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REVIEW

A Glimpse towards Advanced Molecular Diagnostics for the Detection of Phytopathogens

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Phytopathogens have the potential to inflict significant losses on the agricultural system, making precise and rapid detection and diagnostic tools essential for disease management. Identifying plant pathogens on different plant components used to propagate crops is a key aspect of disease prevention. Effective disease control relies on ensuring harmful organisms are absent from crop plants. Molecular-based techniques, particularly those utilizing nucleic acids, have proven sensitive and targeted in detecting phytopathogens on seeds and other plant parts. Polymerase Chain Reaction (PCR) technology has been instrumental in identifying, characterizing, and detecting numerous phytopathogens. DNA-based techniques offer crucial resources for the accurate detection of plant diseases, enhancing the identification of systematic infections. The development of specific primers has been critical for phytopathogen detection in diagnostic studies. Various PCR techniques, such as isothermal amplification-based PCR, SSCP-PCR, and quantitative PCR, serve as innovative tools in molecular diagnostics for pathogen detection and systematic infection identification. Although molecular diagnostic techniques have advanced significantly, some steps still need to be standardized for these methods to effectively aid in disease detection.

Keywords: Amplified Fragment Length Polymorphism, Isothermal Amplification, Molecular Diagnostic Techniques, Phytopathogens,Restriction fragment Length Polymorphism

INTRODUCTION

Phytopathogens are the most important biotic factors that show epidemic disastrous effects on the crop. Numerous adaptations within the biological system lead to evolutionary changes in the forms and types of microorganisms, which can go on to become genera and species. Plant illnesses are linked to about 3000 bacterial species, 8000 fungal species, and 1000 viral species. In the natural environment, phytopathogens can infect plants at any stage, from seed germination to seed maturation, through seeds, the air, and the soil, which can have a significant impact on the development of disease (Hariharan and Prasannath 2021) Seed-borne pathogens cause yield and quality losses, so early diagnosis of seed-borne phytopathogen is necessary to control the propagation of disease (Gupta and Kumar, 2020). This reduces the usage of fungicides, pesticides, insecticides, weedicides, and other chemical agents, as well as economic losses. Blight, root rot, canker, smut, leaf strip, damping off, wilt, grey mould, anthracnose, downy mildew, dwarf bunt, blast, green motile, and mosaic viruses are the most common phytopathogen-caused diseases, resulting in significant losses to economically important agronomical, forest, and agricultural systems. Global food security is determined by crop protection, according to Associated Chambers of Commerce and Industries of India (ASSOCHAM), the annual crop loss is about Rs. 50,000 crore, behind these losses phytopathogens play a major role through inciting the diseases. The pathogen's genetic diversity and extensive dispersion across host species in growing regions are important considerations in defining and creating disease management measures. As a result, having access to genetic diversity data related to the target pathogen is critical for developing management strategies that effectively control disease incidence (Prasanna

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kumar *et al*. 2021). Successful discovery and succeeding framework for quick reaction are pivotal components to battle against phytopathogens. Therefore, disease management requires diagnosis of diseases based on the detection of phytopathogens at species level. In the field of phytopathogenic organism diagnosis, advanced methodologies have been established. After infections, traditional disease diagnostic approaches have used visual signatures such as fungi propagules viz.,conidia, mycelia, sporangia and bacterial colony on the external surface flora, and its colony characteristics or disease symptoms caused by fungal pathogens and bacterial pathogens. These advances are keystones for disease detection and diagnostics certainly utilized methods involving isolation, culturing and re-inoculation, microscopic examination.

followed by biochemical characterization (Umesha and Avinash, 2015), nucleic acid based amplification, and anti-body based method of identification (Martínez-Ruiz *et al*. 2019). These techniques have some drawbacks because it gives only species level identification not up-to genus level. Over and above that detection of phytopathogen and diagnosis has not been effective due to unpredictability from traditional and serological methods. Advanced molecular diagnostic methods can get grip on species level identification so this can overcome problems of conventional methods.

Advanced molecular approaches are low-cost, high-throughput strategies for detecting phytopathogens. Polymerase chain reaction (PCR), real-time PCR, quantitative PCR, multiplex PCR, colony PCR, single strand confirmation polymorphism (SSCP-PCR), restriction fragment length polymorphism (PCR-RFLP), and amplified fragment length polymorphism (AFLP) are examples that are suitable for genotyping of species. Further, isothermal amplification methods include loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), strand displacement method (SDA), rolling circle amplification (RCA), and next-generation sequencing (NGS). Post-amplification methods include DNA microarray, DNA macro array, and hybridization-based methods such as fluorescence *in situ* hybridization etc.

A new and quickly spreading disease infection poses a threat to the world economy. Thus rapid, quick, and accurate method is a key program to detect and identify the phytopathogen. Several methods have been discovered and there are certain pieces of evidence reviewed from this literature. It also summarizes advantages and drawbacks of advanced diagnostics techniques to study the phytopathogens.

Polymerase Chain Reaction Based Methods

The polymerase chain reaction (PCR) technique has been recognized in molecular biology since it was first described in 1985 by Kary Mullis (Ma and Michailides, 2017). This technique is remarkable due to its ability to withstand thermal conditions. PCR consists of three primary phases: initial denaturation, primer annealing, and elongation. Thermal stability is crucial throughout the procedure since DNA must be denatured at 90–95 °C for 5 minutes at the start. The melting temperature (Tm) of DNA refers to the point at which half of the DNA molecules become denatured. During the primer annealing phase, which occurs at 65–75 °C, a specific complementary primer sequence is required. DNA polymerase begins strand synthesis at the primer during this phase. The elongation phase takes place at 72 °C, where the binding of nucleotide sequences occurs. Taq polymerase from *Thermus aquaticus* and Pfu DNA polymerase from *Pyrococcus furiosus* have been found to exhibit great specificity, thermal stability, and dependability (Valasek *et al.* 2005).

Primers, which are nucleotide sequences complementary to the target DNA, play a crucial role in PCR. PCR is a widely used molecular biology technique that enzymatically replicates DNA without the need for live cultures. It requires only a small quantity of DNA and amplifies it exponentially. PCR is commonly used for the detection of hereditary diseases, genetic fingerprinting, diagnostics of infectious diseases, gene cloning, paternity testing, and DNA computing. The PCR assay requires target DNA, complementary primers, nucleotides (A, T, G, and

Table 1: PCR based identification of seed borne bacteria and fungi with specifically designed primers.

Target pathogen	Crop disease	Specific primers	Detection limit	Target sequence	References
X. campestris pv. *campestris*	Cabbage Black rot disease	5'CCCGGCACGAAAAGTGTCAAG3' 5'-CCTTAGCTCGGATTTTGGCC-3'	100ng	hrpF	Roohie and Umesha (2012)
Colletotrichum acutatum	Lupins Anthracnose disease	5' -CCCGAGAAGGCTCCAAGTA-3 5' -CATAAACGCCTAAGAACCGC-3	10ng and 100fg	IGS region	Pecchia *et al* . *(*2019)
Fusarium species	*Coriandrum sativum* Vascular wilt disease	5'-TCCGTAGGTGAACCTGCGC-3' 5'-TCCTCCGCTTATTGATATGC -3	Not mentioned	ITS primers	Ashwathi *et al* (2017)
X. campestris pv. *campestris*	Brassica Black rot	5'-ATGTCGCTCAACACGCTTTC-3' 5'-GTTTTGCGTGTAGCCCTTTGC-3'	1µl of target DNA	hrpF gene	Berg *et al* (2005)

Table 2 : RT-PCR for the detect Phytopathogen and their causal organism

Crop	Causal organism	Detection limit	Target sequence	Reference
Brassica	*Xanthomonas campestris* pv. *campestris*	50 $ng.ml^{-1}$	hrpF	Eichmeier *et al*. (2019)
Tomato	*Clavibacte rmichiganensis sub sp. michiganensis Pseudomonas syringae* pv. *tomato*	50 $ng.\mu l^{-1}$	16-23S rRNA, hrpL genes, and lepA genes	Penazova *et al.* (2020)
Cucumber	*Pseudomonas syringae* pv. *lachrymans*	10ng	gap1 gene	Meng *et al*. (2016)
Mung bean	*Rhizoctonia solani*	1.24 pg	ITS primers	Dubey *et al*. (2016)
Maize	*Peronosclerosporasorghi*	32fg	SCAR primers	Sireesha *et al*. (2018)
Safflower	*Fusarium oxysporum* f. sp. *carthami*	1pg	SCAR primers	Singh and Kapoor (2018)
Maize	*Fusarium* spp. and *Magnaporthiopsis maydis*	20 ng μL^{-1}	ITS primers	Campos *et al*. (2019)
Pea	*Pea seed borne mosaic virus*	1µL	Elongation factor 4 E (eIF4E)	Swisher *et al*. (2020)
Rice seed	*Fusarium fusjikuroi,* and *Magnaporthe oryzae*	6.18ng/µl-0.618fg/µl, 1.37ng/µl-0.137fg/µl	Elongation factor-1alpha	Ortega *et al*. (2018)
Wheat and Barley	*Fusarium graminearum Hypovirus 2*	12,800nts of RNA dependent RNA polymerase	Translation elongation actor-1 alpha	Li *et al*. (2015)

C) for chain extension, and a DNA polymerase enzyme. The enzyme links individual nucleotides in the target DNA with the primers to form PCR products. Primers specify the DNA sequence to be amplified. Analysis of PCR products involves separating the amplified product using agarose gel electrophoresis, which separates DNA based on size and charge. The amplified products can be stained with chemical dyes or labeled with fluorescent dyes on PCR primers prior to amplification (Garibyan and Avashia, 2013).

In the context of plant pathology, PCR is widely used to detect plant pathogens (phytopathogens) such as bacteria, fungi, and viruses. It is also valuable in cloned gene expression studies. Numerous studies on the detection of phytopathogens have been reported (Table 1), including *Fusarium* species (Chehri *et al*. 2011), *Cassava Mosaic Gemini Virus* (Ndunguru *et al*. 2005), *Xanthomonas* spp. (Roohie and Umesha, 2012), and *Ralstonia solanacearum* (Umesha and Avinash, 2015). Besides conventional PCR, advanced molecular techniques such as isothermal amplification-based PCR, single-strand conformation polymorphism (SSCP-PCR), and quantitative PCR (qPCR) have become invaluable tools for pathogen detection. These methods offer enhanced sensitivity and specificity, allowing for effective detection of

Table 3: Detection of Pathogens by using multiplex PCR

Crop	Pathogen	Target sequence	Detection limit	References
Musa acuminata *Musa balbisiana*	*Fusarium oxysporum f. sp. cubense*	Translation elongation factor -1 alpha Intergenic spacer region (IGS)	10 ng μL^{-1}	Dita *et al.* (2010)
Food sample	*Fusarium species*	Trichodiene synthase (tri5) Zinc finger transcription factor (tri6), etc.	1µl-50ng.μl^{-1}	Dawidziuk *et al.* (2014)
Tomato	*Clavibacter michiganensis sub sp. michiganensis* *Fusarium oxysporum sp. lycopersici* *Leveillula taurica* *Begomoviruses*	Ribosomal intergenic sequence	4 or 100ng	Quintero-Vásquez *et al.* (2013)
Solanaceae crop	*R. solanacearum* *X. perforans*	Species specific primers	10-100ng	Umesha and Avinash (2015)
Chrysanthemum	*Stagonosporopsis sp.*	IGS primers	10ng	Vaghef *et al.* (2016)
Rice crop	*Xanthomonas oryzae pv. oryzae, X. oryzae pv. oryzicola*	Hypothetical protein hrp/hrc gene	10ng to 1fg	Cui *et al.*(2014)
	Acidovorax avenae sub sp. avenae	Paraquat-inducible protein ATP synthase subunit beta AtpD	1ng	Boureau *et al.* (2012)
	Pseudomonas fuscovaginae	(atpD) gene		Bangratz *et al.* (2020)
	Burkholderia glumae *Burkholderia gladioli*	16S rRNA gene GTP cyclohydrolase II		
	Pantoea species			
Tomato	*Tobacco mosaic virus* *Tomato mosaic virus*	Species specific primers	100ng	Kumar *et al.* (2011)

Table 4: Experimental studies utilizing the AFLP technique for the genotype analysis

Disease	Causal organism	Detection limit	Pre-selective primers	Selective primers	No. of primer pair tested	Reference
Patch disease	*Rhizoctonia solani (Waiteacircinata)*	100ng	*EcoRI* *MseI*	*EcoRI* *MseI*	4	Amaradasa *et al* (2015)
Fusarium wilt disease to oil palm	*Fusarium oxysporum*f. sp.*elaeidis (Foe)*	400-500ng	*EcoRI* *MseI*	*EcoRIMseI*	3	Adusei-Fosu *et al* . (2019)
Fusarium wilt disease	*Fusarium oxysporum* f. sp. *momordicae*	300ng	*EcoRI* *MseI*	*EcoRI* *MseI*	N/A	Chen *et al*. (2015)
Downy mildew	*Hyaloperonospora parasitica*	N/A	*Taq I* *Ase I*	*Taq I* *Ase I*	N/A	Xiao *et al*. (2016)
Fusarium wilt	*Fusarium oxysporum*f. sp. *cucumerinum*	N/A	*EcoRI/MseI*	*EcoRI/MseI*	N/A	Jaber *et al.* (2020)
	Xanthomonas campestris pv. *vesicatoria*	100-200ng	*Pst I and Taq I*	*PstI-^{33}P* *Taq I-^{33}P*		Noel *et al*. (2001)
Bacterial leaf spot	*Pseudomonas syringae* pv. *tagetis*	300ng	*EcoRI/MseI*	*EcoRI/MseI*	100	Song *et al*. (2015)
Bacterial wilt	*Ralstonia solanacearum*	200ng	*EcoRI/MseI*	*EcoRI/MseI*	7	Mollae *et al*. (2020)

Table 5 : LAMP based detection of plant pathogens

Host	Pathogen	Region or locus	Sensitivity	Assay time minutes	Reference
Rice	*Magnaporthe oryzae*	Elongation factor 1- alpha sequence of *F. fujikuroi* and calmodulin sequence of *M. oryzae*	100 to 999 pg *of F. fujikuroi* and 10 -99pg of *M. oryzae*	45, 60 ^{0}C	Ortega *et al.* (2018)
Potato Tomato	*Alternaria solani*	Histidine kinase gene (HK1)	136 ng to 13.6 fg	60, 63 ^{0}C	Khan *et al.* (2018)
Rice	*Magnaporthe oryzae* *Sarocladium oryzae*	RNA polymerase II large subunit	100fg	20, 60 ^{0}C	Prasanna kumar *et al* (2021)
Chickpea	*Fusarium oxysporum* f. sp *ciceris*	Elongation factor 1 alpha gene	10fg	60, 63^{0}C	Ghosh *et al.* (2015)

systemic infections. Furthermore, the development of several distinct primers has been crucial to the success of diagnostic studies in phytopathogen detection. Molecular diagnostics continue to evolve, offering more robust and reliable methods for identifying and managing plant diseases, thus safeguarding crop health and agricultural productivity.

Real time PCR/ Quantitative PCR (RT-qPCR)

A supplemental approach to the PCR methodology is real-time PCR, also known as quantitative PCR (qPCR). Real-time PCR (RT-PCR), which uses RNA as a template to create complementary DNA (cDNA), is commonly employed to analyze gene expression. This approach is widely used in gene expression studies, RNAi validation, microarray validation, pathogen detection, genetic testing, and disease research. RT-PCR amplification determines the gap sequences between distinct clones of specified primers. In the 1990s, real-time quantitative amplification techniques revolutionized basic and applied studies in plant pathogen detection across various biological fields. qPCR is known for its sensitivity, accuracy, reliability, and high throughput in amplifying target DNA. In nucleic acid research, RT-PCR is considered the gold standard for sensitive and specific detection and quantification.

There are two methods for performing the RT-qPCR assay: the one-step and two-step techniques. The one-step method includes all the necessary components-such as reverse transcriptase enzyme, buffer, and sequence-specific primers-combined in a single reaction. This method offers several benefits, including reduced experimental variation, fewer pipetting steps, minimized risk of contamination, rapid and highly reproducible procedures, and suitability for high-throughput amplification. The two-step technique, on the other hand, involves using separate tubes for different optimized buffers, reaction conditions, and priming techniques. This method allows the generated cDNA pool to be stored for extended periods and used in multiple processes. It also offers flexibility in optimizing reaction buffers, conditions, and primer selection for each reaction.

In RT-qPCR, oligo-dT primers, random primers, and sequence-specific primers can be used. These primers anneal to the template mRNA strand, allowing reverse transcriptase enzymes to initiate cDNA synthesis. Oligo-dT primers, or anchored dT primers, consist of one G, C, or A sequence at the 3' end and stretch the thymine residue to anneal to the polyA tail of mRNA. Random primers, typically 6–8 bases long, anneal to various tRNA, mRNA, and rRNA molecules. Sequence-specific primers, as the name suggests, target only specific mRNA sequences. Common reverse transcriptase enzymes used in RT-qPCR include Maloney murine leukemia virus reverse transcriptase and avian *myeloblastosis virus* reverse transcriptase (Bustin, 2004).

The real-time PCR assay includes three primary fluorescence-based methods for detecting amplicons: TaqMan probes, fluorescence resonance energy transfer (FRET) probes, and molecular beacons. Real-time PCR is quick, sensitive, and highly reproducible, eliminating the need for time-consuming electrophoresis steps. This method has been widely used to detect bacterial plant pathogens such as *Clavibactermichiganensis* subsp. *sepedonicus*, *Ralstonia solanacearum*, *Agrobacterium* spp., *Xylella fastidiosa*, *CandidatusLiberibacter asiaticus*, *Xanthomonas citri*pv. *citri*, and others (Table 2). Real-time PCR has proven highly effective for detecting prokaryotes in plant tissues and has greatly benefited regulatory agencies by allowing the rapid and sensitive detection of confined pathogenic phytobacteria, helping to prevent their spread.

The *hrpB7* gene sequence has been explored for identifying the causal agents of bacterial spot and appears to be conserved among *Xanthomonas* species (Strayer *et al*. 2016). Multiplex real-time PCR assays provide a robust detection method for *Xanthomonas campestris*pv. *campestris*, which causes vascular diseases (Eichmeier*et al*. 2019). In quantitative PCR, the relative number of target DNA and RNA sequences can be estimated by applying a calibration curve that relates the Ct (cycle threshold) value (Meng *et al*. 2016) and Cq (quantification cycle) value (Sireesha *et al*. 2018) using specific primers. The

accuracy and reliability of real-time PCR results are further enhanced through the use of software to analyze data generated by PCR machines.

Multiplex PCR

Multiplex PCR, widely used in molecular biology laboratories, follows the same principles as traditional PCR. However, this technique allows for the simultaneous amplification of different gene segments by employing multiple sets of primers within the same PCR mixture. It is an efficient, time-saving, and cost-effective method for genetic analysis, especially in experiments that require repetition. Multiplex PCR requires a very low quantity of DNA, typically around 10-200 ng. The design of specific primers is crucial for efficient amplification, and several criteria must be met, including primer length. Typically, primers are 18-22 bases long, and their melting temperatures should be similar, ranging between 55 °C and 60 °C. Sequences with a high GC content and high melting temperature are preferred for multiplex PCR (mPCR). It is essential to ensure primer specificity to the target sequence and check for the formation of primer dimers. Primer dimerization within the reaction mixture can lead to nonspecific amplification, which can compromise experimental results.

First described in 1988, multiplex PCR has since been successfully applied to various fields, including DNA testing, gene deletion analysis, mutation and polymorphism analysis, quantitative analysis, and reverse transcription. It is also used in the identification of infectious diseases caused by viruses, bacteria, and parasites. To perform multiplex PCR effectively, certain criteria must be met (Table 3). Primer concentrations should be equimolar, typically ranging from 0.1 to 0.5 µM, although this may vary depending on the loci being amplified. For low-copy-number or high-complexity DNA, the primer concentration should range from 0.3 to 0.5 µM; for high-copy-number or low-complexity DNA, it should be between 0.04 and 0.4 µM.

The concentrations of dNTPs and MgCl2 are also critical: MgCl2 concentration is kept constant at 2 mM, while dNTP concentration is increased stepwise from 0.5 to 1.6 mM. Accurate results are typically achieved with dNTP concentrations between 200 and 400 µM. The buffer concentration is increased to 2X, and the target DNA concentration is kept very low, with 2.5 U of Taq DNA polymerase used per 50 µL reaction volume. The use of adjuvants is also crucial in multiplex PCR, as they help relax DNA and facilitate template denaturation. Common adjuvants include DMSO, glycerol, formamide, and betaine, which are used to maintain concentrations up to 0.8 µg/µL (*Markoulatos et al. 2002*).

Colony PCR Assay

In 1989, Güssow and Clackson successfully applied the colony PCR method for diagnosing bacterial infections (Güssow and Clackson, 1989). Since then, colony PCR has become widely used across various fields of microbiology, including the detection of *Escherichia coli*, *Bacillus subtilis*, *Bacillus coagulans*, *Pichia pastoris*, *Trichosporona sahii*, and *Aspergillus* species (Packeiser *et al*. 2013). Despite its widespread adoption, the application of colony PCR in diagnosing phytopathogens remains limited, with only a few references available. Notably, it has been established for detecting bacterial wilt caused by *Ralstonia solanacearum* (Umesha *et al*. 2012). Additionally, colony PCR is utilized for screening polyhydroxyalkanoate (PHA) producers isolated from the environment (Sheu *et al*. 2000).

Colony PCR is a modified version of conventional PCR that follows the cyclic process of denaturation, annealing, and extension to amplify DNA. It plays a vital role in gene cloning, gene transfer, and genetic engineering research by confirming the presence of inserted DNA. This method is known for its simplicity, high throughput, speed, and cost-effectiveness in detecting the presence or absence of inserted DNA in plasmid constructs.

The process typically involves lysing individual colonies, either by heating them briefly in water or directly in the PCR reaction during the initial heating phase. This heating step releases plasmid DNA, making it available as the template for amplification. After the PCR process, the presence or absence of amplicons is determined

using agarose gel electrophoresis. Colony PCR has become an integral tool in modern microbiological research due to its efficiency and effectiveness in various diagnostic and screening applications.

Single Strand Conformation Polymorphism-PCR

Single-strand conformation polymorphism (SSCP) analysis is a widely used method for detecting genetic variations across different species. Initially developed to identify point mutations in human DNA, SSCP exploits differences in DNA mobility during electrophoresis to find sequence variants. Under non-denaturing conditions, mutant DNA is compared to wild-type DNA. The PCR technique is used to extract and amplify the DNA. The quality, accuracy, and length of the DNA sequences depend on the characteristics of the sample. Fluorescent primers are employed to actively identify the amplified amplicons of interest, and agarose gel electrophoresis is used to verify the purity and concentration of the PCR products.

Variations in the 16S rRNA gene generate distinct banding patterns that help differentiate between species. For instance, SSCP analysis has been used to distinguish plant pathogens such as *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. *vesicatoria*, and *Xanthomonas oryzae* pv. *oryzae* based on their unique banding patterns (Srinivasa *et al*. 2012). Additionally, SSCP-PCR has been developed to differentiate multiple species within the genera of pythiaceous organisms using a single pair of oomycete primers (Kong *et al*. 2005).

Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP**)** is a PCR-based fingerprinting technique initially developed in the late 1990s. AFLP involves five key steps: (a) restriction of genomic DNA followed by ligation of adaptors to the restricted fragments; (b) pre-selective PCR amplification of a subset of the restricted fragments; (c) selective PCR amplification; (d) electrophoretic separation of the amplified DNA fragments; and (e) scoring and interpretation of the data (Paun and Schönswetter, 2012).

As a multilocus marker system, AFLP does not require prior knowledge of sequence data to generate polymorphic markers, making it a versatile tool for investigating the genetic diversity of organisms. The dominant nature of AFLP markers means that it is unable to distinguish between homologous alleles. However, AFLP remains an excellent alternative to traditional methods for accurately and cost-effectively characterizing subtle differences within species, such as in *Rhizoctonia solani* and *Waitea circinata* isolates.

Due to technological advancements, AFLP has evolved into a relatively simple, fast, and reliable method for generating a large number of informative genetic markers. Its applications range from studying the inheritance of agronomic traits in plant and animal breeding to diagnosing genetically inherited diseases, forensic typing, parentage verification, and identifying DNA markers linked to genetic traits. AFLP is highly effective at examining whole genomes for polymorphisms, making it an invaluable tool for analyzing genetic diversity and genome-wide variation. The AFLP process involves digesting genomic DNA with two types of restriction endonucleases: rare-cutters and frequent-cutters. Rare-cutting enzymes recognize sequences of 6–8 base pairs (e.g., *EcoR1*, *Ase1*, *HindIII*, *ApaI*, *PstI*), while frequent-cutters recognize sequences of 4–6 base pairs (e.g., *MseI*, *TaqI*). The use of highly specific restriction enzymes results in the production of a reproducible set of DNA fragments. The choice of enzyme depends on the complexity and methylation status of the genome. After restriction digestion, nucleotide adaptors of 10-30 base pairs in length are ligated to the sticky ends of the restriction fragments using T4 DNA ligase. Amplified fragments are then separated by gel electrophoresis, and the data are analyzed to determine genetic diversity. AFLP is a robust tool for characterizing the genetic diversity of various organisms, as summarized in Table 4.

Random Amplified Polymorphic DNA (RAPD-Dominant Marker)

A single arbitrarily chosen primer and polymerase chain reaction (PCR) can create a simple and

reproducible fingerprint of a complex genome (Welsh and McClelland, 1990). This method, known as Random Amplified Polymorphic DNA (RAPD), is a molecular marker technique that utilizes PCR to amplify specific DNA regions. After denatured DNA is extracted, template molecules are annealed with short, random primers and amplified through PCR. The short primers used in RAPD typically range from 8–10 nucleotides in length. As the number of PCR cycles increases, the specificity and strength of primer binding improve, allowing for the amplification of distinct genomic regions. Following amplification, electrophoresis is used to separate the amplified DNA products.

The resulting banding patterns from RAPD can be useful for the detection of genetic variations. In some cases, specific bands from gels may be excised and sequenced, facilitating further analyses such as PCR, dot hybridization, or other diagnostic procedures. However, RAPD is often regarded as a labor-intensive technique with limitations in terms of infection analysis, as it provides variable results, especially at the sub-species level. Despite these drawbacks, RAPD has been employed to identify conserved regions of genomic DNA in eukaryotic microorganisms. Identification often relies on nucleotide sequences from conserved regions, which are amplified using PCR. RAPD is commonly used in the identification, characterization, and early detection of diseases, particularly those caused by microbes, parasites, and infections. RAPD analysis has revealed significant levels of genetic variability across numerous isolates. Compared to methods such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and Inter Simple Sequence Repeats (ISSRs), RAPD is relatively simple, faster, and easier to analyze. Sub-molecular technologies like Internal Transcribed Spacer (ITS) and RAPD provide the foundational data required for species differentiation and help clarify evolutionary relationships between species. Molecular markers such as RAPD and Single Nucleotide Polymorphisms (SNPs) have been developed for marker-assisted selection, facilitating selective breeding and reducing breeding cycles. RAPD analysis does not require prior knowledge of the target DNA sequence and provides insight into genetic differences within large genomes. These markers enable the rapid detection of genetic differences at the DNA level without the need for phenotyping.

In plant pathology, RAPD has been used for detecting pathogens like *Fusarium* wilt in the field (Lin *et al*. 2010). RAPD has also been proven as a valuable diagnostic tool for identifying fungal pathogen strains in the Mediterranean region. The use of molecular markers aids in studying the distribution of pathogens and enhancing the efficient deployment of available host resistance genes. Markers like RAPD facilitate early detection of pathogen strains and help track changes in the frequency of races due to the use of resistant crop varieties.

RAPD has been utilized for the genetic characterization of phytopathogens such as *Fusarium oxysporum* f. sp. *cubense*, *dianthi*, *pisi*, and *vasinfectum*. It has also been combined with ribosomal DNA sequencing primers to differentiate between yellowing and wilting pathogens (del Mar Jiménez-Gasco*et al*. 2001). Additionally, the development of RAPD with SCAR (Sequence Characterized Amplified Region) markers has been useful for detecting *Phytophthora sojae*, the causative agent of root rot disease in soybean (Xiong *et al*. 2019). RAPD has also been applied in the detection of brown rot of alfalfa caused by *Phomasclerotioides* (Larsen *et al*. 2002), analysis of genetic diversity in *Ralstoniasolani* causing cotton damping-off (Heflish, 2020), and identification of *Verticillium dahliae* pathotypes causing Verticillium wilt in cotton (Pérez-Artés *et al*. 2000). Furthermore, RAPD has been instrumental in characterizing the genetic variability of *Macrophominaphaseolina* and *Fusarium* species (Jana *et al*. 2003).

Restriction Fragment Length Polymorphism (Co dominant Marker)

Restriction Fragment Length Polymorphism (RFLP) is a molecular technique used to detect polymorphisms based on differences in DNA sequences. The process involves the isolation of genomic DNA, followed by its digestion using restriction enzymes. These enzymes cut the DNA at specific recognition sites, generating fragments

of varying lengths. The resulting fragments are separated by agarose gel electrophoresis and then transferred onto a membrane using the Southern blot technique. Hybridization of the membrane with a labeled DNA probe helps determine the length of DNA fragments that are complementary to the probe. RFLP is widely used for identifying variations in homologous DNA sequences, as differences in the presence or absence of restriction sites lead to distinct fragment lengths. An RFLP is observed when the length of the detected DNA fragments varies between individuals.

The RFLP probe was successfully developed to characterize *Fusarium oxysporum* f. sp. phaseoli using a digoxigenin-labeled, 1.3 kb fragment of the D4 probe, which contains a random fragment from *Fusarium oxysporum* f. sp. *dianthus*. RFLP has also been utilized to identify and classify phytoplasmas associated with aster yellows disease in *Portulaca oleracea*, *Stellaria media*, *Daucus carota* ssp. *sativus*, and *Cyclamen persicum*. The technique has been further employed to characterize '*Candidatus Phytoplasma asteris*', which is associated with leaf disease in bitter gourd. In this context, RFLP has proven effective in identifying unknown phytoplasmas by comparing their RFLP patterns to those of known species, offering a reliable method for phytoplasma differentiation across classification systems. Kumar *et al*. (2017) expanded on this by developing virtual RFLP techniques to detect phytoplasmas associated with brinjal little leaf disease. Additionally, PCR-RFLP has been utilized to monitor fungicide resistance in *Cercosporabeticola* populations affecting sugar beets, specifically tracking the presence of G143A and E198A mutations. The technique has been used to directly detect the QoI-resistant cytochrome b allele and the benzimidazole-resistant â-tubulin alleles from leaf tissue (Rosenzweig et al. 2014). RFLP techniques targeting 16S ribosomal and *tuf* DNA have also been employed for the detection of phytoplasma-associated diseases in *Brassica* species (Ahmad *et al*. 2017).

RFLP has been adapted for use in *Cucumis sativus L.*, where it was developed to detect phytoplasma lineages (Usta *et al*. 2017). For viral identification, the HCPro sequence of the Greek lentil *PSbMV* isolate (GR33) was subjected to in silico restriction analysis. Enzymes such as AluI, HaeIII, HhaI, RsaI, Sau3AI, and Tru9I were used to perform virtual gel electrophoresis, allowing for the identification of the pea seed-borne mosaic virus (Giakountis *et al*. 2015). In another study, nuclear rDNA PCR products were digested with enzymes HaeIII, HinfI, AvaI, and MspI, while mitochondrial SSU rRNA PCR products were digested with HaeIII, HinfI, HindIII, and DraI. The resulting restriction fragments were separated by agarose gel electrophoresis, and fragment sizes were estimated through visual comparison with a DNA ladder. For both nuclear ITS and mitochondrial SSU rRNA RFLP types, genetic relationships were inferred based on differences in restriction sites within the amplified sequences (Arruda *et al*. 2005).

Single Nucleotide Polymorphism

Single Nucleotide Polymorphism (SNP) arrays are among the most high-throughput, cost-effective, and automated methods for genotyping. Despite these advantages, SNP identification in complex polyploid genomes presents significant challenges, which have slowed SNP discovery and array development in polyploid species. According to You *et al*. (2018), ploidy plays a critical role in the quality and validation rates of SNP markers in SNP arrays. SNPs have become vital tools for genetic research and molecular breeding due to their ability to associate specific markers with genes controlling important agronomic traits. When plant breeders use SNP markers, they can group individuals into distinct genotypic classes based on the presence or absence of key loci of interest. One of the inherent advantages of SNPs over other molecular markers is their abundance, high throughput capacity, and cost-effectiveness within species. Post-PCR high-resolution melting analysis facilitates automated SNP detection, which makes them co-dominant markers able to distinguish between heterozygous and homozygous alleles.

SNPs are also useful for combining candidate gene techniques with association-based fine mapping to identify genes of interest (Kim *et al*.

2018). Additionally, SNP markers can assess linkage disequilibrium and define haplotypes. The advantages of SNPs over other genetic markers include their high prevalence in genomes and the ability to leverage modern, high-throughput technologies for analysis.A SNP haplotype refers to a specific combination of SNPs that are closely linked within a region of a chromosome, often inherited as a unit from parent to offspring. Data from SNP haplotypes are particularly valuable when closely spaced SNPs fully define haplotypes in a given region. Some haplotypes are tagged by specific SNPs, which are useful for genetic mapping and marker-assisted selection (MAS). SNPs are critical markers in MAS programs, especially for disease resistance in plant breeding, allowing for precise genetic selection.SNP markers are favored for MAS due to their abundance and presence near most genes (Chandra *et al*. 2017). For example, SNPs have been developed to detect Phytophthora root rot, a disease responsible for significant annual soybean yield losses. Screening for polymorphisms between parental cultivars has been used to clarify the genotype of populations and characterize whole genome sequences. Lei *et al*. (2017) developed SNP markers to investigate somatic recombination, comparing parental isolates from wheat and barley genotypic races of *Puccinia striiformis* f. sp. *tritici* and *P. striiformis* f. sp. *hordei*.

Isothermal Amplification Based Methods

Nucleic acid amplification is the most helpful technology in the field of life sciences. Because of the certain limitations of conventional PCR, there is a forthcoming interest in simple nucleic acid amplification methods that can be performed isothermally. Isothermal DNA and RNA amplification methods have been broadly utilized in recent years. Some of the techniques, including rolling circle amplification (RCA), nucleic acid sequence-based amplification (NASBA), strand displacement method (SDA), and loop-mediated isothermal amplification (LAMP) assays, are utilized.

LAMP Based Assay

The Loop-Mediated Isothermal Amplification (LAMP) assay is a powerful and highly sensitive method used to identify bacteria, fungi, and viral pathogens. Compared to conventional PCR, LAMP is notably more sensitive and specific, making it an ideal choice for pathogen detection. It operates on the principle of developing microdevices that incorporate foldable technology, making it suitable for point-of-care applications.LAMP is a single-tube amplification technique that amplifies DNA at a constant temperature of 60-65°C, eliminating the need for thermal cycling. The process involves 4-6 primers that facilitate the amplification cycle. These primers help form a dumbbell-like DNA structure, which is then converted into a stem-loop DNA structure. This structure undergoes amplification, producing large quantities of DNA. The amplification process is driven by strand displacement by a polymerase enzyme, typically Bsm DNA polymerase derived from *Bacillus smithii*.

The major advantage of LAMP is its simplicity and low cost. The DNA is typically detected by visual observation, often using a colorimeter or turbidity to monitor the precipitation of magnesium pyrophosphate, a by-product of the amplification process. The technique can detect DNA at very low concentrations, starting at femtogram levels. LAMP's versatility and high specificity make it suitable for detecting various pathogens. Some successful applications of LAMP in plant pathogen detection include: *Pseudomonas syringae*pv. *tomato*, causing bacterial speck in tomatoes (Chen *et al.* 2020), *Erwinia amylovora*, responsible for fire blight disease in pears and apples (Buhlmann *et al.* 2012), *Pseudomonas fuscovaginae*, leading to sheath brown rot in rice crops (Ash *et al*. 2014). LAMP's high sensitivity, simplicity, and cost-effectiveness make it a valuable tool for rapid pathogen detection, particularly in field settings and for point-of-care diagnostics.

Rolling Circle Amplification

Rolling Circle Amplification (RCA) is an isothermal enzymatic assay that employs DNA/RNA polymerase to synthesize single-stranded DNA or RNA. This method produces repeating units of DNA, resulting in numerous signals in a localized area. One advanced form of RCA is

Hyper-Branched RCA (HRCA), also known as Ramification Amplification (RAM), which utilizes a reverse primer complementary to the RCA product. In RCA, circular oligonucleotides, typically 25-100 nucleotides, serve as templates for the DNA/RNA polymerase. RCA can be employed to develop "padlock" probes for the detection of nucleic acids and proteins. The circular oligonucleotide acts as a vector for producing biologically active RNAs through a process known as Rolling-Circle Transcription (RCT).A significant application of RCA involves using small DNA circles to encode and extend human telomeres. This amplification process leverages the phi29 DNA polymerase, known for its strand displacement activity, which generates both single and multiple sets of primers that anneal to a circular DNA template. This process forms a "ramified" or hyper-branched complex. During the strand displacement process, free single-stranded DNA is synthesized as the newly generated DNA dissociates from the earlier synthesized DNA template (Hariharan and Prasannath, 2021).RCA offers a sensitive and cost-effective method for pathogen identification without the need for DNA sequencing. The amplified fragments can be visualized using fluorescent dyes, and the specificity of probe-template binding can be verified through 1% agarose gel electrophoresis.

Applications of RCA

1.Padlock Probes: RCA padlock probes have been designed to detect polymorphisms in the elongation factor-1 alpha gene for identifying dominant species of *Fusarium graminearum* (Davari *et al*. 2012).

2. Circularization: The circularization of padlock probes, accomplished using T4 DNA ligase or thermally stable ligase, allows for highly sensitive recognition of point mutations in target DNA.

3. Amplification Methods: Circularized padlock probes can be amplified using single-primer RCA with phi29 DNA polymerase or two-primer hyper-branched RCA with Vent (Exo) DNA polymerase.

4. Multiplex Assays: RCA products can be hybridized with different fluorescent dye-labeled probes, enabling multiplex assays to analyze multiple alleles across the entire genome. These assays can be distinguished by detecting specific wavelengths and filters within an exhaustive fluorescence detection system.

Overall, RCA represents a versatile and powerful tool for nucleic acid detection, offering high sensitivity and specificity for various applications in molecular biology and diagnostics.

Nucleic Acid Sequence Based Amplification (NASBA)

Nucleic Acid Sequence-Based Amplification (NASBA) is a technique that amplifies single-stranded RNA templates, converting them into complementary DNA (cDNA) under isothermal conditions. This process occurs at a temperature of 41 °C and utilizes key enzymes including avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase.

Key Features of NASBA

1. Molecular Beacons:The NASBA technique incorporates ongoing observation with molecular beacons, which are single-stranded oligonucleotides with a unique stem-loop structure.

2.The loop is complementary to the target RNA sequence, while the stem consists of double-stranded structures. One strand of the stem is labeled with a fluorophore, and the other is labeled with a quencher.

3. When the loop hybridizes with the target RNA, the stem opens, separating the quencher from the fluorophore, which allows the probe to emit fluorescence.

NASBA is a valuable diagnostic tool for detecting pathogenic organisms, conducting gene expression studies, and assessing cell viability. It is particularly useful because RNA molecules degrade rapidly after cell death, making it a reliable method for identifying viable cells.This technique is applied in various fields, including microbiology, virology, and environmental studies.

NASBA is a rapid, specific, and sensitive method for recognizing viable bacterial cells. It employs a hybridization device that allows for nucleic acid sequence visualization results within 30 minutes.The efficacy of NASBA has been demonstrated with bacterial suspensions subjected to various heat treatments and treated with sodium ortho-phenyl phenate. For instance, the detection of *Xanthomonas citri* subsp. *citri* viable cells was analyzed using specific primer sets targeting 16S rRNA and Internal Transcribed Spacer (ITS) regions to distinguish living and dead cells. Additionally, gumD mRNA was synthesized to evaluate the viability of this bacterium under different bactericidal treatments (Scuderi *et al*. 2010).Overall, NASBA represents a powerful and efficient tool for RNA amplification and detection, with significant applications in clinical diagnostics and environmental monitoring.

LAB-ON-CHIP Method

Lab-on-Chip (LOC) Systems represent a breakthrough in clinical and biological assays by enabling multiple analyses on a single miniaturized platform. These systems integrate processes like sample preparation, cell growth and detection, cell lysis, PCR, and other analytical steps, enhancing detection accuracy while minimizing false positives (Bhattacharya et al. 2007). LOC devices play a critical role in various research fields, including:

·
Genomics: Facilitating rapid genetic analyses.
·
Proteomics: Assisting in protein profiling.
·
Pharmacology: Supporting drug discovery and validation.
·
Drug Delivery: Enabling controlled delivery systems.
·
Point-of-Care Diagnostics: Providing rapid and precise diagnostics outside of traditional labs.

The development of chip-based diagnostic tools is especially promising for the detection of plant pathogens. These devices enable fast, specific and sensitive pathogen detection without requiring extensive laboratory infrastructure. Silicon-fabricated Microdevices, These are commonly used in nucleic acid-based assays for pathogen detection. However, some methods remain time-consuming or require specialized laboratories (Julich *et al*. 2011). PDMS-Based Microfluidic Platforms, platforms made from Polydimethylsiloxane (PDMS) incorporate microfluidic channels and chambers as small as 20 ìl, obtained via replica molding. Such systems have been successfully used to detect pathogens like *Xylella fastidiosa* subsp. *pauca* strain CoDiRO (Chiriacò *et al*. 2018).

Application of LAB-ON-CHIP Method

·
Miniaturization: Enables high-throughput analysis with minimal sample volume.
·
Automation: Facilitates multi-step assays on a single chip, reducing human intervention.
·
Speed: Accelerates the detection process compared to traditional methods.
·
Portability: Allows point-of-care usage, ideal for field diagnostics.

The incorporation of lab-on-chip technologies into plant pathogen diagnostics offers exciting potential for improving the speed and accuracy of pathogen detection in agriculture, enhancing disease management strategies.

CONCLUSION

The advanced molecular diagnostic techniques have increased advantages over the detection and diagnostics of phytopathogens. A precise and advanced diagnostic tool provides emerging and novel approaches to phytopathogen detection. Now a day's agricultural systems faces economical losses due to unconditional effects of phytopathogens. It is strongly believe that the detailed information given in this review will help to carry out further advanced process in the detection of various phytopathogens, which causes disastrous effects to the crop plants. However, molecular based approaches will provide accuracy towards the detection of systematic infections of phytopathogens in the agriculturally important plant species.

Furthermore, it needs easy and less time consuming methods to detect phytopathogens. Quantitative PCR (qPCR) and multiplex-PCR are widely utilized for the quantification and differentiation of causal agents, even when present in minimal concentrations within a sample, making them effective tools for precise pathogen detection. Currently LAMP assay (Loop Mediated Isothermal Amplification) proceeds trend in the phytopathogen detection. Apart from the different molecular diagnostic methods bioinformatics are essential to avoid misrepresentation of results. The challenges are to develop practical approaches to devise point of care technology; biosensor based approach, nano-based technology and should be more effective, time saving, easy analysis of phytopathogen detection.

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Conflict of Interest. Authors declare no conflicts of interest

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