Recent advances in molecular techniques for the identification of phytopathogenic fungi – a mini review

Sidra Aslam⁸, Aisha Tahir⁹, Muhammad Farhan Aslam⁹, Muhammad Waqar Alam⁹, Arshad Ali Shedayie⁹ and Sehrish Sadia⁸

⁸MOE Key Laboratory for Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing, People’s Republic of China; ⁹Molecular Biochemistry Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan; ¹⁰Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), University of Agriculture, Faisalabad, Pakistan; ¹¹Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan; ¹²Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing, People’s Republic of China

ABSTRACT
At present, 1.5 million species of fungi are estimated. Among these less than 5% have been described. Many fungal species cause disease in plants. These diseases cause major economic and production losses in the agricultural industry worldwide. Monitoring plant health and detecting the pathogen early are essential to reduce the disease spread, and facilitate effective management practices. DNA-based methods now provide essential tools for accurate plant disease diagnosis. Recently, effective amplification platforms, probe development, various quantitative PCR, DNA barcoding and RNA-Seq-based next-generation sequencing have revolutionized the research in fungal detection field, and differentiation area. Although the molecular diagnostics techniques have grown extensively over the last couple of decades but still there is a long way to go in the development and application of molecular diagnostics to assist the plant disease diagnosticians. Finally, molecular diagnostic techniques used in plant disease diagnostic clinics need to be robust, reliable, inexpensive and easy to be used that they can compete with, and complement traditional techniques. Challenge now remains with the researchers to develop the practical techniques used for diagnostic setting. Examples of the recent advancement in the molecular techniques for diagnosing the fungi causing plant disease are discussed in the review.

Abbreviations: PCR: polymerase chain reaction; LAMP: loop-mediated isothermal amplification; RCA: rolling circle amplification; NASBA: nucleic acid sequence-based amplification; ITS: internal transcribed spacer; RT: reverse transcriptase; FEB: Fusarium ear blight; qPCR: real-time PCR; SNPs: single nucleotide polymorphisms; HRCA: hyper-branched RCA; FIP: forward inner primer; BIP: backward inner primer; COX I: cytochrome c oxidase I; MBs: molecular beacons.

1. Introduction
Fungi show the greatest eukaryotic diversity on the planet with their conservatively estimated 1.5 million species and they are one of the primary decomposers in the ecosystem (Capote et al. 2012). Fungi behave as both friends and foes. Many species of fungi are beneficial to human, vegetal, animal and environmental health because of its economic, medical and commercial uses. Along with it many of them are harmful because of its ability to act as pathogen and cause diseases. Identification of fungi responsible for plant diseases is the cornerstone of plant pathology discipline (Borman et al. 2008). A lot of studies have described the devastating effects of these fungi on the crop yield. Rice, which is a staple food of world’s half population, is attacked by the Pyricularia oryzae causing rice blast, and it is leading to a per year loss of 10–30% of the crop (Talbot 2003).

Among the 1.5 million fungal species, less than 5% have been described. Indeed the challenge of documenting the remaining 95% is more tractable with molecular techniques than with conventional methods, which only adds 1000 new species per year (Hawksworth 1991). The main goal of all diagnostics field is to identify disease-causing organism (origin of a disease) in such a way which is fast, accurate and reliable. Basic methods used to detect the organism mostly rely on microscopic, cultural and morphological approaches that require extensive time, labor and classical taxonomy knowledge (Nilsson et al. 2011). These approaches, although the cornerstone of fungal diagnostics, can lead to the unreliable results due to the problems in identification. Additionally, experts and specialist with a practice in fungal identification are required for effective results (Chalupová et al. 2014).

Due to the conventional methods limitations, molecular techniques came in use for the investigation of identification and classification problems. A high variety of molecular methods are increasingly becoming valuable tools in all aspects of fungal diagnostics. These techniques include immunological methods, nucleic acid-based probe technology and polymerase chain reaction (PCR) technology. The former methods rely upon phenotypic characters while the latter based on genotypic characters gives fast, highly specific, effective and potentially more accurate results. In contrast to the basic methods, isolation of organism do not require culturing (Badali and Nabil 2012; Spring and Thines 2010).
Table 1. Molecular diagnostics techniques for the identification of phytopathogenic fungi.

| Methods                  | Advantages                                                                 | Disadvantages                                                  |
|--------------------------|-----------------------------------------------------------------------------|----------------------------------------------------------------|
| Conventional PCR         | Gives rapid and Precise results, use when the primer of specific species    | Required much labor and cost                                   |
| Nested PCR               | Use of two sets of primer increase the yield and specificity of amplification of the target DNA | Risk of contamination, because of two cycles of amplification  |
| Multiplex PCR            | Time and money saver by using the several pairs of primers in a same reaction | Interference of primers and probes, reduce sensitivity         |
| Reverse transcriptase    | Gives quantitative data about pathogens, more sensitive than conventional PCR | Formation of each assay is time consuming and requires the expensive equipment and the reagents |
| Real-time PCR (q PCR)    | Automated and no need of post amplification analysis                        | Cost and complexity due to simultaneous thermal cycling and fluorescence detection |
| in situ hybridization    | Maximum use of the short supply tissue                                      | Difficulty in identifying targets that have low DNA and RNA copies |
| FISH                     | Can be used for non-dividing cells                                           | Probe-preparing method is very difficult because it is necessary to tailor the probes to identify the particular sequences of DNA |
| Microarray               | Easy to use because it does not require the large-scale DNA sequencing      | Large amount of mRNA is required                               |
| LAMP                     | Rapid, sensitive and highly specific                                        | Primer design is complex; recognizes only one specific pathogen; risk of sample contamination |
| NASBA                    | Expensive equipment is not needed. Better than RT-PCR                       | Specificity of the reactions is dependent on thermolabile enzymes. Reaction temperature cannot be exceeded than 42°C without compromising it |
| RNA interference (RNAi)  | Ability to simultaneously interrogate thousands of genes                    | Variability and incompleteness of knockdowns and the potential non specificity of reagents |
| Northern blotting        | Detection of RNA size                                                       | Applied only on a small sample of the genes                    |
| SAGE                     | Prior knowledge of the subject’s genome is not requisite                     | Specificity of tag sequence                                    |
| RNA-Seq                  | Increased specificity and sensitivity                                        | Needs expensive equipment. Bioinformatics knowledge requisite for data analysis |

When the morphological characters are not visible, then enzyme-linked immunosorbent assay, an immunological method, was applied for the quantification and detection of fungi. These immunological methods rely on antigen–antibody reaction coupled with a fluorescent dye or enzyme has become available for simple and rapid results (Mostafa et al. 2012; Peruski and Peruski 2003). Although, production of antibodies has generally successful for viruses, it is less well for complex organisms such as fungi. However, it is difficult and expensive to produce highly specific antibodies. Other limitations include: these methods work reliably only at the genus level. Specificity and accuracy is most important in diagnostics applications and this may restrict the use of immunological methods (Borman et al. 2008). Nucleic acid-based methods allow the determination of closely related species and detect the minute quantity pathogen when no visible sign is present. In molecular methods, DNA/RNA probe technology includes southern hybridization, in situ hybridization, fluorescence in situ hybridization, microarray and macroarray. Isothermal amplification technology includes loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA) and nucleic acid sequence-based amplification (NASBA). PCR technology includes multiplex PCR, nested PCR, real-time PCR and reverse transcriptase (RT)-PCR and DNA barcoding has been recently used. Each technique has its own advantages and limitations as shown in Table 1.

In this article, we discuss the advancements in molecular techniques for identification of those fungi which caused disease in plants, as shown in Figure 1. Then, focus will be on ‘fungal DNA Barcoding and RNA-Seq’, which are emerging molecular techniques to identify the pathogenic species so as to monitor the plant diseases.

2. Molecular methods for the detection of fungi

2.1. PCR-based methods

2.1.1. Conventional PCR

The invention of PCR brought a great revolution in the plant pathology field. This technique allows synthesizing of the specific part of DNA in million copies through alternate cycles of denaturation, annealing, elongation by using specific primers. Initially, PCR was highly specific for the detection of diseases caused by bacteria and viruses. Now, it is widely used for the plant pathogen detection as well. PCR depends on the efficacy of DNA extraction and the concentration of deoxyribonucleoside triphosphate. Sometimes, the performance is affected by inhibitors present in sample assay (Fang and Ramasamy 2015). To overcome this issue in plants, cetyl trimethyl ammonium bromide method is widely used with the addition of particular chemical and enzymatic treatments. Several other methods are present to reduce the effect of PCR inhibitors. In phenol–chloroform extraction method, instead of ethanol precipitation, silica matrix purification can be used to recover the DNA (Mancini et al. 2016). Moreover, for the detection of pathogens, PCR technology requires the designing of a primer to initiate the DNA replication process, which could limit the practical applicability of the technique for field sampling of disease. Sometimes a single pair of primer does not give the specific and accurate results so as to overcome this limitation DNA probes and nested primers are mostly used nowadays (Compton 1991). Mycosphaerella musicola and M. fijiensis (which cause leaf spots disease in banana and plantain) are detected by PCR technology (Henson and French 1993). No doubt this approach gives the highly specific results, but it is too much costly and required much labor.

2.1.2. Nested PCR

Nested PCR is used to achieve a high degree of specificity and sensitivity. For example, nested PCR sensitivity is 1000 times greater than single PCR for fungal identification (Yeo and Wong 2002). In this method, two consecutive rounds occur in which a single pair of primer is used to amplify a large region of DNA, then this amplified sequence of DNA acts as a target for the second round by using two internal primers. In this type of PCR, a considerable risk of contamination is measured because two cycles of amplification are to be performed in separate tubes. So, chances to occur false-positive results due to contamination, and intense
labor are major drawbacks of this technique (Rahman et al. 2013). To overcome these limitations, laboratories must follow some strict precautions as to use the separate equipment and place for each PCR cycle (Trtkova and Raclavsky 2006). Orchid disease caused by Phytophthora spp. was detected through nested PCR. Two species of Phytophthora; P. palmivora and P. parasitica were detected by nested PCR which cause the disease (Tsai et al. 2006). P. Cactorum was specifically detected in diseased strawberry plants by using nested PCR (Bhat and Browne 2010).

2.1.3. Multiplex PCR

Multiplex PCR can be proved as time and money saver by using the several pairs of primers in the same reaction that allowing the simultaneous detection of different targeted sequences of DNA. The method has so much importance in plant pathology when plants get infected with more than one pathogen. Different fragments that are specific to target pathogenic fungi were simultaneously amplified, and detected on the basis of their molecular sizes on the agarose gels. DNA-synthesizing accuracy is strongly affected by amplicon size. To avoid this pitfall, primers must be designed carefully along with their relative concentration and annealing temperature (Dasmahapatra and Mallet 2006). Nowadays, padlock probes (PLPs) are used in the multiplex technique for the identification of pathogenic fungi. Multiplex PCR technique has been used for the simultaneous detection of fungal pathogens, such as F. oxysporum, B. cactivora, P. nicotinae and P. cactorum, and causes major disease in grafted Cacti (Cho et al. 2016).

2.1.4. RT-PCR

Since the discovery of Standard PCR, a large number of changes have been developed in its procedure. Some of these changes have expanded the utility and diagnostics capability of PCR in many biological and medical fields (Tang et al. 1997). An important limitation of other PCR types is their inability to differentiate between dead and living fungi. This limitation is overcome by the discovery of RT-PCR. Actually, mRNA in dead cells is degraded; so the detection of mRNA by RT-PCR can be done to check the cell viability (Capote et al. 2012). In this process, firstly, the RNA is reversely transcribed into cDNA by random primers and RT enzyme and then amplified by any PCR-based method. So, RT-PCR is mostly used to detect and diagnose the RNA-containing viruses (such as retroviruses) infections. Diagnosis of RNA-containing viruses can be helpful in developing or checking the effectiveness of antimicrobial vaccines or therapy. RT-PCR has been used to quantify the Fusarium graminearum fungi that cause the Fusarium ear blight (FEB) disease in cereals such as wheat, rye, barley, oat and maize (Brown et al. 2011).

2.1.5. Real-time PCR (Q PCR)

Conventional PCR methods give us only amplification of targeted DNA that is not enough for rapid disease diagnosis. In last few years, a new technique, RT-PCR has been introduced. This technique is an improved form of conventional PCR in which the DNA can be quantified along with the amplification (Mackay 2004). Monitoring of the reactions during amplification steps has been made possible by the use of fluorescent dyes such as SYBR Green I or sequence-specific
fluorescent-labeled probes as the Taq Manprobe (Badali and Nabili 2012). When the fluorescent dye intercalates to DNA, fluorescent signal is generated. This signal increases as the amount of targeted DNA increases after each cycle of amplification (McCourtney et al. 2003). It is less costly to use the fluorescent dye as a monitoring agent but has limitation due to its non-specific nature. Actually the binding of intercalating dye to all present DNA can produce the false results in the form of primer dimer. Then fluorogenic probes came into use due to their high specificity (Atkins and Clark 2004; Bu et al. 2005). These probes are connected with two types of fluorescent dyes, one is the reporter dye that attaches to the 5’ end, and other is quencher dye on 3’ end. Close proximity of reporter and quenching dye prevent the emission of fluorescence. Due to the exonuclease activity of Taq polymerase, reporter dye becomes separate from quenching dye and started to fluorescence (Dasmahapatra and Mallet 2006).

qPCR has been used for the identification and quantification of the disease-causing fungi, such as Aspergillus versicolor, Cladosporium cladosporioides, Stachybotrys chartarum, and Alternaria alternate (Black 2009).

### 2.1.6. Serial analysis of gene expression

Serial analysis of gene expression (SAGE) is a comprehensive and sequence-based method for the quantitative gene expression profiling which allows the identification of multiple transcripts simultaneously. The method based on sequencing and quantification of the 15 bp or the longer oligonucleotides and similarity of sequences against the available genome sequences to find the corresponding expressed genes (Velculescu et al. 1995). This method uses two samples, which are ligated and labeled with the separate primers, and then it is amplified. Then, primers are removed, providing sticky ends that form the concatemers. They are cloned into vector, and sequenced trailed by the wide computational analysis. SAGE has some drawbacks. First, it needs mRNA in a large quantity. Second, sometimes 15 bp tag is not enough to specifically identify the gene of origins with the more complex genomes. SAGE was first used to identify B. graminis on the barley leaves (Dawei and Peng 2014).

### 2.1.7. DNA barcoding

DNA barcoding is a molecular diagnostic technique in which a small segment of DNA is used to identify the species of all domains of eukaryotic life. Barcode regions are universally found in target lineages. It exhibits adequate DNA sequence variation to distinguish different species. In DNA barcoding, standardized sequences of 500–800 base pairs are used to identify species with the markers valid for the wide range of taxonomic group (Krishnamurthy and Francis 2012). For the microbial organisms, such as fungi, bacteria, and algae, this PCR-based method is not only helpful to recognize the cultured species but also used for the identification of species for uncultured taxa from natural environment. Identification of species by using the barcode depends on the number of representative present in the database. By DNA barcoding, fungi can be identified in life-cycle stages not suitable for morphological identification. The efficacy of barcoding relies on this assumption that genetic variations within a species are much smaller than the variations between species. Fungi that are not suited for morphological identification and discovery can be identified and discovered at molecular level by DNA barcoding. In this technique, effective marker is very helpful to identify the poorly understood fungal species diversity in the natural environment (Roe et al. 2010). Full working of DNA barcoding technique is shown in Figure 2.

There are several scientific advantages of DNA barcoding, which include: (i) facilitating the identification of species at any phase of life, (ii) enabling the discovery of species based on the phylogenetic analysis of nucleic acid sequences, (iii) providing insight for diversification of life, (iv) promoting the development of DNA sequencing tools, useful in the field of biodiversity (Savolainen et al. 2005). Reliable barcodes provide standardized and realistic species identification tool for the evaluation of biodiversity and environmental studies. Selection of barcode region is compromised among the prospects to design ideal and universal DNA markers for the PCR amplification. Mitochondrial Cytochrome C Oxidase (COX I) region used for animal barcoding was excluded as an ideal marker for fungi because it cannot amplify fungal DNA due to the presence of large introns. Molecular systematics of fungi are based greatly on the analysis of nuclear ribosomal RNA (rRNA) cistron, consists of small (18S) and large (28S) subunits (Hebert and Gregory 2005). ITS region can be amplified from many fungal taxa by using a limited set of primers (Schoch et al. 2012). Moreover, it has found that differences among species are often higher than those within species. So, ITS is used as a universal barcode region (Schoch et al. 2012; Li et al. 2011). This level of polymorphism makes the ITS region a strong and valid candidate for the fungal DNA barcoding. For taxa, where primary barcodes are often ineffective for identifying pathogenic fungal species, secondary barcodes are continuously being established. It will be the most realistic method for DNA sequence-based identification of phytopathogenic fungi. Simplifying ITS region to either the ITS-1 spacer or ITS-2 spacer only has also gained significant attention in the field of barcoding-based research. ITS2 is also not a coding region, but it has a conserved secondary structure core that helps to launch the data-handling systems. ITS-2 spacer is found to be a very informative secondary barcode for plants (Xu 2016). Use of ITS-2 for metabarcoding could potentially expand the comparative studies among fungi and plants. The largest subunit of ribosomal polymerase II was nominated as a representative protein-coding gene due to the good PCR success result with this gene and its function as a phylogenetic marker in the AFTOL project (Tanabe et al. 2004). Hence, ITS, SSU, LSU EF-1α, and RPB are barcoding markers that have been used for fungal DNA barcoding, as shown in Table 2. Cashew is one of the most exported horticulture crops, especially in Western Africa. Secondary barcodes of glyceraldehyde-3-phosphate dehydrogenase, glutamine synthetase and ApMAT have been used to identify the cashew fungal-associated diseases as well as to distinguish the causal pathogens (Montero et al. 2015).

### 2.2. DNA/RNA probe-based methods (sequencing-independent methods)

DNA/RNA probe methods use the probe to diagnose the plant diseases caused by the microbes such as fungi with great sensitivity and speed. This technology is considered as the backbone to the most of the current knowledge. In these methods, the probe is used for the analysis of nucleic acid without its amplification. Probes are the single-stranded
shorter sequences of DNA that are labeled with the chemiluminescent reporter molecule, or with radio-labeled isotopes, such as 32P, 33P and 35S. These are used to identify the homologous sequence on the targeted DNA. In traditional methods, DNA probes are mostly used as an alternative to PCR for the identification of fungi. But in recent methods, these are mostly used in conjunction with PCR (McCartney et al. 2003).

2.2.1. Northern blotting
Northern blot, which is also known as the RNA blot, is used to transfer the RNA onto a carrier for the identification of pathogenic fungi. The Northern blot is the same as to the Southern blot except that the RNA material is used instead of the DNA. Firstly, the RNA from each tissue should be purified so as to be examined the expression of the gene of interest. The RNA material is then loaded on agarose gel. After that the gel is passed to an electric current that migrates RNA towards the bottom of a gel. Smaller RNAs move faster compared to the larger RNA (Kim et al. 2010). After that, separated RNA fragments are blotted on a special filter paper; therefore each RNA molecule maintains its position relative to all other molecules (Berg 2007). The filter is then exposed to radioactive probes so as to hybridize it to complementary sequences. After that, the filter is put for autoradiography so as to develop the film. Finally, a band should be detected on the autoradiograph if the probe has hybridized to a fragment of RNA on the filter. The Northern blot is beneficial to study the gene expression. First, the position of bands on the blot gives the RNA size. If the size of the RNA is known, it will provide an approximation for the coding capacity of transcripts and also the size of the protein to which it encodes. Secondly,
In situ hybridization has some pitfalls. Firstly, radioactive probes are very costly and hazardous material. It must be handled, transported and disposed very carefully. Secondly, a disadvantage of using in situ hybridization technique is the difficulty in identifying targets that have low DNA and RNA copies (Qian and Lloyd 2003). In situ hybridization has been used for the identification of the fungi such as Blastomyces dermatitidis, Coccioidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum and Sporothrix schenckii (Hayden et al. 2001). ISH is a tool which is used to visualize the infection of plant tissue by the rust fungi. This method has been used to generalize the ISH protocol for the localization of rust fungi in the paraffin-embedded sections of plant material (Ellison et al. 2016).

### 2.2.3. FISH

Due to the drawback of radio-labeled probe-based hybridization, fluorescence in situ hybridization (FISH) came into development, which is used for the rapid characterization of microorganism such as fungi. FISH provides greater speed, resolution and safety and paved way for the development of multiple targets detection simultaneously and quantitative analysis at phylogenetic level (Tsui et al. 2011). In FISH, a fluorescent probe is used that binds only those part of the chromosome with which it shows a greater degree of homology or complementation. Fluorescent probes are prepared by enzymatic incorporation of fluorophore-modified base throughout the probe length (Baschien et al. 2001). Traditional techniques for the identification of microorganism requires that cells must be actively dividing. However, FISH can be used for the non-dividing cells, which make it a highly versatile technique. Non-dividing cells can be recognized by their low level of fluorescence intensity. Different types of probes such as locus-specific probe, centromeric repeat probe and whole chromosome probe can be used. Each of which has its different applications. FISH technique has some pitfalls. In FISH, probe-preparing process is very complicated because it is necessary to tailor the probes to detect the specific sequences of DNA (Volpi and Bridger 2008).

### 2.3. Post amplification technique

#### 2.3.1. Microarray

The concept of microarray technology emerged from the two technical advances. Firstly, the efforts on the DNA sequencing and secondly the focus on expressed component of genome. Microarray DNA chip technology allows the analysis of thousands of mRNAs simultaneously and used to observe the changes in gene expression. This technique is different from above techniques in this perspective that it provides the expression measurements on defined sets of genes (Eshaque and Dixon 2006). In this technology, thousands of DNA probes are arrayed within a small surface area onto a support matrix that includes glass chip or nylon filters. Location of the probe on the chip is called spot (Robinson et al. 2000). These probes are immobilized on the support matrix and targeted
cDNAs applied as a solution to the chip for the hybridization. Quantification of the bounded cDNA is measured by using the radio-labeled probes or fluorescent tags. Signal is produced by the hybridization of the probe to the targeted mRNA that can be detected and integrated by the dedicated software. The dedicated software generates the Gene Expression Profile for each biological sample (Russo et al. 2003). By using this technique, comprehensive understanding of the cell of fungus can be achieved in a single array. Microarray technique is very easy to use because it does not require the large-scale DNA sequencing. Although this technique can monitor the global changes in the gene expression, significant issues are considered in it. Firstly, a large amount of mRNA is required in this technique. Secondly, this study is limited by cost and access (Singh and Kumar 2013). Due to the numerous error-prone steps in the microarray experiment, replications are required to reduce the chances of error in such experiments. This technology is called destructive testing because physical disruption of cells is required to gain access to its gene expression patterns; false microarray data can be produced from the degradation of mRNA. Microarray technology has been used for the identification of the Aspergillus candida species (Singh and Kumar 2013).

2.3.2. Macroarray

Macroarray is also called the DNA array hybridization, or Reverse dot plot. This method uses the sensitivity of the DNA amplification and do not require the radioisotopes (Singh and Kumar 2013). Macroarray is rapidly becoming a standard molecular tool for diagnostic and epidemiological studies in an increasing number of laboratories all over the world. It works on the basis of simultaneous amplification of the related species through PCR. Along with this, it analyses a number of amplified sequences simultaneously in one hybridization reaction. It is a more sensitive technique than PCR alone. In this assay, PCR amplification is combined with hybridization, which increases sensitivity up to 1000-fold or higher than PCR only (Taoufik et al. 2004). This technique has fast turnaround time of 1–2 days compared to radioactive culture methods that require 2–8 weeks for their completion. In this technique the internal probes are designed to differentiate the species. These probes are fixed to the nylon membrane support. Oligo are permanently bound to the membrane by UV cross-linking. Then the PCR-amplified products are hybridized with the spotted series of species-specific probes present on the strips (Tsui et al. 2011; Leinberger et al. 2005). Macroarray in combination with PCR has been used to detect and identify the mycobacterium to the species level (Leinberger et al. 2005). This assay has also been used for the identification of Alternaria alternata, Aspergillus fumigatus, Fusarium solani, Candida albicans and Cladosporium herbarum (Sato et al. 2010). Macroarray technique has been used for the identification of fungal and oomycete pathogens which causes disease in solanaceous crops (Zhang et al. 2008).

2.4. Isothermal amplification-based methods

2.4.1. Rolling circle amplification

For the identification of pathogens, single nucleotide polymorphism (SNP) detection is becoming popular. Detection of SNPs among different genotypes by the conventional real-time PCR has many challenges and drawbacks. So, to overcome these drawbacks, species-specific PLPs (Circularizing oligonucleotide probes) are used. Circularizing oligonucleotide probes are the single-stranded DNA molecules having target recognition sequences of 20-nucleotide present at the both 5’ and 3’ ends, which are connected by the 40-nucleotide long linker sequence (Tsui et al. 2011). Designing of PLPs are first reported by Nilsson et al. in 1994. When hybridized to targeted region, both ends come near to each other and become circularized by the use of ligase, leaving no gaps. The circulized probe spans the entire target region in a manner similar to that of the padlocks, driven by the helical nature of the double-stranded DNA (Wang and Yang 2010). So, we can say that PLPs are useful only for detecting DNA molecules with known sequences. PLPs can be used in PCR, but such probes are so far failed to yield reliable in situ single-copy gene detection. Therefore, Padlock probe is preferably suitable to template a reaction of RCA. RCA is based on rolling replication of short single-stranded DNA circular molecules. The RCA process is isothermal and also called rolling circle replication (RCR). This process requires DNA polymerase, a primer to initiate the replication, DNTPs and DNA binding and unwinding proteins. Most of the groups are using the RCA reaction for signals’ amplification, where the small circular probes serve as the template. In RCA reaction, primer has dual functions, both as RCA signal amplifier and discriminator by being complementary to the DNA-targeted sequence (Kuhn et al. 2002). In RCA, a second primer complementary to the RCA product can be used; so hyper-branched RCA (HRCA) reaction will be generated. HRCA is also in use as an alternative method to PCR for DNA amplification. Recently Major Molecular Types of Cryptococcus neoformans and C. gattii are identified by hyper-branched rolling circle amplification (HRCA) (Trilles et al. 2014). RCA technology is a robust and simple method that can make available a universal platform for the localization of a wide variety of molecules such as nucleic acid sequence (Gusev et al. 2001). Black grain eumycetoma, which represents the most common fungal mycetoma in the whole world, affects the lower extremities, which leads to severe disability. A group of fungi involved in this disease is successfully identified by RCA (Ahmed et al. 2014).

2.4.2. Loop-mediated isothermal amplification

LAMP is a robust and novel nucleic acid amplification method that is considered an alternative to the PCR. It amplifies the targeted nucleic acid under the isothermal conditions with the high specificity. LAMP does not require a thermal cycler to produce the temperature changes instead it requires single temperature for DNA amplification (Tsui et al. 2011). So, it is based on auto-cycling strand displacement amplification of DNA. In this technology, Bst DNA polymerase and a set of four primers that consist of two inner and two outer primers are used, which recognize total six unique sequences on the targeted DNA. Two inner primer are referred to as forward inner primer (FIP) and backward inner primer (BIP), while outer primers are F3 and B3 (Fakrauddin 2011). Each BIP and FIP consists of two distinct sequences according to sense and anti-sense strand of targeted DNA. One of the inner primers is used to begin the LAMP reaction while the other for self-priming. LAMP reaction is processed in a heat block or water bath at 65°C for 1 h. Then amplification product is detected by using the dye SYBR Green 1 as well as by electrophoresis. Final product has many inverted repeats
of targeted sequence which exhibit cauliflower-like structure with multiple loops. LAMP is 10 times more sensitive and accurate than the conventional PCR (Ren et al. 2009). LAMP technology does not require expensive equipment to get a high level of accuracy, and it has less number of preparation steps than the conventional, and qPCR. Due to high-amplification efficiency, up to 10^3 copies of a target part can be attained in less than 1 h of incubation. Another advantage over the PCR in many cases is that the inhibition reactions occurred less in LAMP than that in PCR. In LAMP, no time is lost for thermal changes as it happened in PCR. For PCR, an expensive thermal cycler is required while LAMP reaction required just a single tube (Fakruddin 2011). But several factors are present there which affect the accuracy of LAMP reaction such as the usage of specific DNA polymerase, which is critical for LAMP efficiency. Annealing of the four primers to targeted DNA is also important for the accurateness of LAMP. LAMP is useful for the detection and diagnosis but not for cloning purpose. However, a main drawback of LAMP technology is the use of indirect evaluation methods such as Mn2+ dye, gel electrophoresis, SYBR Green I dye, hydroxynaphthol blue dye, composite probe method and turbidimetric method, which cannot be differentiated between desired products, and nonspecifically amplified product, thus leads to false positives. The usage of molecular beacons (MBs) solves this problem by producing the fluorescence signals when it binds to target DNA. Hence, it acts as a direct detector of amplification product. Optimal conditions for the MBs (such as beacon length of 25–45 bp, reaction temperature of 60–65°C and beacon concentration of 0.6–1 pmol/μL) are found as an assessment tool in LAMP. A novel method based on MB-LAMP has been validated, which provides the direct detection of LAMP product, as shown in Figure 3. MBs are fluorescent nucleic acid probes which have a hairpin structure. The hairpin structure avoids the fluorescence because quencher is physically closed to fluorophore. Moreover, binding to LAMP product sequences changes the MB spatial configuration, which separates fluorophore and quencher at both the ends of a single strand of nucleic acids, and desorbing fluorescence (Liu et al. 2017). Ascochyta blight disease (which is caused by Ascochyta rabiei L. fungi) in chickpeas has been diagnosed using LAMP method. Comparison of conventional PCR with LAMP not only exhibits the greater accuracy, sensitivity and specificity for the detection of A. rabiei, but also used the simple equipment, and required the less operational time (Das et al. 2012). LAMP has been successfully used to detect the presence of thermodependent dimorphic fungus, Para- coccioides brasilensis (Endo et al. 2004). This technique is also used to identify the pathogenic fungus such as Ochroconis gallopava (Tsui et al. 2011). Penicillium marneffei has also been successfully diagnosed by using lamp technique (Sun et al. 2010). Recently, Ophiostoma clavatum, a Primary blue Stain fungus, is identified by using LAMP (Villari et al. 2013).

### 2.4.3. Nucleic acid sequence-based amplification

NASBA is a very sensitive, isothermal and transcription-based amplification system that is specially designed for RNA detection. Some NASBA systems can also amplify the DNA. It is also well known as self-sustained sequence replication (3SR). This tool is not only useful in basic research but also in the application-oriented fields, such as clinical medicine development, and infectious diseases diagnosis (Sergentet-Thevenot et al. 2008).

Numerous amplification methods have already been established, such as PCR (Saiki et al. 1992), LAMP (Lee et al. 2009) and RCA (Lizardi et al. 1998). None of these methods can amplify the RNA directly with high sensitivity. NASBA provides many advantages over the other techniques of mRNA amplification. It can amplify more than 10^9 copies of the nucleic acid sequence in just one and half an hour by the action of three enzymes. NASBA is an isothermal reaction that is performed at 41°C, which excludes the need for a thermal cycler and can facilitate the production of point-of-test devices (Fakruddin et al. 2012). An important benefit of NASBA is the production of single-stranded RNA amplicons, which can be used directly in another round of amplification or can be queried for detection without denaturation, or strand separation (Chang et al. 2012). Several studies have reported that the amplification power of NASBA is comparable to, or even better than that of the RT-PCR (Chang et al. 2012). RNA being the genomic material of many RNA viruses, an RNA-based amplification technique in contrast to the PCR keeps away from an additional reverse transcription step, thus minimizing the contamination risk and lowering hands-on time. It assists in better RT-PCR reaction as it provides the faster amplification kinetics that is especially suitable for the detection of Retroviruses. It can measure the replication of DNA viruses by detecting late mRNA expression. It supports detection of human mRNA sequences.
without DNA contamination risk (Lauri and Mariani 2009; Fakruddin et al. 2012). Gene expression studies can be carried out without the intron-flanking primers or DNAses. There are also some disadvantages of NASBA. First of all, RNA integrity is the foremost cause of concern for the NASBA, and also for other RNA amplification techniques. Although the amplification reaction itself is isothermal at 41°C, only a single melting step before the amplification reaction is required, which allows 416 annealing of primers to the target (Compton 1991). Moreover, due to the dependence of specificity of the reactions on thermo labile enzymes, reaction temperature cannot beat 42°C without compromising it. Finally, the length of the targeted amplified RNA sequence should be between 120 and 250 nucleotides (Fakruddin et al. 2012).

2.5. RNA interference

RNA interference (RNAi) is used as a latest technology to identify and control the phytopathogenic fungi. It is an efficient technique that can be used in a highly tissue-specific way to fight mycotoxicogenic fungi, which causes the infection in crop plants (Panwar et al. 2012). Positive transgenic RNAi execution depends on many factors, which includes: (i) designing the vectors in such a way, so as to produce the double-stranded RNAs (dsRNAs) that will make the small-interfering RNA (siRNA) species for the ideal gene silencing, (ii) accessibility of plentiful target siRNAs at infection place, (iii) efficient uptake of siRNAs by fungus, (iv) siRNA half-life, (v) amplification of the silencing effects. RNAi eliminates the negative consequences of current disease control and fights the alarming rise of the fungicide resistant plant pathogens (Ishii and Holloman 2015). RNAi absolutely knocks down the genes by using the event of intrinsic cellular defense. Detection of the dsRNA or hairpin RNA (hpRNA) by the fungal cells leads to the targeted transcripts by using the sequence homology important for the degradation or silencing (Nakayashiki et al. 2005). To identify the unique fungal targets, cell-specific and dual RNA sequencing data should be provided. Then hpRNA or dsRNA can be modified for a definite transcript which can directly limitize the fungal pathogenesis. RNAi technology has a benefit of the cell’s natural machinery, which is assisted by the short-interfering RNA molecules, to successfully knock down the expression of a gene of interest. Major disadvantage of RNAi is the possibility of off-target effect, which lead to genes silencing, which tolerate the partial complimentarily to the sense or anti-sense strand of targeted gene. RNAi has been used in Zea mays for the identification of Aspergillus flavus fungus (Masanga et al. 2015). It has also been used to identify pathogenic Fusarium graminearum in Hordeum vulgare (Chen et al. 2006).

2.6. RNA-Seq-based next-generation sequencing

RNA-Seq is a newly developed deep-sequencing technology. Generally, a large population of RNA is changed to cDNA library with adaptors that linked to one or both end. After that, each fragment with or without amplification is sequenced in a high-throughput way to get small sequences from one end as in single-end sequencing or both ends as in pair-end sequencing. Reads are usually 30–400 bp, depending on DNA sequencing technique used. Library is prepared to see how closely the RNA sequencing results reveals the original RNA transcripts are mostly determined in the library preparation step. To create a RNA-Seq library, the fragmentation of either the RNA or the cDNA is required to allow the processing through next-generation sequencing. Developed mRNA should be primed for RT reaction by the use of either random primers or oligo primers. The benefit of using oligo(dT) is that the majority of cDNA produced should be poly-adenylated mRNA; hence more of the sequences obtained should be informative (Mortazavi et al. 2008).

Three most generally used next-generation sequencing platforms for the RNA-seq are SOLiD and Ion Torrent, both advertised by the Life Technologies and Illumina's HiSeq (Dawe and Peng 2014). Following sequencing, the resultant reads are either aligned to reference genome or assembled de novo without genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and level of expression for each gene. While most of RNA-Seq analysis data depend on the alignment of the reference genome sequences, new softwares such as Rnnotator and Trinity assemble the RNA-Seq data into the transcriptomes without referring a reference sequence by assembling the short adjacent reads from the RNA-sequencing data. These approaches allow the discovery of new transcripts, and fair detection of the transcripts from numerous sources, permitting more effective application of the RNA-Seq for the detection of transcripts and classification of transcriptomes in the non-model organism (Grabherr et al. 2011).

Through this technology, the fungus Magnaporthe oryzae is identified, which causes rice blast disease in rice (Soanes et al. 2012). The fungus Verticillium dahliae is identified in tomato, which causes Vascular wilt disease (de Jonge et al. 2012). RNA-seq could be used to find the inclusive changes in fungi within a plant or could be used to identify the new pathogens. The potential appropriateness of mRNA-seq data for the recognition of nucleotide differences is able to reveal the plant pathogenic fungal pathogenicity genes those are mutant in their protein-coding transcriptome. Technologies are making us closer to the capability to use RNA measurements for the plant disease diagnostics (Metzker 2009).

3. Conclusion

In previous years, despite the availability of many technologies, many challenges were remained to identify the unculturable fungi, so as to detect the cryptic species, and to differentiate fungal communities’ diversity involved in causing a disease in the plant. At that time, nobody knows how many fungal species exist. But at present, more and more diagnostic laboratories are using molecular techniques to detect, and identify the diseases caused by plant pathogenic fungi (Ahmed et al. 2014; Kim et al. 2010). Better understanding of pathogenicity factors, rapid and accurate detection of fungal pathogens to the species or strain level are the crucial prerequisite for disease surveillance and development of novel disease control strategies. Current technologies such as DNA barcoding and RNA-Seq-based next-generation sequencing helped to face and overcome these challenges. We hope that new developments will boost the adoption of these new technologies for the diagnosis and study of plant disease (Chang et al. 2012; Tsai et al. 2006). Molecular
techniques can be also developed based on the different fungicide mechanisms to rapidly detect resistant isolates. Furthermore, a timely detection of resistance levels in populations of phytopathogenic fungi in a field would help the growers formulate proper decisions on resistance management programs to control plant diseases (Chalupová et al. 2014).

Acknowledgements

Aslam and Tahir design the structure of review paper. Aslam wrote the paper. Sadia and Shedayi helped in collecting the material. The authors are thankful to Deng-Ke Niu for their kind help.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China [grant numbers 31671321, 31371283].

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