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A Review on Disease Detection, Pathogen Identification and Population Genetics in Fungi

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Abstract
Based on knowing the fungi how much they are important in causing holistic impacts on human welfare, the present review initiated with reviewing the different disease detection and pathogen identification methods of plant pathogenic fungi and over viewing the population genetics of fungi. Currently, more and more diagnostic laboratories and inspection agencies are using molecular methods for detection and identification of diseases caused by plant pathogenic fungi. Better understanding of fungal-plant interactions, pathogenicity factors, rapid and accurate detection of fungal pathogens to species or strain level are a crucial prerequisite for disease surveillance and development of novel disease control strategies. Molecular technology increases understanding of the biology and population structures of plant pathogens, provides quick and accurate answers to epidemiological questions about plant diseases, and supports disease management decisions. Both domestic and international plant quarantine stations must be facilitated with high throughput, rapid and sensitive methods for detection of quarantine pathogens. The methods of population genetics offer powerful tools to elucidate the life histories of important plant pathogens and address fundamental questions about the biology of these organisms. Increased knowledge of the population biology of pathogens is likely to lead to better management of disease in agricultural ecosystems. Eventhough every pathogen detection methods starting from conventional up-to the most sophisticated real-time PCR, they have their own advantages and drawbacks, but selecting of the most appropriate one based on its multi-dimensional importance is crucial at this time. In Ethiopia, most of the detection methods are based on the conventional one, but these methods are not as effective as molecular detection methods. So developing and utilizing these more effective, time sever and easy assays of fungal disease detection and pathogen identification methods is important.
Keywords: Agricultural Ecosystems, Fungal-Plant Interactions, PCR, Pathogenicity Factors, Real-Time PCR

1. INTRODUCTION

A fungus is a member of a large group of eukaryotic organisms classified as a separate Kingdom: Fungi (Whittaker, 1969). Kingdom Fungi contains a very large biodiversity of organisms such as yeasts, moulds, rusts, smuts, truffles, morels and mushrooms (Alexopoulos *et al*., 1996; Stajich *et al*., 2009). More than 69,000 species have been described, but the total number of existing fungi may be more than 1.5 million species (Hawksworth, 1991). Of these species of fungi, only around 5-10% are able to culture artificially (Manoharachary *et al*., 2005). Many fungal species are important plant and human pathogens (Agrios, 2005). Fungi play critical roles in human and animal health, agriculture and food industry, biotechnology, and as model organisms for basic scientific inquiries (Xu, Undated).

Fungi have an enormous impact on human welfare by destroying valuable crops as devastating pathogens or producers of mycotoxins (Xu *et al*., 2006). Fungal pathogens including oomycetes have been responsible for several destructive diseases such as potato late blight, wheat stem rust, rice blast and grapevine downy mildew that have ruined the economy of several countries resulting in famine and migration of millions of humans to other countries to escape starvation and ultimate death (Narayanasamy, 2011). Fungi outnumber all other types of pathogens that attack plants and cause a very serious economic impact on agricultural production due to their ability to induce diseases of cultivated crops that result in important yield losses (Paplomatas, 2006). Microbial plant pathogens including fungal pathogens have the ability to infect a few or a wide range of plant species causing varying magnitude of quantitative and qualitative losses in crops cultivated in different ecosystems (Narayanasamy, 2011). Global losses caused by crop diseases have been estimated to range from 9% to 14.2% of potential yield (Orke *et al*., 1994). The estimates of losses made later indicated that about 14.1% of produce may be lost due to crop diseases with a monetary value of $220 billions per annum, the developing countries suffering more losses compared with developed countries (Agrios, 2005).

Among fungi, those that are soil-borne are more difficult to handle for a number of reasons. Soil is a very complex environment that bears difficulties in the identification, isolation and quantification of pathogens. In this respect, detection and enumeration of soil fungal inhabitants have always been challenging issues through the years (Kowalchuk, 1999). Developing direct detection assays is challenging because fungal pathogens can exist as multiple species complexes or at very low concentration in clinical and natural environments. Different molecular genotypes/varieties can also exist within species and may have different pathogenic profiles and virulence levels to the host. In addition, unculturable and non-sporulating fungi remain a major challenge when

studying biotrophic, endophytic, and mycorrhizal groups. Therefore, novel techniques are required when attempting to detect fungi in the environment (Tsui *et a*l., 2011). Over the years, several classical approaches have been developed to detect and identify fungi that cause diseases of plants. Selective media have been devised to exclude the large number of contaminant organisms (mostly saprophytes) and allow growth of target fungi. However, in most cases (especially for soil-borne microorganisms) recovery has been associated with the method of choice, contaminants as better competitors often outgrow the target pathogen, more than one species share common morphological characters while the bias of the researcher who uses the method is an unavoidable piece in the microbial detection puzzle (Termorshuizen *et al*., 1998). Rapid and accurate detection of fungal pathogens to species or strain level is often essential for disease surveillance and implementing a disease management strategy (Tsui *et a*l., 2011). Better understanding of fungal-plant interactions and pathogenicity factors is a crucial prerequisite for the development of novel disease control strategies. Recent advances in sequencing and genomic techniques have made it possible to monitor gene expression changes at the whole-genome level, which has impacted all aspects of biological sciences (Xu *et al*., 2006).

In phytopathology early identification of the causative agent of disease is paramount in order to recognise the pathogen, and implement regulations involving control and quarantine (Atkins and Clark, 2004). The more knowledge about the genetic structure of populations of plant pathogens is also needed to implement effective control strategies (Wolfe and Caten, 1987). Conventional methods often rely on identification of disease symptoms, isolation and culturing of environmental organisms, and laboratory identification by morphology and biochemical tests. These methods, although the cornerstone of fungal diagnostics, can lead to problems in identification, resulting in incorrect interpretation, diagnosis and ultimately treatment. The methods rely on experienced, skilled laboratory staff, the ability of the organism to be cultured, are time consuming, non-quantitative, prone to contamination and error and in the case of plant and medical pathology often delay treatment (Atkins and Clark, 2004).

During the last decades, the advent of molecular biology promised to offer radical alternatives in the detection and enumeration of fungal pathogens. At the same time, the acquisition of DNA sequences provided information that led to the identification of new and unknown species. These data, together with classical characterization of fungi in the field, opened new insights into the range of fungal functions and interactions mostly within terrestrial communities (Bahnweg *et al*., 1998). New, rapid screening methods are being developed and increasingly used in all aspects of fungal diagnostics. These methods include immunological methods, DNA/RNA probe technology and polymerase chain reactions (PCR) technology (Atkins and Clark, 2004). Molecular technology increases understanding of the biology and population structures of plant pathogens, provides quick and accurate answers to epidemiological questions about plant diseases, and supports disease management decisions (Michailides *et al*., 2005). Based on the importance of knowing the fungi how much they are important in causing holistic impacts on human welfare, the present review initiated with the objectives: to review the different disease detection and pathogen identification methods of plant pathogenic fungi and to overview the population genetics of fungi.

2. DETECTION AND IDENTIFICATION OF PLANT PATHOGENIC FUNGI

Historically, the detection and identification of fungi has relied on a combination of microscopy and culture-based techniques. The microscopic examination of clinical specimens using histopathologic studies or direct fluorescent stains allows for rapid detection; however, these methods lack sensitivity and specificity (Hayden *et al*., 2001; Kaufman *et al*., 1998; Schwarz, 1982) and typically allow for a preliminary identification only (Wengenack, and Binnicker, 2009). Rapid progress in understanding plant disease is dependent on efficient methods for analyzing plants and their pathogens (Tzeng *et al*., 1992).

Detection of microbial pathogens in crop plants and other host plant species and also in the environment such as soil, water and air may be required in order to (i) determine the presence and quantity of the pathogen(s) in a crop to initiate preventive or curative measures; (ii) assess the effectiveness of cultural, physical, chemical or biological methods of containing them; (iii) certify seeds and planting materials for quarantine and certification programs; (iv) quantify the pathogen population in the location concerned and for relating to consequent yield loss; (v) assess variations in pathogen infection in germplasm collections to select sources of resistance to disease(s) in question; (vi) identify rapidly new pathogens or strains of existing pathogens to restrict their further spread; (vii) study the taxonomic and evolutionary relationships of plant pathogens; (viii) resolve the components of complex diseases induced by two or more pathogens; and (ix) study the intricacies of interactions between plants and pathogens to have an insight into the phenomenon of pathogenesis and gene functions (Narayanasamy, 2011). Over the last 30 years, several techniques have been developed which have found application in plant pathogen diagnosis; these include the use of monoclonal antibodies and enzyme-linked immunosorbant assay (ELISA), which drastically increased the speed in which pathogen antigens could be detected *in vivo*, and DNA-based technologies, such as the polymerase chain reaction (PCR) which enable regions of the pathogen's genome to be amplified several million fold, thus increasing the

sensitivity of pathogen detection. Despite such advances, cultural diagnosis still predominates, largely due to the technical experience & costs associated with the more recent techniques (Fulton, 1997).

Where little is known of the genetic constitution of a particular fungus, direct analysis of polymorphisms in DNA can provide useful information on variation both within and between species. Several detection methods were used with varying degrees of success to differentiate pathovars or subgroups within taxa of plant-pathogenic fungi (Abdennadher, 1995). For example, RFLP analyses of nuclear DNA have proved to be a powerful method for studying variation within a range of species (Hulbert and Michelmore 1988; McDonald and Martinez 1990). However, detection of polymorphysms by techniques involving Southern hybridization analysis is time consuming and laborious. An alternative approach has been to utilize the PCR (Saiki *et al*., 1988) to examine for example, length variation in mini- and micro-satellite arrays (Love *et al*., 1990).

2.1. *Immunodiagnostic Assay*

The principal aim of an immunodiagnostic assay is to detect or quantify the binding of the diagnostic antibody with the target antigen. There are a number of different ways of detecting antibody/antigen binding, but often these involve coupling the antibody to an enzyme that can be used to generate a colour change when a substrate is added. Antibodies are molecules, produced by mammalian immune systems, that are used to help identify invading organisms or substances. If antibodies can be generated that recognise specific antigens associated with a given plant pathogen they can be used as the basis of a diagnostic tool. The use of antibody technology is well established in medical diagnostics and interest in its use in plant pathology has been increasing over the last decade (Ward *et al*., 2004).

Enzyme linked immunosorbent assay (ELISA), a technology developed in the 1970s, is the most commonly used diagnostic technique that uses antibodies (Clark and Adams, 1977; Voller and Bidwell,1985). It involves an enzyme-mediated colour change reaction to detect antibody binding. This is usually done in a microtitre plate where the degree of colour change, usually measured in a computer-controlled plate reader, can be used to determine the amount of pathogen present. These assays have the advantages of being simple, cheap and suitable for processing many samples (Ward *et al*., 2004). Since immunodiagnostic methods are most efficient they are successfully employed exclusively for the detection and diagnosis of Karnal bunt (Majumder *et al*., 2013). Kumar *et al*. (2008) developed antibody methods such as microtitre enzyme linked immunosorbant assay (ELISA), immunofluorescence staining test (IFST), seed immunoblot binding assay (SIBA), dyed latex bead agglutination test and immunodipstick assay. These immunoassay systems could be allowed for reliable quantitative assessments in a high throughput manner in regulatory laboratory facilities if developed in an appropriate rapid format in the form of kits such as immune-dipstick and immune-lateral flow assays. The immunodiagnostic assays for field use are inexpensive, rapid and do not require highly trained personnel (Majumder *et al*., 2013).

The simplest format is the plate-trapped antigen ELISA (PTA-ELISA) (Figure 1). In this assay, the microtitre plate wells are directly coated with the test sample. This is followed by incubation with a specific antibody which binds to the target antigen. In some assays the specific antibody is conjugated to the enzyme (direct detection), and in others the specific antibody is detected by a second generic antibody, e.g. anti-rabbit or anti-mouse, which is conjugated to the enzyme (indirect detection). A more commonly-used format is the double antibody sandwich (DAS-ELISA, Fig. 1). Here, specific antibodies are used to coat the microtitre plate, which then trap target antigen from the test sample. An enzyme-labelled specific antibody conjugate is then used for the detection (Ward *et al*., 2004). There are other less commonly used variations of ELISA such as competition assays (Kitagawa *et al*., 1989; Lyons and White, 1992). Micro-titre ELISA is being used for early detection of Karnal bunt pathogen in the host when the infection levels are very low (Varshney, 1999). SIBA was developed by Kumar *et al*. (1998) to check out the teliospore load on seeds at the time of vigour testing. The principle behind this technique is that when the infected seeds are kept on the nitrocellulose membrane, the teliosporic antigens are adsorbed and diffused according to the teliosporic load on the nitrocellulose sheet. The coloured imprint develops and appears to have direct correlation with the grade or severity of infection on bunted grains (Majumder *et al*., 2013).

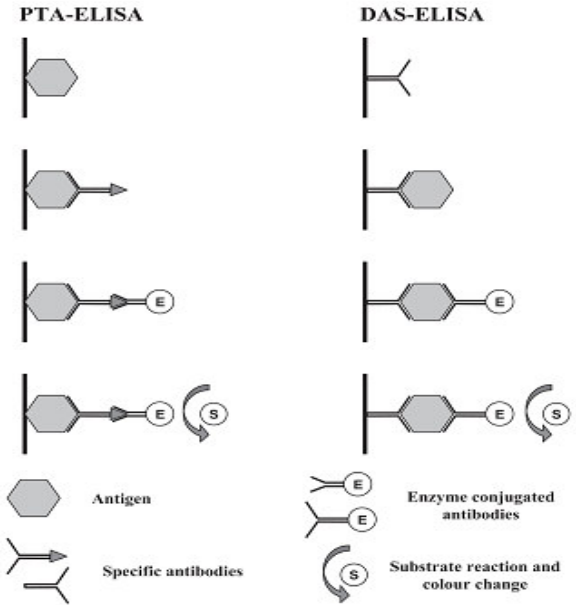

Figure 1. Schematic diagram illustrating two of the most common ELISA formats: Plate-trapped antigen (PTA-ELISA), here shown using an indirect detection system and Double antibody sandwich (DAS-ELISA). The reactions are described in detail in the text (Ward *et al*., 2004).

2.2. Nucleic Acid Based Techniques

Among the tools available for pathogen detection, nucleic acid based techniques are widely recognized as one of the most useful and efficient methods for detection (Majumder *et al*., 2013). Nucleic acid-based methods (using probes and/or PCR) have increasingly been used in recent years to develop diagnostic assays for plant pathogens (Schots *et al*., 1994; Ward, 1994; Martin *et al*., 2000). Molecular assays offer a promising alternative to routine methods by reducing the time to identification, increasing sensitivity, and enhancing laboratory safety. However, a lack of test standardization and limited validation data for many fungal nucleic acid tests has hindered their general acceptance and broad implementation into clinical laboratories (Wengenack, and Binnicker, 2009). Commercially available nucleic acid hybridization probes, under the brand name AccuProbes, were introduced in 1992 by Gen-Probe, Inc. for use with a limited number of fungi (Beard *et al*., 1993; Sandin *et al*., 1993).

Nucleic acid based detection techniques, particularly those that rely on the PCR, typically are rapid, potentially very sensitive and highly specific (Majumder *et al*., 2013; Ward *et al*., 2004). Most assays developed for bacteria and fungi have detected pathogen DNA, which is easier to prepare, and more stable than RNA (Ward *et al*., 2004). However, proper implementation of these techniques poses challenges, ranging from the significant cost of the technology to the need to understand the techniques well enough to be able to interpret (Majumder *et al*., 2013). Nucleic acid hybridization assays involve the selection, cloning and chemical labeling of sequences specific to the target organism. These are then used as probes to detect RNA or DNA of the pathogen in extracts or tissue squashes of plant material. The assay may involve immobilization and detection of nucleic acid on a membrane or, in some instances, utilize a microplate format similar to that used in immunoassays (Umek *et al*., 2001). The development and use of nucleic acid hybridization assays to detect and identify plant pathogenic fungi has been limited, although species-specific DNA probes have been developed towards several *Fusarium species*, including *F. culmorum, F. graminearum* and *F. avenaceum* (Knoll *et al*., 2002). Hybridization is relatively insensitive and there are few instances where this method has been used to detect fungi directly in extracts from plant tissues. Hybridization assays are sufficiently sensitive for the identification of fungi cultured from plant tissue (bio-amplification) but this, of course, incurs the problems associated with selection during isolation and greatly increases the time required to complete the analysis (Mostafa *et al*., 2012).

2.3. PCR-Based Assays

The polymerase chain reaction (widely referred to as PCR), a technique introduced in the mid-1980's by Kary Mullis (1990). PCR is an in vitro method of nucleic acid synthesis by which a particular segment of DNA can be amplified which detect organism specific DNA/RNA sequence (Majumder *et al*., 2013).The development of PCR technology relies on three fundamental steps: (1) the selection of a specific target region of DNA/RNA to identify the fungus,(2) extraction of total community DNA/RNA from the environmental sample and (3) a method to identify the presence of the target DNA/RNA region in the sample (Atkins and Clark, 2004). The

procedure takes place in three essential steps: the initial melting of the double strands of the DNA follows the annealing (hybridization) of two synthetic oligonucleotides (primers) with sequences complimentary to the ends of the target fragment and the cycle is completed by the final primer extension (polymerization) by the DNA polymerase (Paplomatas, 2006). It involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of DNA, annealing of the primers to their complementary sequences and extension of the annealed primers with a thermostable DNA polymerase (Mullis, 1987). PCR is used to create large amount of copies of DNA. Several attempts have been made to develop species-specific PCR primers for fungal plant pathogens (Hensen 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).

The PCR technology has produce many procedures for typing strains and species, some of which have become standard methods for species and strain identifications (Xu, Undated). The PCR has been found a widespread use throughout plant pathology, and a significant number of PCR-based assays have been developed for use with species associated with the production of mycotoxins (Parry and Nicholson , 1996). PCR can also be used in a one- or two-step reverse-transcriptase (RT) PCR protocol to detect genes that are being expressed rather than to detect the presence of the DNA that encodes a particular gene. Reverse transcriptase is used to generate a DNA copy (cDNA) from RNA (mRNA or total RNA) present in a sample. The cDNA is then subjected to PCR using primers designed for the target sequence. When designed to genes characteristic of a particular species or to genes involved in mycotoxin biosynthesis and assayed in a real-time or competitive PCR format the mRNA level of target genes can be estimated in a manner similar to that for DNA (Alexander *et al*., 1997). Although PCR is a highly sensitive technology, the presence of inhibitors in the plant tissues and soil, greatly reduces its sensitivity. The inhibitors are believed to interfere with the polymerase activity for amplification of the target DNA. Another problem with the conduct of PCR arises from the possible DNA contamination leading to false negative results (Narayanasamy, 2011).

PCR-based molecular methods and sequencing of ribosomal DNA have been used successfully to identify the richness of the species in different environments (Van den Koornhuyse *et al*., 2002), and have provided insight into the ecological processes that affect the structure and diversity of fungal communities (Gomes *et al*., 2003; Artz *et al*., 2007). The internal transcribed spacer (ITS) region has become a validated DNA barcode marker for the identification of many fungal species (Seifert, 2008). PCR is undoubtfully the most important technique in diagnostics and has found wide application as a powerful molecular tool mostly due to the development of thermo tolerant DNA polymerases and automated thermocyclers (Paplomatas, 2006). PCR is preferred over classical or other molecular techniques in the diagnosis of plant pathogens for a number of advantages that makes it very popular (Henson and French, 1993). PCR methods are easy to set up and have the advantage of requiring only minute amounts of starting material or template DNA. Although simple in concept, PCR methods have unrivaled, often overlooked complexity. The source of this complexity includes multi-ionic interactions, kinetic constants, and enzymatic activities etc. These factors can repeatedly affect the reactants in a typically small PCR reaction volume over an extended time period. Despite these potential problems, many methods have been developed and are widely used (Xu, Undated). There are some common PCR-based strain typing techniques which are used to detect and identify fungal pathogens. Including: Random amplified polymorphic DNA (RAPD), PCR fingerprinting, Microsatellite loci, PCR-RFLP of known genes, Single-strand conformation polymorphism (SSCP), Amplified fragment length polymorphism, Restriction fragment length polymorphisms, DNA sequencing and, Real-time PCR.

2.3.1. Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPDs) analysis has attracted a lot of attention after it's advent during the 90's (Williams *et al*., 1990). In RAPD analysis, genomic or template DNA is primed at a low annealing temperature (30–38°C) with a single short oligonucleotide (ca. 10 bases) in the PCR. Multiple PCR products of different electrophoretic mobility are typically generated (Williams *et al*., 1990). RAPD analysis detects two types of genetic variations: (i) in the length of DNA between the two primer binding sites, and (ii) in sequence variation at the priming regions. Nucleotide substitutions in the region of PCR primer binding, particularly at the 3′ ends, can prevent binding of the primer to the DNA template. As a result, this band will be missing in a PCR reaction. Similarities in banding profiles among strains (i.e., the number and mobility, but not the density of the bands) can be calculated and used to infer strain relationships. When multiple primers are screened, RAPD analysis can be very sensitive to detect variation among isolates that cannot be observed using other methods (Xu, Undated).

RAPD markers have become popular because this PCR technology is relatively easy to implement (Williams *et al*., 1990), and the necessity of small amounts of genomic DNA (Paplomatas, 2006). The greater importance of RAPDs to plant pathologists, is it can be assayed using very small amounts of fungal biomass, making them an ideal tool for obligate biotrophs such as rusts and mildews. Because a large number of amplicons can be screened in a relatively short period of time, RAPDs are especially useful in differentiating

clonal lineages for fungi that reproduce asexually. Furthermore, RAPD data are easy to interpret because they are based on amplification or nonamplification of specific DNA sequences (amplicons), producing a binary data set that is easy to enter into a spreadsheet for analysis (McDonald, 1997). Although technically fast and simple, there are some disadvantages to RAPD. The major drawback is irreproducibility. RAPD analysis can detect minute variation among strains because, as noted above, even a single nucleotide mismatch in the priming region may prevent annealing and the absence of a characteristic band on gels. Small differences in any aspect of PCR conditions that affect binding of the primer may have similar effects; consequently, RAPDs are sensitive to the vagaries of the testing procedure. This problem can be minimized if strains under study are treated identically. When multiple strains are compared, the same PCR buffer, the master mix (includes all four nucleotides, primers, appropriate ions, and DNA polymerase) and the same thermal cycler and PCR running program should be used at the same time (Xu *et al*., 1999a; 2000a).

Despite their drawbacks, RAPDs are powerful tools that are especially useful for fungi that are obligate parasites or that have a population structure composed of clonal lineages. PCR-based genetic markers that can detect more than two alleles and that exhibit codominance, such as SCARs and microsatellites (Groppe *et al*., 1995), are likely to replace RAPDs as studies of fungal population genetics become more sophisticated. As advanced PCR-based markers become available, it may become possible to amplify specific DNA sequences from soil or root samples and make a direct assessment of the genetic structure of populations of soilborne fungi without first making pure cultures. But if a fungus can be cultured readily on artificial media, then RFLPs offer many advantages for population genetics studies (McDonald, 1997). Random amplified Polymorphic DNA (RAPD) analysis has been used by a large number of workers to study variability within and between species, including many *Fusarium* species (*F. graminearum*, *F. cerealis (F. crookwellense)*, *F. venenatum*, *F. torulosum*, *F. sambucinum and F. proliferatum*) (Carter *et al*., 2002). RAPDs is also applied to the carnation wilt fungal pathogen *Fusarium oxysporum f. sp. dianthi* and they were able to identify specific banding patterns that were subsequently used as probes to distinguish between races of the pathogen (Manulis *et al*., 1994). In another study, genetic relationships could be inferred among the wheat bunt fungi using RAPD markers (Shi *et al*., 1995), *Alternaria* species pathogenic to crucifers could be differentiated on the basis of RAPD profiles (Sharma and Tewari, 1998).

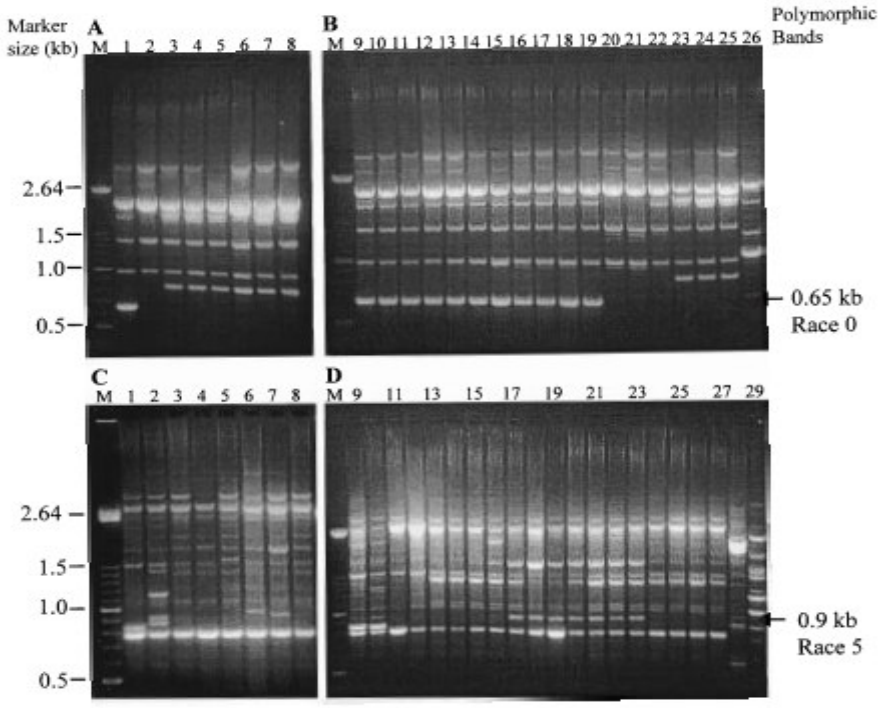

Figure 2. RAPDs generated by primer OPF-16 (A, B) and OPF-10 (C, D) using a DNA bulk of *F. oxysporum f. sp. ciceris* isolates of a race (A, C) and DNA from individual isolates representative of the race (B, D). DNA products were separated on a 1.5% agarose gel and stained with ethidium bromide. Numbers on the left side are the sizes of the 0.1-kb DNA ladder XIV (Boehringer-Mannheim) (lane M). Numbers on the right side are the sizes of the marker bands that identify races 0 (A and B) and 5 (C and D). A and C: lanes 1–8, DNA bulks for each of *F. oxysporum f. sp. ciceris* races 0, 1B/C, 1A, 2, 3, 4, 5, and 6. B: lanes 9–19, race 0 isolates Foc- 7802, -9018 (JG62), -82108, -8717, -7952, -8207, -9032, -9601, -91118, -9604, -82118; lanes 20–22, race 1B/C isolates Foc-USA 3–1 (JG62), -1987 W-17, -9602; lanes 23–26, race 1A isolate Foc-7989, race 5 isolate Foc-8012, race

6 isolate Foc-9093 (PV1), and nonpathogenic *F. oxysporum* isolate Fo-9009, respectively. D: lanes 9–12, race 0 isolates Foc-7802, -91108, -9018 (JG62), -9601; lanes 13–16, race 1A isolates Foc- 9027 (PV1), -9165, -9166, -7989; lanes 17–23, race 5 isolates Foc-8012, -8508, -9035, -USA 1–1 (JG62), -USA 14201, -9094 (JG629), -1987-W6–1; lanes 24–27, race 6 isolates Foc-TONINI, -9023, -9164, -9093 (PV1); and lanes 28–29, nonpathogenic *F. oxysporum* isolates Fo-8250 and -9009, respectively (Jimenez-Gasco *et al*., 2001).

2.3.2. Restriction fragment length polymorphisms (RFLP)

Restriction polymorphisms have been used to discriminate species and strains of fungi as well as other biological taxa. One approach is to digest genomic DNA with a restriction enzyme and directly examine the resulting bands in agarose or polyacrylamide gels after electrophoresis. Depending upon the size of the genome and the frequency of restriction recognition sites in the genome, it may be possible to directly compare digests of whole genomic DNAs from different species/strains. In complex genomes such as those in fungi, this direct comparison can only detect differences in high copy number DNA molecules, e.g. the ribosomal DNA genes and mitochondrial DNA. For low copy number genetic elements, it is almost impossible to observe restriction site polymorphisms through simple digestion and electrophoresis on agarose or polyacrylamide gels (Xu, Undated). The efficiency of RFLP detection varied with the number of restriction fragments to which the probes hybridized (Tzeng *et al*., 1992)

The most widely used RFLP method is a DNA–DNA hybridization-based technique that involves cutting genomic DNA with restriction endonuclease(s), separating the DNA fragments in agarose gels with electrophoresis, transferring DNA onto membranes, and hybridizing the membranes with labeled specific probes (Xu *et al*., 1997, 1998). RFLPs generated only through total genomic digests or with additional Southern hybridization using repetitive elements are challenging for interpretations. This is because these kinds of banding patterns are fingerprints and are difficult to relate to specific alleles of individual loci. In addition, when the number of bands is high, the accuracy in determining the number and size of DNA bands will be limited and affected by the electrophoretic conditions (Xu, Undated). RFLPs are most useful for fungi that can be grown easily in pure culture. Because probes can be chosen to show a high degree of specificity to the target fungus, some contamination by DNA from plants, other fungi, or bacteria can be tolerated. The properties of codominance and multiple alleles make RFLP markers advantageous compared to RAPDs for most studies in population genetics. An added advantage is that other labs can reproduce results using the same battery of probes and restriction enzymes, allowing work to be shared and compared among several labs (McDonald, 1997).

One disadvantage of RFLPs is that they require relatively large amounts (5 to 10 µg) of DNA from each individual, so the fungus must be grown in pure culture prior to DNA extraction. Of greater concern for many plant pathologists, RFLP analysis requires more technical expertise, including cloning, Southern blotting, and labeling of probes. Once the compulsory techniques have been mastered, RFLP analysis can proceed very rapidly. As an example, we routinely collect data for two RFLP loci from 300 individuals in a week. Another disadvantage of RFLPs is that they require expensive nylon membranes for Southern blotting. If only a few loci are assayed, the cost of materials and supplies for RFLP analysis can be expensive compared to RAPD analysis. However, repeat hybridizations of the same blot brings costs down very quickly, in our lab to less than 30 cents per locus per individual (McDonald, unpublished data as sited in McDonald, 1997).

RFLPs were used in nuclear and mitochondrial (mt) genomes in many studies of plant-pathogenic fungi. Because RFLPs are based on DNA-DNA hybridization, they are technically more difficult than RAPDs but offer the advantage of being more reproducible. Like RAPDs, RFLPs are easy to interpret. In addition, they are co-dominant and exhibit a potentially unlimited number of alleles per locus (McDonald, 1997). RFLPs can potentially provide a large number of genetic markers (Botstein *et al*., 1980; Michelmore and Hulbert, 1987). Such molecular markers have several advantages over classical genetic markers for many applications, including the development of genetic maps (Hubert *et al*., 1988). For RFLPs detected by Southern hybridization with probes targeted to single copy DNA markers, the interpretations are straightforward and data can be used in a variety of ways. Single copy RFLP markers are excellent for addressing population and evolutionary genetic questions in diploid or dikaryotic fungi (Xu *et al*., 1997, 1998).

2.3.3. Amplified fragment length polymorphism (AFLP)

The development of amplified fragment length polymorphism (AFLP) method has had a significant impact in its relatively short history. AFLP is a powerful method for fingerprinting strains and for generating a large number of dominant markers for the analysis of genetic crosses (Vos *et al*., 1995). AFLP usually involves two PCR steps. The first step is the pre-amplification step that uses unlabelled primers with single selective nucleotide in the primer. After the first step, the reaction mixtures are diluted for second PCR amplifications. In the second amplification, additional selective nucleotides are often added to enhance specificity. The selective second step often uses fluorescently or radioactively-labeled primers (Xu, Undated).

In the study of entomopathogenic fungi, the AFLP relies on the initial digestion of the entire genomic DNA with restriction enzymes into fragments of variable size. Specific adaptors are then attached to the sticky ends, and the fragments are amplified by PCR with primer pairs that anneal to the adaptors. The fragment sizes

can be scored on polyamide gels or if they are labelled with flourescent tags the fragments can be sized more objectively on a capillary sequencer. This fingerprinting method is more reproducible and has recently been used to characterise genotypes of *Beauveria bassiana* and *Metarhizium anisopliae* (de Muro *et al*., 2003; de Muro *et al*., 2005; Inglis *et al*., 2008). AFLP has several powerful advantages over the other methods. Many more fragments can be generated and analyzed in a simple reaction. It can detect restriction site variations as well as insertions and deletions within a genomic region. Different enzymes and/or selective extension nucleotides can be used to create new sets of markers. Therefore, AFLP can provide an almost limitless set of genetic markers. In addition, the fragments are stable and highly reproducible since they are amplified with two specific primers under stringent conditions (Xu, Undated). AFLP has been used to develop a species-specific assay for *A. ochraceus* that did not cross-react with other *Aspergillus* or *Penicillium* species tested (O'Brian *et al*., 2003).

2.3.4. PCR fingerprinting

The continuous improvements of the PCR technique led to its linking with various approaches for DNA fingerprinting (Paplomatas, 2006). PCR fingerprinting is similar to RAPD, except that primers are longer (>15 bases) and annealing temperatures are higher and PCR conditions more stringent. Most PCR fingerprinting primers are designed from repetitive DNA sequences (Xu *et al*., 1999a; 2000a). Commonly used PCR fingerprinting primers in fungi include M13, which is derived from the core sequence of phage M13; T3B, which originates from the internal sequences of tRNA genes; and TELO1, which is based on fungal telomere repeat sequences. Because of more stringent reaction conditions, PCR fingerprinting is generally more reproducible than RAPDs. Nonetheless, it suffers the same problems of interpretation as RAPDs. However, under standardized conditions, PCR fingerprinting has proven quite reliable for discrimination and the identification of species and strains (Xu, Undated).

2.3.5. Microsatellite

One emerging technique exploits the hypervariability of DNA regions composing multiple tandemly repeated units of di-, tri- or multiple nucleotides. This hypervariability can be caused by either strand slippage during DNA replication or unequal crossing-over during meiosis, both can occur much more frequently than nucleotide substitutions. Useful microsatellites can be located by probing a genomic library with simple repeated sequences or by searching databases of gene sequences. PCR primers flanking these repeat regions can be developed and PCR products can be run on polyacrylamide gels to detect differences in repeat numbers (Field *et al*., 1996). One potential drawback of this technique is that because multiple alleles are often found at a single locus, the relationships among alleles can be difficult to decipher, and alleles may be identical by convergence, not by descent (Xu, Undated). Microsatellite markers can be used to specifically target a fungus, e.g. *Metarhizium anisopliae*, in DNA extracted from bulk soil and thereby establish whether the fungus is present in the soil sample and how many alleles are present (Meyling, 2008).

2.3.6. PCR-RFLP of known genes

With increasing knowledge of genes and genomes from fungi, the supply of single-copy genes for genotyping is now feasible for many fungal species. These gene sequences can be used to investigate the variability among strains and the history of populations and species. One fast application is to design PCR primers to amplify a particular gene from representative strains followed by digestion of the amplified products with an array of restriction enzymes to screen for variability. Variable restriction sites can then be used to screen a larger sample of isolates (Xu, 2002). The gene specific PCR in combination with restriction digestions can generate excellent co-dominant markers that are highly stable and reproducible, ideal for both haploid and diploid organisms (Xu *et al*., 1999b). The study carried by Godoy *et al*. (2004), were used PCR-RFLP to demonstrate a high heterogeneity in the genotypes of the isolates of *F. solani* causing keratitis in Brazil. These PCR-RFLPs have been also used for the characterisation of both *Beauveria* and *Metarhizium* species (Bidochka *et al*., 2001).

2.3.7. Single-strand conformation polymorphism (SSCP)

SSCP is a promising technique that allows efficient detection of nucleotide substitutions in short fragments (<500 bp) of DNA. SSCP analysis typically involves the amplification by PCR of a unique segment of genomic DNA, melting the PCR products, and running the single strands on a non-denaturing polyacrylamide gel (Hauser *et al*., 1997). The detection system can be accomplished by either radioactive labeling of DNA during the PCR amplification step or by silver staining of DNA after gel electrophoresis. Polymorphic differences in strand mobility result from the effects of primary sequence changes on the folded structure of a single DNA strand. The primary sequence differences alter the intra-molecular interactions that generate a three-dimensional folded structure. The molecules may thus move at different rates through a non-denaturing polyacrylamide gel. Because these conformational variations are subtle, the success of any particular SSCP experiment depends heavily on the following two factors: (i) the particular DNA fragments being investigated, including the primary DNA sequence organization and the size of the DNA fragments, and (ii) the optimization of experimental conditions to maximize differential migration among fragments. Investigators have used a variety of methods to improve the resolving power of SSCP, including adding glycerol to polyacrylamide gels, reducing temperatures, and increasing the length of the gels or the duration of gel electrophoresis. Nonetheless, differentiation among

polymorphic molecules on a polyacrylamide matrix is not entirely predictable, and the method can result in false negatives, ambiguous results and experimental artifacts (Xu, Undated).

2.3.8. Heteroduplex

The analysis of heteroduplex is dependent on conformational differences in double stranded DNA. In this technique, PCR products from two different strains in equal quantities (e.g., from wild and mutant DNA samples) are combined in a non-denaturing buffer (Olicio *et al*., 1999). The DNA is melted at high temperature (e.g. 95°C) and is then slowly cooled to room temperature. During the cooling process, the complementary single strands from the same origin strain anneal to form homoduplex DNA, and the complementary single strands from different origins also re-anneal but form heteroduplex DNA. The mismatch in the heteroduplex DNA causes the re-annealed double strands to have a different flexibility and three-dimensional shape than homoduplex DNAs. As a result, the mobility of heteroduplex DNAs will be slower than that of homoduplex DNA. The running and detection conditions for heteroduplex analysis are similar to that for SSCP. Heteroduplex analysis works well for fragments with 200–600bp in length (Xu, Undated).

2.3.9. Real-time PCR

The process of quantifying target DNA has recently been simplified considerably with the advent of real-time PCR. This method avoids the usual need for post-reaction processing, as the amplified products are detected by a built-in fluorimeter as they accumulate. This is done by using non-specific DNA binding dyes (e.g. Sybr Green) or fluorescent probes that are specific to the target DNA (Wittwer *et al*., 1997). The principle underlying real-time PCR is that the larger the amount of target DNA present in the sample being tested, the quicker the reaction progresses and enters the exponential phase of amplification. The amount of PCR amplicon produced at each cycle is measured, using the fluorescent dyes or probes, and for each sample tested the cycle threshold (Ct) is calculated. This is the cycle number at which a statistically significant increase in fluorescence is detected (Figure 3). The Ct increases with decreasing amounts of target DNA. A calibration curve relating Ct to known amounts of target DNA is constructed and used to quantify the amount of initial target DNA in an unknown sample. Software supplied with real time PCR machines is used to rapidly analyse the results (Ward *et al*., 2004).

Recently, a TaqMan real-time PCR (Heid *et al*., 1996) method was developed to quantify different species of *Fusarium* in wheat kernels (Waalwijk *et al*. 2004). It is possible to detect several targets simultaneously by using probes with different fluorescent reporter dyes (Weller *et al*., 2000). Such assays can be used to quantify different organisms, polymorphisms or single point mutations. Real-time PCR methods are not yet widely used for plant pathogen diagnostics but many assays have already been developed for detection of fungi, bacteria, viruses and viroids (Schaad *et al*., 1999; Boonham *et al*., 2000; Mumford *et al*., 2000; Fraaije *et al*., 2001, 2002; Schaad and Frederick, 2002).

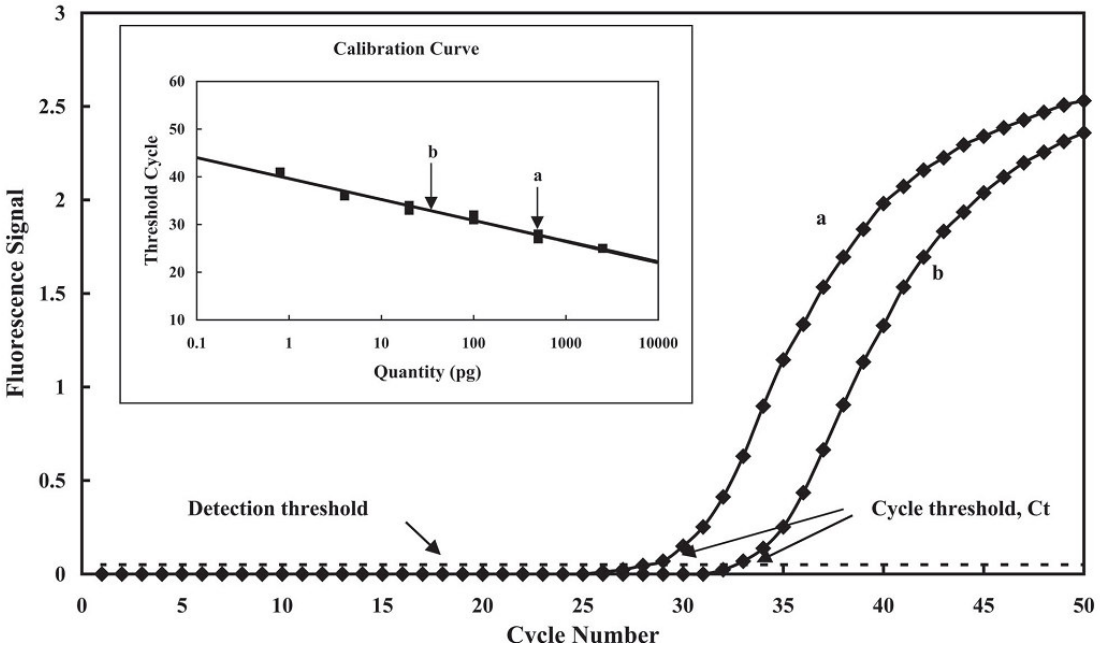

Figure 3. Real-time PCR. Time course of fluorescence signal, using a TaqMan probe, for two samples containing *Septoria tritici* DNA (curve a is from the more concentrated sample). The detection threshold used to define cycle threshold, C, is shown as a broken line. The Ct values for the samples are indicated on the figure.

Inset: calibration curve relating the initial quantity of target DNA to threshold cycle and showing the values of Ct for samples a and b. The larger the threshold cycle the smaller the amount of target DNA in the sample (Ward *et al*., 2004).

In addition to simplifying quantification, real-time PCR has a number of other advantages over conventional PCR. It is faster and a higher throughput is possible. Post reaction processing isunnecessary, eliminating the risk of carryover contamination. It can be more specific than conventional PCR, if a specific probe is used inaddition to the two specific primers. The high specificity of the probes used mean that it is good for detecting single nucleotide polymorphisms (SNPs). These are where the specificity is determined by a single base pair change in the DNA, as is often the case (for example, when distinguishing between fungicide resistant and sensitive isolates). It is sometimes difficult to design conventional PCR methods that will detect such small differences reliably (Ward *et al*., 2004).

Figure 4: The portable Smart Cycler TD. Real-time PCR assays can be conducted at remote/field locations using the Smart Cycler. The Smart Cycler processing block has 16 independently-controlled reaction sites (I-CORE modules) allowing different cycling parameters to be run simultaneously (Frederick and Snyder, 2001).

3. POPULATION GENETICS OF FUNGI

Population genetics is the study of the structure of populations and of the evolutionary processes that shape these structural patterns. “Genetic structure” refers to the amount and distribution of genetic variation within and among populations. Defining the genetic structure of populations is a logical first step in studies of fungal population genetics because the genetic structure of a population reflects its evolutionary history and its potential to evolve (McDonald, 1997). The evolutionary processes include mutation, gene flow, recombination, selection, and drift. Population divergence resulting from such evolutionary processes, as well as from hybridization or vicariance (fragmentation of the environment that can lead to fragmentation of populations), eventually results in speciation (Carboneand Kohn, 2004).

The development of molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNAs (RAPD) has revolutionized fungal systematics (White *et al*., 1990). Genetic markers and sampling designs are most suitable for examining population genetic structure. Although isozymes and other electrophoretically based markers continue to be useful, DNA nucleotide sequence is the gold standard because of its high information content and reproducibility (McDonald, 1997). Markers can provide resolution on different temporal scales, for example, nucleotide sequence variation to examine ancient patterns of population divergence (Carbone and Kohn, 2001a) and DNA fingerprints to resolve population genetic structure on a more recent time scale (Carbone and Kohn, 2001b). RFLPs, are a DNA fingerprints which are used to resolve population genetic structure, but AFLPs or microsatellites are also expected to evolve rapidly and therefore represent recent evolution (Carbone and Kohn 2004). In the absence of any prior knowledge of pathogen population structure, hierarchical sampling strategy can be used as a starting point which is proposed by McDonald (1997).

For most questions in population genetics, it is best to use genetic markers that are selectively neutral, highly informative, reproducible, and relatively easy (inexpensive) to assay. Isozymes, RFLPs, and RAPDs have been used extensively in eucaryotes (McDonald, 1997). Genetic diversity among individuals in populations has been identified using electrophoretically-based markers, such as allozymes, for at least thirty years (Scribner *et al*., 1994). More recently, markers have been developed by means of random amplified polymorphic DNAs (RAPDs), restriction or amplified fragment length polymorphisms (RFLPs or AFLPs) in nuclear or mitochondrial DNA, DNA fingerprints, electrophoretic karyotypes, microsatellites, and minisatellites (Carbone and Kohn 2004). A major limitation in the genetic interpretation, as loci and alleles, of electrophoretically-derived markers is that co-migrating bands shared by two individuals do not necessarily reflect descent from a common ancestor; identity by allelic state does not necessarily indicate identity by descent (Lynch, 1988). Consequently, these markers are not optimal for phylogenetic reconstruction although they have been useful in

systematics for discriminating between species, and in population genetics for typing strains (McEwen *et al*., 2000; Taylor *et al*., 1999a), estimating gene diversity (Linde *et al*., 2002; McDonald *et al*., 1995) and determining genotype diversity (Ceresini *et al*., 2002; Chen and McDonald, 1996; Kohn *et al*., 1991; Kumar *et al*., 1999; Milgroom *et al*., 1992).

It is best to use the widest practical array of genetic markers to obtain the most comprehensive understanding of the genetic structure of populations. To obtain an accurate assessment of population genetic structure, using 6 to 12 unlinked marker loci distributed across many chromosomes is recommended (McDonald, 1997). Bonnen and Hopkins (1997) as a first used a molecular technique RAPD analysis to examine the intra-species variation of *V. fungicola*. They tested 66 isolates collected over a 45-year period. All isolates were compared by examining colony morphology, fungicide sensitivity, virulence, geographic region, and RAPD grouping. The range of variation in the tested isolates indicated that the population was very homogeneous. Bidochka *et al*., (1999b) examined phylogenetic relationships in the genus *Verticillium* using PCR reaction. The authors performed sequence analysis of many *Verticillium* species (including *V. fungicola* isolates) using the internal transcribed space 1 (ITS1) region and a portion of the relatively more conserved nuclear small subunit of ribosomal RNA (rDNA). They reported the phylogenetic data of genus *Verticillium* are polyphyletic groups based on similar morphological characteristics and have their origin in traditional taxonomy (Piasecka *et al*., 2010).

4. CONCLUSION

Currently, more and more diagnostic laboratories and inspection agencies are using molecular methods for detection and identification of diseases caused by plant pathogenic fungi. Better understanding of fungal-plant interactions, pathogenicity factors, rapid and accurate detection of fungal pathogens to species or strain level are a crucial prerequisite for disease surveillance and development of novel disease control strategies. The development of more versatile robust and cost effective systems, allowing for greater sensitivity and specificity, elevated throughput and detection of multiple microbes will continue over the coming years. Universal applicability of a new nucleic acid based assay may lead to abandon traditional reliable methods. For some laboratories, the need for chemicals, equipments and lack of skill workers are drawbacks in adopting PCR-based detection methods for routine detection. Both domestic and international plant quarantine stations must be facilitated with high throughput, rapid and sensitive methods for detection of quarantine pathogens. The methods of population genetics offer powerful tools to elucidate the life histories of important plant pathogens and address fundamental questions about the biology of these organisms. Increased knowledge of the population biology of pathogens is likely to lead to better management of disease in agricultural ecosystems. Eventhough every pathogen detection methods starting from conventional up-to the most sophisticated real-time PCR, they have their own advantages and drawbacks, but selecting of the most appropriate one based on its multi-dimensional importance is crucial at this time. In Ethiopia most of the detection methods are based on the conventional one, but these methods are not as effective as molecular detection methods. So we should have to develop and utilize these more effective, time sever and easy assays of fungal disease detection and pathogen identification methods.

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