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MOLECULAR MARKERS IN DIAGNOSIS AND MANAGEMENT OF FUNGAL PATHOGENS: A REVIEW

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ABSTARCT:

Fungal pathogen incites very serious diseases in plants and responsible for considerable yield losses. A precise identification of fungal phyto-pathogens is essential for designing effective management strategies. During the last decades, the advent of molecular biology promised to offer radical alternatives in the detection and enumeration of fungal pathogens. At the same time, the acquisition of DNA sequences provided information that led to the identification of new and unknown species. These data, together with classical characterization of fungi in the field, opened new insights into the range of fungal functions and interactions mostly within terrestrial communities but also for fungal plant pathogens as a whole. The present review focuses on the application of various molecular techniques especially molecular markers in the detection, identification, Characterization and quantification of fungal pathogens that incites diseases in plants.

INTRODUCTION:

The trend for globalization in agriculture creates even higher demands in various aspects of plant protection. With the advancement of international commerce that practically cancelled the geographic boarders between countries, requires more strict legislation to check the movement of plants and plant materials. Phytosanitary inspections and quarantine regulations became more stringent demanding increased control measures not only between countries but also within the territories of a certain country. Selection of healthy and disease free propagation materials to the prevention of spread of a pathogen to another country, screening of mother plants for certain pathogens, monitoring of resistance phenotypes of a fungus to certain agro chemicals are among challenges encountered on a routine base. Furthermore, research frontiers have been widened to more detailed and in depth studies

of host-parasite interactions, disease resistance, pathogens population structure. Thus, the development of methods capable to detect and identify pathogens in plant materials in a fast, accurate and sensitive manner has been necessitated more than ever.

All disease management programmes need a simple, safe and rapid and cost effective method of pathogen detection. Though visual identification of plant disease is the most rapid and cost effective method of disease diagnosis, but it is difficult for inexperienced personal and is also limited particularly to disease affecting aerial parts of the plants. Other method of pathogen identification is based on observed morphological characteristics by microscopic examinations of the diseased tissue, this method also require highly specialized taxonomists, which are now rarely available.Various problems associated with micro scopical detection of plant pathogens can be overcome by protein/ Nucleic acid based detections. These methods are very robust and repeatable. Protein based assay ELISA may be very useful in fungal pathogen diagnosis [1]**.**The nucleic acid based detections can be used at any developmental stage of plant since all living cells contain entire set of genome and not affected by environment. The Polymerase chain reaction based assays may be used as tool for fungal detection [2]**.** The advent of molecular biology has offered a number of revolutionary new insights into the detection and enumeration of phyto-pathogens and also has started to provide information on identifying unknown species from their DNA sequences. Non radioactive gene probes labels can be preferred for labeling specific gene probes while detection of fungal pathogen [3]. Molecular data, combined with classical characterization of fungi in the field, provide new aspects about fungal functions and interactions within terrestrial communities [4]**.** Recent advances in the detection of plant pathogens using immunological or serological and nucleic acid-based techniques has enabled the major genera and species of disease-causing organisms to be quickly and reliably identified. The application of these technologies in plant pathology has greatly improved our ability to detect plant pathogens and is increasing our understanding of their ecology and epidemiology

Although molecular methods, such as polymerase chain reaction (PCR) and molecular hybridization, are routinely used in the diagnosis of human diseases, they are not yet widely used to detect and identify plant pathogens because of cost and other constraints. The presents review presents some of the diagnostic tools currently used for fungal plant pathogens and describe some novel applications.

MOLECULAR MARKER TECHNOLOGY (M.M.T): The science of plant genetics traces back to Mendel's classifical studies on garden peas. Since then researchers have been identifying, sorting and mapping single gene markers in many species of higher plants. In early part of twentieth century, scientist discovered that Mendelian factors controlling inheritance which we now call genes were organized in linear fashion on chromosomes. Actually the first genetic map was produced by Sturtevant with segregation data derived from studies on drosophila. The markers on first genetic map were phenotypic traits scored by visual observation of morphological characterstics of the flies. Generally a marker must be polymorphic that is it must exist in different forms so that the chromosome carrying the mutant gene can be distinguished from the chromosome with normal gene by the form of marker it also carries. This polymorphism in the marker can be detected at three levels i.e phenotypic level (by Morphological markers), difference in proteins (by Biochemical markers) or difference in the nucleotide sequence of DNA (by Molecular markers)

MORPHOLOGICAL MARKERS:

Morphological markers generally match to the qualitative traits that can be scored visually. They have been found in nature or as the result of

mutagenesis experiments. These markers are highly influenced by environmental factors.

BIOCHEMICAL MARKERS (e.g. Isozymes): Biochemical markers are proteins produced by gene expression. These proteins can be isolated and identified by electrophoresis and staining. Isozyme the different molecular form of same enzyme, that catalyzes the same reaction, are proteins. They are revealed on electrophoregram through a coloured reaction associated with enzymatic activity. They are the products of various alleles of one or several genes .The first molecular markers that initially were used extensively to study systematic of plants, animals and insects were isozyme (or isoenzyme) [5]. Isozyme analysis was initially utilized to study the taxonomy of plant pathogenic fungi [6,7]**.** The method involves extraction of crude proteins and separation by electrophoresis on starch, non-denaturing poly acrylamide gels (PAGE) or isoelectric focusing. Isozyme zones are visualized after supplying the appropriate substrate necessary for the specific activity of each enzyme. The resulting Isozyme banding patterns (zymograms) are used to infer genetic relationships based on interpretations of banding polymorphisms assuming that isozyme zones correspond to equivalent loci .Analysis of isozyme variation found application in distinguishing fungi bearing overlapping morphological or cultural characters, such as species of the genus *Phytophthora*. By comparing 18 Isozyme loci, three species of *Phytophthora* (*P. cambivora*, *P. cinnamomi*, *P. cactorum*) could be separated and subsequently the systematic of twelve papillate *Phytophthora* species were re-evaluated [8]**.**

Because isozymes are post transcriptional markers, their expression is influenced by environmental changes leading to polymorphisms that might not reflect real differences at the molecular level. This was the basic reason that application of isozyme in studying fungal plant pathogen variation was rather limited although in general they may provide satisfactory levels of polymorphic loci.

D.N.A BASED MOLECULAR MARKERS: Molecular markers are also called DNA markers**.** It is a DNA sequence that is readily detected and whose inheritance can be easily monitored. The use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit applied purpose. A molecular marker has some desirable properties like

- It must be polymorphic
- Co-dominant inheritance
- Should be evenly and frequently distributed
- Should be reproducible
- Should be easy fast and cheap to detect.

The development of protocols for extracting DNA from various sources i.e. pure fungal cultures, infected plant tissues, infested soil or air samples, spun-off the possibilities of developing molecular diagnostics initially through nucleic acids hybridization. DNA probes were among the first molecular techniques applied in the detection, identification and phylogenetic analysis of fungal pathogen [9, 10].DNA isolation is crucial step in DNA based molecular markers study. Generally fungal biomass is collected through liquid culture and crushed with help of liquid nitrogen to isolate genomic DNA. but For unculturable organisms (such as powdery or downy mildew pathogens), DNA is isolated from spores directly collected from infected plant tissues If DNA of soil-borne pathogens is extracted directly from soil samples, attention must be drawn to co-extraction of humic acids or other inhibitory substances. No single marker meet all these requirements so there is need to develop a wide range of molecular markers.

CLASSIFICATION OF MOLECULAR MARKERS (First way):

1. Non PCR based molecular markers: e.g. R.F.L.P and R.L. G .S
2. PCR based molecular markers: R.A.P.D & modifications, S.S.R, A.F.L.P & modifications, I.S.S.R, E.S.T & S.N.P etc.

Table No. 01:

Class	Marker system	Abbreviation	Remarks	References
First Generation Molecular Markers	Restriction Fragment Length Polymorphism	RFLP	Based on restriction digestion and hybridization with probe	[11]
	Sequence Tagged Sites	STS	RFLP probes sequenced and converted in to PCR based STS markers	[12]
	Random Amplified Polymorphic DNA	RAPD	Random primers for PCR amplification	[13]
	Sequence Characterized Amplified Regions	SCAR	RAPD marker termini sequenced for designing longer primer	[14]
	Arbitrary Primed PCR	AP-PCR	RAPD primers of 10-15 bases in length for discrete amplification	[13]
	DNA Amplification Fingerprinting	DAF	Single random primer of 5 bases short length	[15]
Second Generation Molecular Markers	Simple Sequence Length Polymorphism	SSLP	Based on tandem repeat flanking sequence	[16]
	Variable Number of Tandem Repeats	VNTRs	Based on tandem repeat sequence hybridization by probe	[17]
	Random Amplified Micro satellite Polymorphism	RAMPO	Random primers used for amplification and then hybridized with micro satellite oligonucleotides probe	[18]
	Cleaved Amplified Polymorphic Products	CAPs	PCR amplified products digested by restriction enzymes	[19]
	Inter Simple Sequence Repeat	ISSR	Single primer based on SSR motif	[20]
	Amplified Fragment Length Polymorphism	AFLP	Detection of genomic restriction fragment by PCR amplification	[21]
	Allele Specific Associated Primers	ASAP	Specific allele sequenced and primers designed for amplification	[22]
Third Generation Molecular Markers	Expressed Sequence Tag markers	ESTs	Sequencing of random DNA clones	[23]
	Single Nucleotide Polymorphism	SNP	Non-gel based marker system and DNA sequence differs by single base	[24]
	Miniature Inverted Repeat Transposable Elements	MITE	Non autonomous transposable elements with strong target site preference	[25]

CLASSIFICATION OF MOLECULAR MARKERS (Second way):

VARIOUS TYPES OF MOLECULAR MARKERS USED IN FUNAGL DIAGNOSIS AND MANAGEMENT:

1. <u>Restriction Fragment length Polymorphism (RFLP):</u> It is non PCR based marker. It was first discovered by Grodziker for mutant strains of Adeno virus. Botstein et al used this for construction of genetic map first time. It was the first technology that enabled the detection of polymorphism at DNA sequence level. RFLPs have found wide application in the detection and characterization of fungi [26, 27, 28, 29]**.** In this method DNA is digested with restriction enzymes, which cuts the DNA at specific sequences, electrophoresed, blotted on a membrane and probed with labeled clone. Polymorphism in the hybridization pattern is revealed and attributed to sequence difference between individual pathogen.

Advantages:

- It is rapid as compared to conventional linkage maps
- Mapping does not necessarily depend on gene function
- Ideally every cistron can be mapped

Disadvantage:

- Require highly pure & sufficient DNA.
- Time consuming &costly method
- Require technically qualified persons

2. <u>Random Amplified Polymorphic DNA (RAPD):</u> This marker system was developed by Welsh & McClelland in 1991. RAPD is performed in conditions resembling those of PCR using genomic DNA from the target fungal pathogen and a single short oligonucleotide (generally 10 mer). The DNA amplification product is generated from a region that is flanked by a part of 10 b.p priming site

in the appropriate orientation. A particular fragment generated for one individual but not for other represents DNA polymorphism and can be used as a genetic marker.

Advantages:

- Need small amount of DNA(15-25 ng)
- Involves non radioactive assays
- Require simple experimental set up.
- Does not involve hybridization step
- Provides rapid screening for DNA sequence based polymorphism at many loci.

Disadvantages:

- Sensitive to change PCR conditions
- Polymorphism is inherited as dominant recessive character. This causes a loss of information relative to markers which show co dominance.

Random amplified polymorphic DNA (RAPDs) analysis has attracted a lot of attention after its advent during the 90's [30] **.** Manulis , *et. al., (1994),* applied RAPDs to the carnation wilt fungal pathogen *Fusarium oxysporum* f. sp. *dianthi* and they were able to identify specific banding patterns that were subsequently used as probes to distinguish between races of the pathogen [31]. In another study, genetic relationships could be inferred among the wheat bunt fungi using RAPD markers [32].Globally RAPD markers are also reported to be useful in diagnostic studies of pathogens [33, 34, 35] **.**

3. Amplified Fragment length Polymorphism (AFLP): This technique is a combination of RFLP & RAPD. This involves major steps as following:

- Cutting of DNA with Restriction Enzymes
- Double stranded oligonucleotides adapters are ligated to the ends of DNA fragments.
- Selective amplification of sets of restriction fragments is usually carried out with P^{32} labeled primers designed according to sequence of adaptors plus 1-3 additional nucleotides.
- Gel electrophoresis and analysis of amplified fragments.

Advantages:

- This marker system is technically more sensitive than previous marker systems.
- This system also shows high reproducibility
- This discriminates heterozygous from homozygous if we use a gel scanner.
- Can be used for population mapping.

Disadvantages:

- This marker system is comparatively coastally than previous one.
- Also require more amount of DNA per reaction
- Technically skilled persons are also required to perform such activities.

This method allows specific co amplification of a high no. of restriction fragment. Nearly 50-100 fragments are amplified and detected on denaturing poly acrylamide gels. This is highly sensitive method for detecting polymorphism throughout the genome and it is becoming increasingly popular. This marker system generate a large no. of restriction fragment bands thereby facilitating the detection of polymorphism. Skrede *et. al.,2012, Chen et. al.,2012* have reported AFLP markers. [36, 37]

4.Allele Specific Associated primers (ASAP): In this specific allele either in homozygous or heterozygous state is sequenced and specific primers are designated for amplification of DNA template to generate a single fragment at stringent annealing temperature. In this marker system higher primer concentration is used and amplified product is analyzed on poly acrylamide gel followed by auto radiographic detection. Billard *et. al.,* 2012 have identified fungal pathogen using ASAP marker system [38].

5.Simple Sequence Repeats (SSR): The term micro satellite was coined by Litt & Lutty in 1989. This is also known as Micro satellite marker system. These are present in genome of all eukaryotes. These are tandemely repeats of mono, di, tri, tetra and penta nucleotides with different length of motif (A,T, AT,AGG etc.). This SSR length polymorphism at individual loci is detected by PCR using locus specific flanking region primers where the sequence is known. SSR are ideal DNA markers

for genetic mapping and population study of pathogens, because of their abundance. Janny et. al., 2006 **,** have studied ectomycorrhizal fungi using SSR markers [39]. *Chen et. al.,2012* have also reported SSR marker in their study [37].

6.Inter Simple Sequence Repeats (ISSR): This marker system is based on SSR marker system. In this marker system primers based on micro satellite are utilized to amplify inter SSR DNA sequences. Huang *et. al.,* 2012 have identified plant pathogenic fungi isolated from Atractylodes sp. using Internal Transcribed Spaces (ITS) regions [40].

7.Expressed Sequence Tagged Marker (EST: Term introduced by Adams et al in 1991. Such markers are obtained by partial sequencing of random c-DNA clones. Such markers are obtained by partial sequencing of random c-DNA clones and are useful in cloning specific genes of interest and synteny mapping. This is also used in full genome sequencing and mapping Programmes and isolation of genes. Dracatos *et. al.,* 2006, Zhong, *et. al.,* 2009 have studied EST markers [41, 42].

8. **Single Nucleotide Polymorphism (SNP):** This marker system belongs to third generation of marker systems. The frequency of occurrence of SNP in a genome is generally one SNP in every 100-3000 bp. The SNPs can be detected by two ways one is gel based assays and other is non gel based assays. Xu *et. al.,* 2007, Sha *et. al.,* 2008 have reported SNP markers [43, 44].

9. **Cleaved Amplified polymorphic Sequences (CAPS):** Konieczny and Ashubel (1993) first adapted the CAPS procedure for genetic mapping by developing a set of CAPS markers for use with *Arabidopsis.* In this marker system PCR amplified DNA fragments are digested with a restriction enzyme rendering restriction site polymorphism. The digested restriction fragments are subjected to gel electrophoresis (agarose) followed by ethidium bromide staining based visualization of bands. Mc Cleary *et. al.,* 2009 have reported CAPS markers in their study [45].

Advantages:

- Small quantity of DNA is required for analysis.
- The size of cleaved and uncleaved amplification products can be adjusted arbitrarily by the appropriate placement of PCR primers.
- Can be readily assayed using standard gel electrophoresis (agarose).
- Procedure is technically simple but the results are robust because an amplification product is always obtained.

10. Sequence Characterized amplified Regions (SCARs): A SCAR is a genomic DNA segment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucleotide primer.SCARs are inherited in a co dominant fashion in contrast to RAPDs which are inherited in a dominant manner. SCAR marker system detects only one locus and the use of longer oligonucleotide primers allows a more reproducible assay than the one obtained with the short primers used for RAPD analysis. Hermosa *et. al.,* 2001 and Luongo *et. al.,* 2012 have reported SCAR markers for fungal study [46, 47].

CONCLUSION: Precise identification and diagnosis of plant pathogens during early stages of infection can help a lot in better management of the diseases. Soil borne pathogen being most difficult to identify during initial stages of infection can be successfully detected by molecular methods. Currently more sensitive methods like Real Time PCR and Microarrays are being used. Real Time PCR has emerged as one of the promising and very sensitive method of plant pathogen detection. Generally pathogen variability is studied by using differential host reactions, culture characteristics, morphological markers and biochemical tests. These markers distinguish pathogens on the basis of their physiological characters. i.e pathogenicity and growth behaviors are and are highly influenced by

the host age, inoculums quality and environmental conditions. Use of different hosts for pathotyping of plant pathogens is a time consuming and laborious process. Moreover, differential hosts are available only for a few host pathogen systems thus limiting the analysis of pathogen variability. In such cases use of molecular markers has been advocated for characterization for genetic variability in phyto pathogenic microbes. In many ways plant pathologists are faced with more difficult diagnostic problems than are in their counter parts in human and veterinary medicine. Plant pathologists deal with many crop species and hundreds of pathogens ranging from viriods through parasitic plants and have access to fewer products to assist in diagnosis. Agriculture lacks the extensive and highly developed infrastructure as medical field for disease diagnosis which includes a wide array of practioners and supporting laboratories where sophisticated tests can be run properly. Only a handful of companies worldwide have developed products for plant disease diagnosis. However many new techniques have been bring forward during the last two decades and becoming available for application in practical disease diagnosis.

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