectors could play an important role in the 'Zero Hunger' mission by reduction of crop losses via development and right implementation of priority based disease management system in sustainable agriculture. Since mid of eighteen century (1845-49), it is reported that the potato late blight (*Phytophthora infestans*) had affected the well-being of nations, left a deadliest disaster is called 'the Great Irish famine', a scourge that had caused a death toll of 1.0-1.5 million people in Great Britain and Ireland (Cox and Large, 1960). After that Sigatoka leaf spot (Stover, 1980) and Panama disease (Ploetz, 2005) of banana and beet curly top disease of sugar beet (Harveson, 2015) also resulted in massive scale of economic losses world-wide. A devastating yield loss in rice crop up to 40-90% was occurred due to epidemic of rice brown leaf spot (*Helminthosporium oryzae*) in Bengal resulted in historic famine in 1943 (Padmanabhan, 1973). Early and reliable detection of such diseases is crucial in order to reduce the huge economic losses as well as valuable human lives. On the other hand, early disease detection in crops is very imperative, because practice of using chemicals or biocides as a curative measure after a severe infection with high incidence and severity causes enormous negative impact on environment and biological ecosystem (Padaria *et al.*, 2016, Sharma *et al.*, 2017). However, use of resistant cultivars for controlling plant diseases as an initial preventive measure is the best option (Sharma *et al.*, 2010, Sharma *et al.*, 2016a). But where resistant varieties are not available, the strategies for integrated disease management are highly dependent on the availability of fast, sensitive and specific disease diagnostic methods (Ghosh *et al.*, 2016, Ghosh *et al.*, 2017, Tarafdar *et al.*, 2018).

About 80000 diseases have been recorded in plants throughout the world, of them majority are associated with soil-borne diseases. It is important fact that diagnose of large share of soil-borne diseases is often difficult, as symptomless host plants at early stages of infection is more challenging. A large number of diseases are spreading or introducing into a new area *via*. propagative materials e.g. seed, rhizospheric soil of seedling, propagules etc. (Biswas *et al.,* 2009, Katan, 2017). Hence, early and speedy detection of soil-borne pathogens is prerequisite for planting materials. Because it could be the part of a controlling measure through effectively linking this tool with a safeguard agricultural systems to prevent further spreading of the disease in an area where the disease was absent. Besides, rapid identification of any destructive pathogen is having a significantly impacts on plant disease management, as it could prevent the outbreak of certain diseases. Traditionally, the most prevalent techniques used to identify plant pathogens relied upon culture-based morphological approaches. These methods are laborious, time-consuming, and require wide knowledge of classical taxonomy. Other restrictions include the difficulty of some species to be cultured *in vitro*, and the incapability to accurate quantification of the pathogen (Ghosh *et al.,* 2015, Sharma *et al.,* 2015b). In combination of classical knowledge of the pathogen and molecular detection strategies could solve these limitations with improved accuracy and reliability.

A large number of molecular methods have been used to detect, identify and quantify a long list of soil-borne pathogens of plants (Ghosh *et al.,* 2016, Tarafdar *et al.,* 2018, Sharma *et al.,* 2015b). Molecular methods have also been applied to the study of the genetic variability of pathogen populations, and even for the description of new fungal species (Judova *et al.,* 2012). In general, these methods are much faster, more specific, more sensitive, and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise. Additionally, due to its high degree of specificity, molecular techniques can distinguish closely related organisms at different taxonomic levels (Ghosh *et al.,* 2016, Ghosh *et al.,* 2017). Here, we review the most important tools for molecular detection of various soil-borne plant pathogens and its implementation in agriculture. We have also reconnoitered how the pathogen detection techniques are advancing to next generation with consideration of upsurge troubles in on-spot and real-time diseases diagnosis. Since on-site disease diagnosis give additional advantages, as it could assist in faster diagnosis even without taking any help of diagnostic laboratory, simultaneously it could help in making decisions on diseases measure in fields at early-stage according to its importance.

MAJOR PATHOGEN GROUPS ASSOCIATED WITH SOIL-BORNE DISEASES

A few of very common soil-borne diseases including seedling, vascular and root rot diseases cause huge losses to many crops. Soil-borne pathogens include fungi, oomycetes, nematodes, viruses. Although they are very diverse, these pathogens share some basic features related to being soil-borne. They survive and act in the soil, at least during part of their lives. Consequently, they are heavily influenced by the soil's abiotic and biotic components. However, the different pathogens favour different type of environments. For instance, some pathogens prefer damp conditions (Sharma *et al.,* 2015b), whereas others like dry environment (Sharma *et al.,* 2015a). Again, some group of pathogens are acidophilic and populate in low soil pH, whereas some groups thrive in high soil pH. In general, the pathogens associated with soil borne plant diseases can be classified into six major groups *viz*. viruses, fastidious prokaryotes e.g. mycoplasma-like organisms (MLOs), spiro-plasmas and rickettsiae-like organisms (RLOs), bacteria, fungi, protozoa and nematodes. Among those, fungal pathogens are more common. There are an estimated 1.5 million fungal species (Hawksworth, 2001), of which over 8,000 are known to cause disease in plants. Most of them infect root leading to eventual death of the plants by root rot, wilting, yellowing, stunting and dieback. Some common root rot fungi include: *Rhizoctonia*, *Fusarium*, *Pythium*, *Phytophthora* and *Cylindrocladium*. Some fungi like *Sclerotinia* and *Sclerotium* cause stem, collar and crown rot of the plant at ground level. Bacteria are less abundant soil borne pathogen than fungi. Some soils borne bacterial pathogens are *Erwinia*, *Rhizomonas* and *Streptomyces* causing soft rot corky root and scab diseases. Soil borne viral diseases are rare as it requires living plant tissue, but they are known to hitch a ride on fungi or nematodes and flow in on water. Pathogenic nematode or roundworms species can be particularly pervasive in soil. They are problematic pathogens mainly for root crops, like carrots. Some nematodes feed on the tips of roots and causing branching and swelling of the roots.

NEED OF MOLECULAR IDENTIFICATION

Plant pathogenic soil-borne fungi, bacteria, phytoplasmas and viruses cause extensive damage in a varied range of crop species worldwide. The damage is often serious to crop production resulting in huge economic losses every year. Use of conventional methods for identifying soil-borne pathogens is reliant on understanding of visual symptoms, pathogen isolation and culturing of the pathogen in the laboratory (Sharma *et al.,* 2015b). Diagnosis of the diseases by this route is often time consuming and impractical when quick results are needed. In addition, many times visual inspection fails experienced and skilled persons are required for correctly identification (Sharma *et al.,* 2015b). Many times certain pathogens remain latent in plant tissue without showing any symptom. In these cases, visual inspection fails to detect the presence of pathogen in plant like fungus, bacteria or virus (Tarafdar *et al.,* 2012, Singh *et al.,* 2013, Tarafdar *et al.,* 2013), and thus complicate in timely disease management decisions.

Many plant pathogens are highly identical to each other morphologically and indeed difficult to identify. One best example is *Macrophomina phaseolina* and *Phoma* spp. For distinguishing, it needs extensive taxonomical knowledge. Sometimes it is also difficult and time-consuming to discriminate between populations of the same pathogen that have specific properties, e.g., fungicide resistance, toxin production, or differences in virulence. Traditionally, for virus detection it was essential to perform time-consuming indexing or for bacteria to be cultured for one or more days at a certain temperature on the appropriate medium in order to identify the colonies using biochemical and physiological tests. For large number of samples, this process was obviously not suitable. Moreover, the necessity for rapid detection techniques of high accuracy is especially required for quarantine pathogens because the risk of the disease and the spread of the inoculum. Newer methods that are increasingly being applied to the diagnosis of plant pathogens include immunological methods, DNA/RNA probe technology, and polymerase chain reaction (PCR) amplification of nucleic acid sequences. These techniques have several potential advantages over conventional diagnostic methods in that they are more accurate, faster, and can be used by any personnel, with no specialized taxonomical expertise. Even more important, these techniques allow detection of non-colourable microorganisms. Furthermore, molecular identification techniques are useful in revealing new diseases of unknown etiology. These technologies not only reveal the presence of pathogen but could even be used for accurate quantification of its biomass. The comparative analysis of genomic sequences allows the phylogenetic reconstruction of the pathogen relationships at different taxonomic levels (Biswas *et al*., 2012a, Biswas *et al.,* 2012b, Sharma *et al.,* 2012b).

NUCLEIC ACID BASED DETECTION TECHNIQUES

A. DNA/RNA Sequencing

The nucleotide sequence of particular gene like *ITS, 18S rDNA, Cox 1, Cox 2, B-tubulin, Ef-1a* is known to be unique and conserved to the particular microbial species and it can be easily identified by analysis of this region. The Sanger sequencing method has been partially supplanted by several "next-generation" sequencing technologies able to produce a high number of short sequences from multiple organisms in short time. Enormous sequencing technologies offer intense increases in cost effective sequence throughput, having a incredible impact on genomic research. Pyrosequencing is a DNA sequencing tool based on the sequencing-by-synthesis principle. The pyrosequencing technology has not been widely applied for the control of fungal plant diseases yet. However, 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 oryz*ae. Another application of this new sequencing technology is the rapid generation of genomic information to identify putative single nucleotide polymorphisms (SNPs) to be used for population genetic, evolutionary, and phylogeographic studies on non-model organisms. Thus, Broders *et al.* (2011) designated the sequencing, assembly and discovery of SNPs from the plant fungal pathogen *Ophiognomonia clavigignenti-juglandacearum*, for which virtually no sequence evidence was earlier available. Moreover, Malausa *et al.,* (2011) described a high-throughput technique for isolating microsatellite markers based on coupling multiplex microsatellite enrichment and 454 pyrosequencing in diverse organisms, such as *Phytophthora alni* subsp. *uniformis*.

B. DNA fingerprinting

Fingerprinting methods permit the screening of random regions of the pathogen genome for recognizing species-specific sequences when conserved genes have not enough deviation to successfully identify species or strains (Patil, 2018). Fingerprinting analyses are usually used to study the phylogenetic structure of fungal populations. However, these methods have been also useful for identifying specific sequences used for identify the pathogen at very low taxonomic level, and even for differentiate strains of the same species with virulence, different host range, and compatibility group.

PCR-Restriction Fragment Length Polymorphism (RFLP). Considerable progress has been made in the use of DNA-based methods for detection, identification, and classification of soil-borne plant pathogens. In particular, specific and sensitive detection methods have been developed, mainly based on polymerase chain reaction (PCR). PCR-amplified nuclear ribosomal DNA (rDNA) makes it possible to distinguish, characterize and classify soil-borne pathogens on a phylogenetic basis, using restriction fragment length polymorphism (RFLP). For example, (Camele *et al.,* 2005) detected and differentiated 10 *phytophthora* species infecting different crops through extensive RFLP of PCR-amplified rDNA, thus permitting selective detection of these *Phytophthora* spp. PCR primers specific to the genus *Phytophthora* were used to amplify and further digest the resulting amplicons yielding a specific restriction pattern of 27 different *Phytophthora* species (Drenth *et al.,* 2006).

PCR-RFLP analysis of the ITS region demonstrated the presence of different anastomosis group within isolates of *Rhizoctonia solani* (Pannecoucque and Hofte, 2009). It also allowed the distinction of pathogenic and non-pathogenic strains of *Pythium myriotolum* (Gómez-Alpízar *et al*., 2011). Sharma *et al*. (2012a) have been used to reveal the genetic polymorphism within populations of *M. phaseolina* isolated from chickpea targeting PCR-amplified rDNA.

Random Amplified Polymorphic DNA (RAPD). Random amplified polymorphic DNA (RAPD) technology is a simple, rapid and inexpensive technique utilizing short synthetic oligonucleotides of random sequences as primers to amplify small amount of total genomic DNA under low annealing temperatures by PCR. A semi distinctive profile pattern is observed on resolving the resultant PCR product. Profile of amplified DNA depends on nucleotide sequence homology among the template DNA and oligonucleotide primer at the end of each amplified product. It produces substantially more robust polymorphic amplification products per experiment than other marker systems and their application does not need any prior sequence information. For this reason, RAPD markers have been found to be very suitable for studies on the genetic structure of fungal populations (Nasir and Hoppe, 1991). The smallest of changes in the genome of the organism can be analyzed by using this marker. RAPD have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species (Nasir and Hoppe, 1991). The analysis of DNA products generated through RAPD has provided information on variation and segregation of genetic traits among strains. RAPD fingerprinting also offers several advantages that may be useful in studying for maespeciales and races of *Fusarium oxysporum* (Belabid *et al.,* 2004). Also, RAPD analysis has been used to characterize strains of many *Fusarium*, *Alternaria* and *Rhizctonia* spp (Kini *et al.,* 2002).

Amplified Fragment Length Polymorphism (AFLP). The amplified fragment length polymorphism (AFLP) is a PCR-based tool used in genetic research, DNA fingerprinting, and in the practice of genetic engineering. In the process of AFLP, two restriction enzymes are used to cut the total genomic DNA into fragments and the resultant strands are then ligated with double-stranded nucleotide adapters. A division of restriction fragments is then selected for amplification. Primers having restriction site sequence and additional nucleotides at the 3′ end are used as selective complementary agents to the adapter, in order to amplify the fragments. This method has an added advantage that it requires very little amounts of DNA templates as compared to other fingerprinting methods such as RAPD and inter-simple sequence repeats. AFLP produces substantially more robust polymorphic amplification products per experiment than other marker systems and their application does not need any prior sequence information. For this motive, AFLP markers are found to be very suitable for studies on the genetic assembly of fungal populations (Sharma *et al.,* 2012a, Gargouri *et al.,* 2006). AFLP markers have found the widest application in analyses of genetic variation below the species level, particularly in investigations of population structure and differentiation including estimation of F_{ST} analogs and genetic variation within populations (Sharma *et al.,* 2012a). Such analyses are critical for conservation genetics, and the swiftness with which AFLP markers can be generated potentials that these markers can deliver vital information under the intense time constraints frequently needed by pending conservation decisions. Apart from problems of population structure and variation, AFLP markers have been applied to evaluate gene flow and dispersal. The high resolution of AFLP markers also allows testing for clonal identity among individuals (i.e. absence of recombination), and thus permits implications about sexual versus asexual modes of reproduction (Majer *et al.,* 1998).

Simple Sequence Repeats (SSR). Simple sequence repeats (SSRs), also known as Microsatellites or short tandem repeats (STRs), are motifs of one to six nucleotides repeated several times in all eukaryotic genomes. The variations present within the number of tandemly repetitive units confer a high polymorphic banding pattern, which can be identified through PCR, using locus specific flanking primers.

They are known for best and ideal markers, widely used in soil borne pathogens for identifying genetic alterations between or within the closely linked species.

These nucleotide units can vary in repeat number between individuals and their distribution in the genome is nearly random. Using primers flanking such variable regions PCR products of diverse lengths can be obtained. So, the microsatellites are extremely adaptable genetic markers that have been widely used for DNA fingerprinting. The advantages of SSRs are that they are highly polymorphic, codominant, and multiallelic several thousand potentially polymorphic markers are available. For genome analysis and genetic mapping SSR markers have become an apt choice in managing such wide range applications.

SSR have been widely used for the study of the genetic diversity of soil borne plant pathogenic fungi within species e.g. *Ceratocystis fimbriata* (Steimel *et al.,* 2004), *Macrophomina phaseolina* (Reznikov *et al.,* 2018), *Puccinia triticina* (Szabo and Kolmer, 2007), *Sclerotinia subarctica* and *S. sclerotiorum* (Winton *et al.,* 2007); and for genetic map construction, e.g. Zheng *et al.* (Zheng *et al.,* 2008) constructed a genetic map of *Magnaporthe grisea* containing of 176 SSR markers. In other testing, microsatellite markers specific for *Phytophthora ramorum* were used to discriminate between A1 and A2 mating types isolates of this pathogen from two different geographic origins (Prospero *et al.,* 2004).

C. Polymerase Chain Reaction (PCR) Based Detection

It is an efficient and cost-effective molecular tool to copy or amplify small segments of DNA or RNA. PCR associates the principles of complementary nucleic acid hybridization with those of nucleic acid repetition that are applied recurrently through frequent cycles. It results in the high amount of production of the specific target DNA/RNA sequences by a factor of 10^7 within a short span of time.

This in-vitro amplification technique can amplify a single copy of nucleic acid target by using 2 synthetic oligonucleotide "primers" that link to the target genomic sequence, which are extended by a Taq polymerase (a thermostable DNA polymerase). This technique developed by Kary Mullis in 1983, is now a common and important technique used in agriculture for a variety of applications. These comprise DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of diseases. The different type of PCR use in pathogen detection are described in below section.

Multiplex PCR. It allows the simultaneous and sensitive detection of different DNA or RNA targets from a single reaction. It can be designed to verify the presence of more than one pathogen in plant material by identifying for common specific sequences in two or more of them. It is useful in plant pathology because different soil borne pathogen frequently infects a single host and consequently sensitive detection is needed for the propagation of pathogen-free plant material. There are several examples of simultaneous detection of several pathogens in a one go by multiplex PCR from host like wheat (Sun *et al.,* 2018), strawberry (Li *et al.,* 2011), turfgrass (Asano *et al.,* 2010). However, the sensitivity of this technique is influenced by the number of targets to be detected, mainly due to the number of different primer pairs instead of the total amount of primer present in the cocktail.

Real-time PCR. Detection and pathogen quantification is an important aspect with respect to plant disease management. Quantification based on culturing techniques is considered relatively inaccurate and in some cases even unreliable, the development of real-time PCR has been a powerful development with regard to pathogen detection and quantification (Tarafdar *et al.,* 2018). Real-time PCR differs from classical end-point PCR by the measurement of the amplified PCR product at each PCR cycle. Since the development of the exponential phase of the reaction is monitored, real-time PCR allows accurate template quantification. Increasingly, real-time PCR is being used for plant pathogen diagnosis, including detection and quantification of different plant pathogenic fungi, oomycetes, bacteria, nematodes and viruses as well as biocontrol agents. Recently, detection methods based on real-time PCR have been developed to identify and diagnose a number of phytopathogenic fungi (Schena *et al.,* 2004, Lievens *et al.,* 2005). These techniques, which enable pathogenic fungi to be identified by a specific increase of fluorescence during PCR amplification, are more sensitive than conventional PCR (Lees *et al.,* 2002), reduce the risk of false positives and promote multiplex and quantitative analyses.

Colony PCR. It is a fast and reliable method for crude mycelium-based amplification and designed for quick screening using the ITS1-5.8S-ITS2 region of the fungal ribosomal DNA cluster. PCR success rate is generally high. A broad application of this method should lead to a simplification of molecular taxonomic analyses and thus allowing for more extensive, sequence-based analyses of fungal isolates. The results are directly obtained from fungal hyphae without any previous DNA extraction or other prior manipulation.DNA of fungal genera *Cladosporium*, *Geomyces*, *Fusarium*, and *Mortierella*can be amplify with high success. DNA of soil-borne yeasts was always successfully amplified. *Absidia*, *Mucor*, *Trichoderma*, and *Penicillium* isolates had noticeably lower PCR success (Walch *et al.,* 2016). Mutualistic Basidiomycota and Ascomycota were also successfully amplified directly from cleaned mycorrhized root tips without previous DNA extraction and a direct PCR in combination with species-specific primers allowed for a fast identification of *Tuber melanosporum* fruiting bodies (Bonito, 2009).

Nested PCR. It is a modification of PCR designed primarily to improve the sensitivity and specificity. It involves the use of two primer sets to undertake two successive PCR reactions in which the product of a PCR is subjected to a second round of amplification using primers core to those engaged for the first round (Kamolvarin *et al.,* 1993).

This method significantly reduces the cross contamination risks and, due to the low volume of sample used, is unaffected by the presence of PCR inhibitors. Nested PCR based assays are more rapid, sensitive, specific and accurate and have been often implemented for the routine diagnostics of a variety of pathogens such as *Phytophthora nicotianae* (Grote *et al.,* 2002), *Botryosphaeriaceae* species (Ni *et al.*, 2012), *Botryosphaeria dothidea* (Ma and Michailides, 2007), *Pilidiella granati* (Yang *et al.,* 2017).

Nucleic Acid Sequence Based Amplification (NASBA). It is a sensitive, isothermal, transcription-based amplification system specifically designed for the detection of RNA targets. It is also known as "self-sustained sequence replication" (3SR) and transcription mediated amplification (TMA). It uses three enzymes avian myeloblastosis virus reverse transcriptase, RNase H and T7 RNA polymerase leading to main amplification product of single stranded RNA. Products of NASBA are single stranded and can be applied to detection formats using probe hybridization without any denaturation step. It provides a rapid analytical method, especially for at line monitoring of pathogen presence. This technology has been applied for detecting the soil-borne bacteria *C. michiganensi*s subsp. *Sepedonicus* and *R. solanacearum* (Szemes and Schoen, 2003).

PCR-ELISA. It combines an immune-detection to detect and quantify specific PCR products directly after immobilization of DNA on a microtiter plate. It detects nucleic acid instead of protein, is a much more sensitive method compared to conventional PCR method, with shorter analytical time and lower detection limit. It involves direct incorporation of labeled nucleotides in amplicons during PCR-amplification, their hybridization to specific probes and hybrid capture-immunoassay in microtiter wells. This technique is potentially automatable and does not require expensive equipment, and thus can be fundamental in laboratories without access to a real-time thermal cycler.

Similar methodology for species identification can provides a molecular technology to progression in large number of samples and still identifies the fungi with a high level of assurance, may greatly reduce the resources and the time. This technique has been successfully used to detect plant pathogenic fungi (Bailey *et al*., 2002, Somai *et al.,* 2002) with more specificity than regular PCR.

Ligase Chain Reaction (LCR). It is an amplification process that differs from PCR in that it involves a thermostable ligase to join two probes or other molecules together which can be amplified by using standard PCR cycling. LCR can also be used to amplify template molecules that can be successfully ligated for a purpose of assessing ligation effectiveness and producing a huge amount of product with even greater specificity than PCR. It was evaluated as an amplication method for an in vivo mutation assay. Specifically, the ligase was tested for its ability to selectively amplify a DNA sequence mutated at a single base (Kälin *et al.,* 1992). Tooley *et al.* (Tooley *et al.,* 2002) developed LCR assay with a set of LCR primers that differentiated *Phytophthora infestans, P. mirabilis,* and *P. phaseoli* from all other *Phytophthora* species.

D. DNA Hybridization Techniques

With the advent of techniques for the isolation, purification, cloning and hybridization of DNA from various microorganisms, DNA probes are among the first molecular markers applied in the detection, identification and phylogenetic analysis of soil brone pathogens. Species-specific DNA probes generated from cloned random DNA fragments derived from genomic DNA that is digested with various restriction endonucleases had a number of advantages over classical approaches. Because of their high specificity, a pure culture of the target organism is not necessary. For instances, cloned chromosomal DNA probes were applied to detect various *Phytophthora* spp. in soil and host tissue, particularly *P. parasitica* (Goodwin *et al.,* 1989). As presence of high copy numbers and ability to produce more simple restriction fragment patterns, mitochondrial DNA (mtDNA) probes were reported to be used to differentiate between *Phytophthora* species that display overlapping variability of morphological characters like *P. cryptogea* and *P. drechsleri* (Mills *et al.,* 1991). This technology has also been applied to distinguish among special forms of the soil-borne fungal pathogen *Fusarium oxysporum*, the causal agent of vascular wilt of a large number of plant species (Bridge *et al.,* 1997) and *Macrophomina phaseolina*, the causal agent of damping off, seedling blight, collar rot, stem rot, charcoal rot and root rot in various economically important crops (Babu *et al.,* 2007).

Fluorescent in situ hybridization (FISH). Fluorescence in situ hybridization (FISH) is a technique applied for bacterial detection that combines the simplicity of microscopy observation and the specificity of hybridization. Its use in detection of plant pathogenic fungus is recent and is dependent on the hybridization of DNA probes to species-specific regions of fungal ribosomes. They are suitable as diagnostic targets as ribosomal RNA holds functional sequences that are common to all species but also sequences that are very specific to individual species, and FISH only needs to recognize this specific information. The probes hybridize with a three-dimensional protein/RNA structure. The sensitivity of the FISH technique is identical to that of amplification tools and, in theory, FISH can identify single cells. This high sensitivity is the result of the high affinity and selectivity of DNA probes because FISH takes place under very stringent hybridization conditions, where a difference of one nucleotide in a 15–20 oligonucleotide probe is adequate to categorize binding. Furthermore, FISH maintains the structural integrity of the pathogen.

The first FISH probe targeting a living microorganism was designed by Li *et al.* (1996) for *Aureobasidium pullulans* on the phylloplane of apple plantlets; this was the first time that a living pathogen had been visualized by FISH. Examples of circumstances in which the method has been used are very different.

Most probes have been intended to target the 18S or 28S rRNA gene, and their specificity needs to be kept under review as sequence databases expand.

Reverse Dot Blot Hybridization (RDBH). Reverse Dot Blot Hybridization (RDBH) also known as macroarray, is a technique based on hybridization of amplified and labelled genome regions of interest to immobilized oligonucleotides spotted on a solid support platform. It is now considered a powerful and practical technique for the detection and identification of bacteria and other microbes, such as plant pathogenic fungi (Le Floch *et al.,* 2007, Sholberg *et al*., 2005). Amplicons of the target gene region(s) are amplified by PCR, labelled with digoxygenin (DIG) and subjected to the DNA hybridization procedure (Fessehaie *et al.,* 2003). A positive reaction between an amplicon and a perfectly matched oligonucleotide generates a chemiluminescent signal which can be detected by X-ray film or a digital camera in dark rooms. Apprehended images are then examined on a computer program such as GenePix Pro (Molecular Devices, Sunnyvale, CA).

The design of species or group-specific oligonucleotides is a crucial step in this procedure since it defines the sensitivity and specificity of the assay (Urakawa *et al.,* 2002). It is generally decided that the length of the oligonucleotide, the type, number, and position of SNPs contained in an oligonucleotide, determine its discriminatory prospective for DNA hybridization. Most DNA macroarrays that have been developed are based on a solo region for the detection of a specific taxonomic group. Among these regions, various genome regions, such as ribosomal DNA spacers (ITS), mitochondrial genes (e.g. cytochrome oxydase c subunit 1, cox1) and some protein coding regions (β-tubulin, EF-1α, etc.), were chosen to target fungi (Le Floch *et al.,* 2007). Oligonucleotides with higher specificity are often intended from polymorphic sites located at indels existing in multi-sequence alignments (Tambong *et al.,* 2006).

E. Isothermal Amplification Techniques

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method developed by Tsugunori *et al.* (2000). This technique has been widely used because of its high specificity, simplicity, efficiency, and quickness. LAMP involves two long outer primers and two short inner primers that recognize the six specific sequences in the target DNA. The first inner primer containing sense and antisense sequences in the DNA will hybridize the target sequence and initiate DNA synthesis. Also, the outer primer carries out the strand-displacement DNA synthesis and yields a single stranded DNA which works as a template for the second inner and outer primers fabricating a DNA molecule with a loop structure.

This makes it ideal for point-of-care detection of plant pathogens in the field (Fukuta *et al.,* 2013) and an alternative and reliable method for the detection of microbial pathogens and diagnosis of plant diseases (Ghosh *et al.,* 2016, Ghosh *et al.,* 2017). The advantages and simplicity of LAMP assay is that the reaction could be easily judged as positive or negative by naked eye through assessing of increased turbidity or colour change (Ghosh *et al.,* 2017), and for that it does not require any expensive instruments like thermal cycler.

In general, these methods are much quicker, more exact, more profound, and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise. Additionally, due to its high degree of specificity, molecular techniques can distinguish closely related organisms at different taxonomic levels (Ghosh *et al*., 2017).

F. Rolling Circle Amplification (RCA)

Amplification of circular DNA using the principle of isothermal amplification is known as rolling circle amplification (RCA). RCA involves using a DNA polymerase with strand displacement activity (such as ϕ29 DNA polymerase) to extend a single primer annealed to a circular DNA template. The strand displacement activity allows the newly synthesized DNA to displace the previously generated DNA releasing ssDNA. This enzymatic process of primer extension combined with strand displacement generates a long single stranded DNA containing a repeated sequence complementary to the circular template.

Rolling circle amplification has been widely used for plant pathogen detection. Several techniques have been used in combination with RCA such as RFLP and direct sequencing to identify and classify plant pathogen efficiently with significantly lower effort and cost than conventional technologies. Necked eye visualization of RCA product has been developed for 40 *Fusarium* strains by adding fluorescent dye to the reactions (Davari *et al.,* 2012). Ligation of padlock probes followed by RCA has also been developed for identification of fungal pathogens (Najafzadeh *et al.,* 2011).

PROTEIN BASED DETECTION TECHNIQUES

Serological detection systems use specific antibody developed in animals in respond to specific protein of the pathogen. Soil borne bacteria can be detected if bacterial antigens are used to developed antibody. In fact, such methods were used as monotonous analytical tool. That Involves use of specific antibodies to perceive their analogous antigens in the test samples. Antibodies are composed of immunoglobulin (Ig) proteins produced in the body of an animal in response to the presence of antigens, which are usually foreign proteins, complex carbohydrates, polynucleotides or lipopolysaccharides. Each antibody is specific to a particular antigen and will bind to it. Diagnosis using serological methods has many advantages. Though antibodies may take several weeks to produce, they are generally steady for long periods if stowed correctly and produce results quickly. They have wide application for general and specific recognition of unique epitopes of many soil borne microorganisms but have been under-utilized in the diagnosis of plant pathogens other than viruses and bacteris.

Tests using antibodies have improved greatly. They are now suitable for both laboratory and field conditions, can identify strains within species, are sensitive to the nano gram level and take less time to carry out. There are certain restrictions to the use of antibodies in pathogen identification. Firstly, the nature of the cross reactions between heterologous antibody-antigen complexes are not well understood so the degree of relatedness between crosses reacting isolates cannot be estimated. Secondly, diagnosis is based on only part of the organism's structure such as the coat protein of a virus, which represents only a small proportion of the information about the virus. Thirdly, serology is only suitable when the antiserum is ready or when an antigen is available for producing an antiserum. Finally, serology is of no use for identifying previously un-described soil-borne pathogens. A serological diagnosis is organized by detecting the rising titers of antibody or detection of IgM. By serological methods most of the common soil borne bacterial infections is diagnosed. Many serological methods have been reported including tissue blot immunoassay (TBIA) enzyme-linked immunosorvent assay (ELISA), and quartz crystal microbalance immunosensors (QCMI).

A. ELISA

The enzyme-linked immunosorbent assay (ELISA) is another method for identification of soil borne pathogen based on antibodies and color change in the assay. In this method, the target antigens from the viruses, bacteria and fungi are made to specifically bind with antibodies conjugated to an enzyme. The detection can be visualized based on color changes resulting from the interaction between the substrate and the immobilized enzyme. The performance of ELISA can be improved greatly with the application of specific monoclonal and recombinant antibodies, which are commercially available.

Specific monoclonal antibodies have been used in ELISA to achieve lower limits of detection in the order of 10^5–10^6 CFU/mL (López *et al.,* 2003). For plant disease detection, tissue print-ELISA and lateral flow devices that enable detection have been fabricated for on-site detection. However, the sensitivity for bacteria is relatively low (10^5–10^6 CFU/mL) making it useful only for the confirmation of plant diseases after visual symptoms appear but not for early detection before disease symptoms occur (López *et al.,* 2003).

B. Lateral Flow Devices

Perhaps, now days lateral flow devices (LFDs) is one of the most readily available farmer's friendly diagnostic tools. The advantages of these devices are that they are simple to use and results are quick, usually in less than 10 min. LFDs are most useful for diagnosis of plant viral diseases and those LFDs are commercially available. The LFDs are mainly based on the serological specificity of polyclonal or monoclonal antibodies to particular targeted pathogens. The sensitivity of LFDs varies with target and type of antibody used. An LFD-based test using an IgM monoclonal antibody to detect *Rhizoctonia solani* can detect as little as 3 ng ml^{-1} of antigen, equal to the sensitivity of standard ELISA procedures (Thornton, 2008). This study was particularly interesting because it targeted a soil-borne plant pathogenic fungus, whereas most commercial LFD-based tests target plant viruses and bacterial pathogens for which specific antibodies are generally widely available. Development of species-specific antibodies to fungi has been a greater challenge but, as noted above, has been successfully achieved for some targets.

BIOCHEMICAL METHODS OF PATHOGEN DETECTION

All organisms have distinct biochemical features, and these can be used for identification. Some characteristics are shared by large groups, while, at the opposite end of the scale, others are unique to individual populations within the species. The characterization of pathogen is therefore paramount in determining the taxonomic level to which an organism defined. Bacteria have long been identified based on metabolic functions such as their ability to metabolize certain substrates and, more recently, by analysis of their fatty acid profiles. Additionally, soluble protein analysis by gel electrophoresis has been adopted for both bacteria as well as fungi. All methods rely upon gene expression, and since this may be regulated by environmental factors, care has to be taken to standardize these. Goor et al. (Goor *et al.,* 1986) were among the first to explore the applicability of the "galleries" of biochemical tests confined in the API (appareilsetprocédésd'identification) systems to the identification of phytopathogenic strains of *Erwinia* and *Pseudomonas*. Those useful for distinguishing strains of *Erwinia* were API 20E, API 50CHE, and the oxidase, enzyme, and aminopeptidase systems. *Pseudomonas* strains were distinguished by using API auxanographic systems 50AO, 50CH, and 50AA. Biology is a substitute to the API system and gave better difference of 204 bacterial pathogens associated with a sheath rot complex and grain staining of rice in the Philippines (Cottyn *et al.,* 1996). Using this system, it was found that all the reported strains of *Pseudomonas fuscovaginae* were positive for the production of 2-ketogluconate, but strains of *Acidovoraxavenae* and *Burkholderiaglumae* were negative (Cottyn *et al.,* 1996). In contrast, *B. glumae* was positive for the production of acid from inositol but negative for the production of 2-ketogluconate, and *A. avenae* was negative for both these reactions. De Laat *et al.* (1994) identified the causal agent of bacterial leaf rot of a species of aloe (Aloevera) as *Erwinia chrysanthemi* biovar 3 on the basis of its ability or failure to metabolize a number of substrates as well as its agglutination by an antiserum prepared against a defined strain of the organism. Similarly, Pernezny (Pernezny *et al.,* 1995) were able to define the bacterial species causing a severe outbreak of bacterial spot in lettuce fields in Florida as *Xanthomonas campestris* on the basis of substrate utilization, the pathovar existence defined as vitians by its fatty acid profile. In some instances organisms may be identified by their production of unusual metabolites.

For example, strains of *Aspergillus flavus* that were aflatoxigenic were recognized by their production of volatile C15H24 compounds such as alphagurjunene, trans-caryophyllene, and cadinene. These compounds were not produced by nontoxigenic strains (Zeringue *et al.,* 1993). Fatty Acid Profiles (FAME Analysis) identification of bacterial pathogens of plants by fatty acid methyl ester analysis is usually performed on pure cultures of the organism. About 40 mg of wet cells is saponified and methylated. The fatty acid methyl esters (FAME) are extracted in an ether–hexane mixture and analyzed by gas chromatography. Areas of the resulting peaks on the chromatograms are calculated and compared with profiles of known reference strains by computer programs (Roy, 1988). For example, the organism responsible for an outbreak of bacterial spot of lettuce was defined as *Xanthomonas campestris pv. vitians* as the fatty acid profiles of the strains collected from the field matched this pathovar most closely (Pernezny *et al.,* 1995). In a more extensive study, Wells *et al.* (1994) were able to differentiate the five species of *Erwinia* of the amylovora group as well as the four species of the herbicola group. Electrophoresis of soluble proteins from plant pathogens often gives rise to complex patterns, and these can be used for identification purposes. Instead of using a general protein stain, such as Coomassie Blue, a particular protein dye, which, for example, might have enzymatic activity, may be revealed by appropriate staining methods. MacNish et al. (MacNish *et al.,* 1994), by staining for pectic enzymes, were able to place 4250 Australian isolates of *Rhizoctonia solani* in 10 groups, which they termed zymograms.

RECENT ADVANCES IN PATHOGEN DETECTION

A. Indirect Ways of Pathogen Detection

There are various ways of indirect pathogen detection methods some of them are thermography, and fluorescence imaging. Thermography allows imaging the differences in surface temperature of plant leaves and canopies, which is also a promising tool to monitor the heterogeneity in the infection of soil borne pathogens. In Thermography, thermo-graphic cameras can capture emitted infrared radiation and color difference can be analyzed. Previous reports have demonstrated that the loss of water in plants regulated by stomata would be affected by plant pathogens (Hillnhütter *et al.,* 2011). The resulting disease can be monitored through thermo-graphic imaging and the amount of water transpired can be determined, without the external temperature influences (Oerke *et al.,* 2006). Whereas, In Fluorescence Imaging technique, the chlorophyll fluorescence is measured on the leaves as a function of the incident light and the change in fluorescence parameters can be used to examine pathogen contagions, based on changes in the photosynthetic apparatus and photosynthetic electron transport reactions (Bürling *et al.,* 2011). Using this technique, spatial and temporal alterations of chlorophyll fluorescence were analyzed for exact detection of leaf rust and powdery mildew infections in wheat leaves at 470 nm (Kuckenberg *et al.,* 2009).

Hyperspectral imaging is also an emerging technology to detect the plant pathogen in an indirect way it can be used to obtain useful information about the plant health over an extensive range of spectrum between 350 and 2500 nm. It is increasingly being used for plant phenotyping and crop disease identification in largescale agriculture. This technique is highly robust and provides rapid analysis of imaging data. Hyperspectral techniques are used for plant pathogen detection by measuring the changes in reflectance resulting from the biophysical and biochemical characteristic alterations upon infection. *Magnaporthe grisea* infection of paddy, *Phytophthora infestans* infection of tomato and *Venturia inaequalis* infection of apple trees have been identified and reported using hyperspectral imaging techniques (Delalieux *et al.,* 2007, Zhang *et al.,* 2003).

B. On-site Direct Diagnosis of Plant Diseases

Now days the developed different advanced on-site diagnosis techniques are very simple and very useful to the growers for making timely decisions and earlier implementation of this technique for enabling correct disease management strategies that could be reduced the impact of the disease. On-site testing provides additional advantages for providing a rapid result without sending the sample to a diagnostic laboratory that may be some distance away and also can engage the grower if such a diagnostics are performed 'field-side' in their presence. For instances, the on-site molecular detection of *Spongospora subterranean*, a soil-borne pathogen of potato is as achieved by using a rapid and simple protocol, called fluorogenic probe-based assay which is comprising of magnetic bead-based nucleic acid extraction and followed by qPCR using portable real-time PCR. This portable real-time PCR approach is favourable when it compared to a laboratory based system because it can detect the pathogen even when the pathogen colonization in host is very low as few as 100 copies of DNA from *Spongospora subterranea.* The developed portable real-time PCR can serve as an alternative to laboratory-based approaches and a useful on-site tool for pathogen diagnosis.

X-ray crystallography is now one of the advanced techniques which is utilizing for detecting specific pathogen based on specific protein which is secreted by the pathogen or host during interaction. In recent studies, based on Pik sensor protein, the devastating rice blast pathogen (*Magnaporthe oryzae*) was detected in Iwate Biotechnology Research Centre (Japan) using X-ray crystallography facilities from Diamond Light Source. This has been first time utilization of crystallographic based method to detect the pathogen in molecular level by following the gene-for-gene model.

C. Use of Next-generation Sequencing (NGS) in Plant Pathogen Detection

During early stage infections in plants caused by different fungal/oomycete pathogens, most of the time it is not detectable until symptoms are developed in host plants.

So many serological and molecular techniques which are already discussed above are generally used for detecting these pathogens. But next-generation sequencing (NGS) is most potential reliable as a diagnostic tool, due to its capacity in targeting unique and multiple loci of pathogens in an infected plant metagenome (Sharma *et al.,* 2016b). NGS has extensive potential for identification of important eukaryotic plant pathogens. But a drawback of this method is to assemble and analysis of huge amounts of sequence, because it is laborious and time consuming. This problem could be overcome by utilizing targeted genome capture (TGC) oligonucleotide probes to enrich specific nucleic acids in heterogeneous extracts and can therefore increase the proportion of NGS reads for low-abundance targets. The Electronic probe Diagnostic Nucleic acid Analysis (EDNA) is a potential technique for greatly simplifying detecting fungal and oomycete plant pathogens by utilising metagenomes. The EDNA has a better accuracy for detecting fungal and oomycete plant pathogens as the electronics probes completely depend on matches between queries and metagenome reads.

CONCLUSION

Plant pathogen diagnostic techniques have contributed significantly to our ability to detect and investigate in the laboratory and, most recently, directly in the field.

The current state of the art techniques demonstrate reproducible sensitivity and are generally much faster than conventional techniques. Better understanding of pathogenicity factors, rapid and accurate detection of fungal pathogens to the species level are prerequisite for disease surveillance and development of novel disease control strategies. Moreover, a timely detection of resistance levels in soil borne fungi in a field would help the growers formulate proper decisions on resistance management programs to control diseases. However, since no single method satisfies all or even most of the emerging criteria for faster, effective, reproducible and sensitive results, there is still an obvious knowledge gap in research in this field.

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Conflicts of Interest

The authors declare no conflict of interest.

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