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REVIEW ARTICLE

MOLECULAR DIAGNOSTIC APPROACHES FOR PLANT PATHOGENS DETECTION AND DISEASE MANAGEMENT

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ABSTRACT

Every year huge crop losses occur due to different pathogens and disease. The traditional method of pathogen detection, which is still in practice, through visual examination is not always precise. Early detection of plant pathogens prior to severe infection is very crucial which is possible through molecular diagnostic approaches and nucleic acid-based tests. As the genetic materials are the ultimate information storage sites in living organism, their exploration through the use of nanotechnology provides the path forward for the three Ds of genomic analysis of pathogens: Diversity, Detection, and Disease diagnosis. Molecular detection method is not only precise and accurate but also faster and easier approach. Pathogen detection through PCR based tests, microarray technology, multiplexing, gene sequencing, genetic markers play a pivotal role in timely detection of causatives and take proper action to prevent the pandemic in plant population and safeguard against possible risks and famine. It is of utmost importance to prioritize such methods to detect plant pathogens, to increase our understanding of ecology and epidemiology and to prevent the spread of inoculum prior to disease spread. The application of novel diagnostic methods to inoculum detection will guide towards better understanding of the temporal and spatial dynamics of epidemic development, and open up new opportunities for disease forecasting and management.

KEYWORDS

Pathogens, nucleic acid, genetic-marker, multiplexing, pandemic.

1. INTRODUCTION

Various agricultural crops are being threatened by a wide variety of biotic stresses every year leading to decrease in production and reduction in quality of yield. About 42% of the world's total agricultural crop is destroyed yearly by diseases and pests (Alvarez, 2004). Farmers often face one or more than one pest or disease and new pesticide-resistant pathogenic strains attacking the same crop. In plant science also, molecular diagnostic technologies can be adopted to identify the microorganisms, pathogens prevalent in plants as like that in medical sectors. With the automation of molecular data acquisition, we can monitor the pathogens and beneficial microorganisms prevalent in soil, air, and water.

Mostly plant pathogens are identified through visual examination in a tradition way. This is often possible only after major damage has already been done to the crop. To save plants from irreparable damage by pathogens, we need to be able to identify an infection even before it becomes visible. Pathogens produce proteins and toxins to facilitate their infection, before disease symptoms appear. Many plant pathogens have similar morphological characters which makes it complex for their identification and are time consuming and requires extensive knowledge in taxonomy. Molecular detection techniques can generate accurate results rapidly enough to be useful for disease management. We need a synthesis of population genetics and epidemiology, resulting in a population biology which would increase the benefits of studying genetic variation in populations of plant pathogens. It is necessary to conduct extensive research in food and economic crops as they are major source for feeding this rapidly increasing population. Any pandemic or epidemic when occurred in those crops in course of time, will affect large world population. Many would go under famine. Late Blight of Potato that caused a famine in Ireland, in 1846, and the Downy Mildew of Grapes that almost caused economic ruin for the wine industry in the Mediterranean, beginning in 1865 are some cases of the devastating losses caused by pathogens.

Technology is an essential component of any scientific endeavor. Computer modeling could be applied to systems analysis and improve disease management in plants. Technological advances in the late 1980s and 1990s led to the development of easily accessible genetic markers such as Restriction Fragment Length Polymorphism (RFLPs), Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphism (AFLPs), microsatellite (Milgroom, 2001). These molecules play vital role in the development of plant diagnostic kits. These kits are designed to detect plant diseases early, either by identifying the presence of the pathogen in the plant or the proteins produced by either the pathogen or the plant during infection. Using Real Time PCR, it is possible not only to detect the presence or absence of the target pathogen, but also possible to quantify the number of the pathogen in the sample. The polymerase chain reaction (PCR), the exponential amplification of a target DNA strand catalyzed by a thermo stable DNA polymerase has become the foundation of Nucleic Acid-based pathogen detection (Vincelli and

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Tisserat, 2008). Monoclonal antibodies, enzyme-linked immunosorbent assay and DNA-based technologies have been the basis for molecular detection in modern plant pathology (Lévesque, 2001). Enumerating the pathogen upon detection is crucial to estimate the potential risks with respect to diseases development and provide a useful basis for diseases management decisions.

Genomics and biosystematics research are generating fast-growing databases that can be used to design molecular assays for the identification of a large number of pathogens and beneficial organisms. The rapid increase in the popularity of this area has been made possible by the easy accessibility of genetic markers, as a result in advances in technology of molecular biology. Scientists in agriculture genomic research are sequencing all the genes of only a few selected economically important crop or pathogen species. Genomics research is providing the data needed to generate microarrays containing all of the genes of a particular species which are used to establish which genes are overexpressed or silenced following a given treatment (Kn and Raju, 2012). A comprehensive taxonomic molecular database is needed to better understand variation in gene sequences by filling in some gaps between the few sequenced genomes available.

2. Approaches Towards Plant Pathogen Detection

Automated DNA sequencing and microarray studies for gene expression are generating data faster than they can be fully analyzed. Without proper identification of the disease and the disease-causing agent, disease control measures can be a waste of time and money and can lead to further plant losses. Many plant pathogens differ from their closest relatives by only a few bases on different genes. One of the most active post genomic research areas is the characterization and detection of Single-Nucleotide Polymorphisms (SNPs). These polymorphisms are responsible for differences in alleles and reflect how similar genes can generate different phenotypes. These new "genome-enabled" technologies are pushing DNA detection to its ultimate limit, the single base pair difference.

To ensure that one has the DNA of the pathogen in the sample aliquot to be used for the molecular detection assay, it will be necessary in some situations to concentrate the pathogen propagules or its DNA beforehand. Methods such as sieving to concentrate spores of a certain size range from the soil might still have a use. Immunocapture of the pathogen propagules or DNA trapping at the bottom of 96-well plates or on the inside surface of capillaries through which a significant sample volume is passed are likely to become commonly used methods prior to the detection assays (Kn and Raju, 2012). Polymerase Chain Reaction (PCR) is an enzyme-driven process for amplifying short regions of DNA in vitro. PCR is a highly sensitive technology. The method relies on knowing at least partial sequences of the target DNA earlier and using them to design oligonucleotide primers that hybridize specifically to the target sequences. Although more costly, real-time PCR offers several advantages over conventional PCR, such as the provision of data in real-time, a much greater quantitation range, greater sensitivity, reduced risk of sample contamination during PCR setup due to laboratory contamination with amplicon, and a greater amenity to multiplexing (Vincelli and Tisserat, 2008).

A relatively new molecular tool that can increase detection sensitivity is Multiple Displacement Amplification (MDA) (Andres et al., 2006). MDA exponentially amplifies DNA using random hexamer primers, resulting in more or less complete genome amplification for essentially all DNA present in the sample. By performing MDA prior to PCR on a sample containing trace amounts of pathogen genomic DNA, one can exponentially increase its quantity, potentially increasing the likelihood of pathogen detection. New variants of PCR, such as simple or multiplex nested PCR in a single closed tube, cooperative- PCR and real-time monitoring of amplicon or quantitative PCR, allow high sensitivity in the detection of one or several pathogens in a single assay (Kn and Raju, 2012). The latest development in the analysis of nucleic acids is micro-array technology, but it requires generic DNA/RNA extraction and pre-amplification methods to increase detection sensitivity (Vincelli and Tisserat, 2008).

However, its sensitivity is greatly affected by the presence of inhibitors which prevent or reduce amplification (Yang and Rothman, 2004). Hence, stringent conditions are necessary in conducting the assay and proper negative controls must be included in the test. Sensitivity and specificity problems associated with conventional PCR and RT-PCR can be reduced by using nested PCR-based methods, based on two consecutive rounds of amplification. PCR concept based on the simultaneous action of four or three primers, has also been developed. This technique named Co-operational amplification (Co-PCR) can be performed easily in a simple reaction increasing the sensitivity level and using ten times less reagent than in conventional PCR (Kn and Raju, 2012). The sensitivity observed is at least 100 times higher than that achieved with RT-PCR and similar to nested RT-PCR. The simultaneous detection of two or more DNA or/and RNA targets can be afforded by duplex or multiplex PCR in a single reaction with several specific primers included in the PCR cocktail. Multiplex PCR is very useful in plant pathology because different bacteria or viruses frequently infect a single crop or host (Kn and Raju, 2012).

It is the most current molecular diagnostic assays used in plant pathology. Because crops can be infected by numerous pathogens which are often present in plants as complexes, it is desirable to develop assays that can detect multiple pathogens simultaneously. Multiplex PCR-based strategies involve the use of multiple primer sets in the same reaction. Multiplex nested RT-PCR method in a single tube, combines the advantages of the multiplex PCR with the sensitivity and reliability of the nested PCR, saving time and reagent costs because two reactions are sequentially performed using a single reaction cocktail. Competitive fluorescence PCR (CF–PCR) is used to simultaneously differentiate between virus strains and multiple virus infections. Extension occurs only where the 3' nucleotide is complementary. Only primers that generate amplicons fluoresce and the wavelength emitted identifies the primers that have been extended (Cuppels et al., 2006). Microarrays are generally composed of thousands of specific probes spotted onto a solid surface usually nylon or glass. Each probe is complementary to a specific DNA sequence provides a signal that can be detected and analyzed.

The DNA Microarray technology, originally designed to study gene expression and generate single nucleotide polymorphism (SNP) profiles, is currently a new and emerging pathogen diagnostic technology, which in theory, offers a platform for unlimited multiplexing capability. As an example of the power of the technique, developed a microarray that simultaneously tests for the presence of most known *Pythium* species (Tambong et al., 2006). Having the capability to use arrays to simultaneously test diseased plant tissue for dozens or hundreds of pathogens would be most useful in cases when the diagnostician may not know which species-specific detection assay to apply. Oligonucleotide arrays offer greatly expanded multiplexing capability compared to conventional PCR, real-time PCR. Because the sample DNA is amplified by PCR, sensitivity of arrays is typically high, and the number of pathogen-specific tests that can be run on the array is essentially unlimited. PCR amplicons can often be sequenced relatively inexpensively and rapidly, given the availability of automated sequencers in many laboratories.

Genetic databases allow rapid comparison of one's sample sequence to extensive and growing libraries of sequences. Routine sequencing is likely to play an increasingly important role in species identification, through the application of DNA bar-coding. The advances in research that will result from the sequencing of many plant pathogen genomes, especially now in the era of proteomics, represent a new source of information for the future development of sensitive and specific detection techniques for these microorganisms. Like genomics research, sophisticated bioinformatic analysis tools will be needed to map pathogen distribution and analyse the complex interspecific and population interactions that we will be able to monitor. Excellent data mining and analysis skills will be essential to make judicious use of these new technologies and to draw the proper conclusions from the large amount of data generated. A group researchers searched for the sources of primary inoculum of *Phytophthora infestans* that caused late blight foci and epidemics in potato fields in a region in The Netherlands (Zwankhuizen et al., 1998).

They combined a traditional epidemiological approach of studying the locations of disease foci and disease gradients in relation to potential sources with a population genetic approach of studying the spatial distribution of pathogen genotypes. By asking which pathogen genotypes (DNA fingerprints) were found in commercial fields and comparing them to genotypes found in potential inoculum sources, the researchers were able to infer possible inoculum sources with a high degree of confidence. A group researcher sampled isolates of *Alternaria alternata* from various citrus cultivars (Peever et al., 2000). By using population genetic analyses of RAPD markers, they showed that *Alternaria* was significantly genetically differentiated among different host cultivars. This result suggests that there is restricted gene flow of pathogens among cultivars despite their close geographical proximity.

3. Sensitivity and Specificity

Sensitivity and specificity are numeric measures of effectiveness of a detection system (Peruski and Peruski, 2003). Diagnostic specificity is

defined as a measure of the degree to which the method is affected by non-target components present in a sample, which may result in false positive responses. Diagnostic sensitivity is defined as a measure of the degree to detect the target pathogen in the sample, which may result in false negative responses (Malorny et al., 2003). Too low sensitivity often leads to false negatives. Thus, a high degree of diagnostic accuracy is characterized by the ability to detect, true and precisely the target microorganisms from a sample without interference from non-target components (Papp et al., 2003). Closely related microbial species often differ in a single (Single-Nucleotide Polymorphism) to a few bases in such genes. However, the high degree of specificity of nucleic acid-based detection techniques, achieved through the use of PCR primers, hybridization probes, or detector oligonucleotides, allows detecting such SNPs (Consolandi et al., 2001). Since closely related pathogens might have a different host range or display a completely different pathogenicity, this is an extremely important trait.

Obviously, the specificity of nucleic acid-based techniques is determined by the sequences that are targeted to enhance specificity. Strategy to select target sequences for detection of plant pathogens involves the screening of random parts of the genome to find diagnostic sequences. This can be achieved by several techniques, including random amplified polymorphic DNA and amplified fragment length polymorphism. Since the location of possible useful sequences in the genome is a prior unknown, there often are few sequence data available for comparison to other organisms in order to guarantee specificity. As a consequence, extensive screening is required to ensure specificity of the potential marker. Array hybridization technology offers the possibility to add a multiplex aspect to PCR-based detection. In theory, DNA arrays, originally designed to study gene expression or to generate SNP profiles, can be used to detect an unlimited number of different organisms in parallel (Lievens et al., 2005). The virtually unlimited screening capability of DNA arrays, coupled with PCR amplification, results in high levels of sensitivity, specificity, and throughput capacity (Lievens et al., 2005).

4. Opportunities

Traditional method of disease and pathogen detection involves visual examination which is often time consuming, not always sensitive and specific enough and precise. Such method requires skilled and experienced person in the field of plant disease and pathogenicity. Also, such traditional method is not suited for analysis of a large number of samples. Modern molecular diagnostic methods and nucleic acid-based detection of plant pathogens ensure accuracy in detection. PCR offers several advantages as organisms do not need to be cultured before their detection. This method is highly sensitive and precise. It enables a single target molecule to be detected in a complex mixture and it is also rapid and versatile. Biological assays are simple, requires minimal knowledge of the pathogen. Species like *Meloidogyne spp* which are difficult to identify by microscopy without considerable expertise can be easily diagnosed using molecular methods.

Similarly, such method is equally applicable for organisms that grow slowly or fail to sporulate in culture. Species that are morphologically similar to related non-pathogenic species can also be accurately identified. Molecular diagnostic method makes it quite easier for identification of life stages that are immature, such as nematode eggs or juveniles include *Phytophthora ramorum*, the cause of sudden oak death, Xanthomonas *citri* strains causing citrus canker and *Tilletia indica*, cause of Karnal bunt of wheat (Mavrodieva et al., 2004). Nucleic acid-based diagnosis is helpful for determining the geographic origin or genetic relationships among pathogen strains, without the requirement of personnel skilled in morphological identification of fungi (McCartney et al., 2003). Use of molecular biotechnology makes it easier and quicker for monitoring of pathogen biotypes that are particularly aggressive or that represent discrete races and for detecting fungicide resistance including quantification of fungicide resistance alleles in a pathogen (McCartney et al., 2003; Kim et al., 2003).

Nowadays, when using molecular techniques, comprehensive screening of samples is made possible because of recent developments in automated high throughput DNA extraction systems and because of the introduction of 96- to 384-well plate PCR systems. In addition, the development of DNA arrays for plant pathogen diagnosis has enabled screening of multiple pathogens in a single assay, eliminating the need of performing several singleplex assays. In general, a multiplex assay, like a DNA array-based test, is the most cost effective per sample because the use of a singleplex assay often requires multiple consecutive analyses to determine and confirm the cause of a disease, hence increasing the price per sample analyzed. Whether through marker assisted breeding, improved molecular diagnostics or the other methods discussed, more research and an enhanced understanding will result in the development of methods that can be applied in the future.

5. Cautions and Caveats

However, different requirements have to be met before new detection methods are implemented in practice. Economical and technical demands need to be duly considered for carrying out molecular diagnostic methods. It should be possible to perform the test with a minimum of taxonomical expertise and at a minimum of cost. Prior to the introduction of nucleic acid amplification methods, in particular PCR, nucleic acid-based diagnostics mainly involve the use of specific probes to report the presence of a certain organism (Fanelli et al., 2007). However, these methods often led to “false negatives” because of too low sensitivity. High sensitivity also causes one of the potential pitfalls of PCR technology: the slightest carry-over contamination can give rise to “false positive” results. The problem of false positives arising from dead pathogen cells is not unique to PCR.

For example, a false negative may result if the pathogen is only present as resistant resting structures from which DNA may not be readily extractable, such as oospores (Martin et al., 2000). False negatives may also occur as infected tissues age in the field, since pathogen populations may decline and secondary organisms may invade (Cuppels et al., 2006). Therefore, stringent conditions and controls are necessary, such as guarding the reagents and samples for accidental DNA contamination via aerosols, running negative controls simultaneously with the test samples, and having separate dedicated areas for pre and post-PCR handling (Andres et al., 2006). Mutations at the primer binding site; even a single-base mismatch may prevent DNA amplification (Singleton, 2000).

Similarly, collection of an appropriate sample is regarded as the basic step in the testing process. Since only 1 to 2 µl of a sample extract is typically used in a Nucleic Acid based test, great care must be exercised in assuring that the extract is prepared from carefully selected representative tissue samples most likely to be colonized by the pathogen of concern (Alvarez, 2004). As different pathogens often colonize different tissues and portions of plant organs, it is required to separately process and test several samples even if they are collected from the same organ. As the amplification of target DNA that occurs in PCR is exponential, the technique is extremely sensitive to contamination (Louws et al., 1999). Hence this problem must also be given due care. Organic and inorganic compounds present in host tissues, components of microbial cells, and other materials can all inhibit PCR (Louws et al., 1999). Practices for mitigating PCR inhibition are varied and include minimizing host tissue pieces used in DNA extraction, sample dilution 10 to 1,000-fold, concentration of pathogen cells by centrifugation, addition of inhibitor-binding agents (Lievens and Thomma, 2005).

6. Conclusion

The lowest risk pathogens are those with strict asexual reproduction, low potential for gene flow, small effective population sizes, and low mutation rates. Early detection of pathogens, e.g., before crops are infected or symptoms have developed, is essential to prevent diseases, spread of the inoculum, and economic losses. Since many pathogens are difficult to identify using morphological criteria, these techniques often lead to incomplete or even wrong diagnoses. Therefore, institutions that provide diagnostic services should intensively search for generic diagnostic tools that can be executed relatively easily and interpreted by technicians with a general education in molecular biology. New molecular detection methods need to be accurate, sensitive, reproducible, and cost effective. However, molecular approaches are still not being used routinely in epidemiology, plant breeding, or disease management. While nucleic acid-based assays provide an excellent opportunity for rapid and precise detection, currently their success largely depends on well-equipped laboratory facilities. It is possible to develop rational and integrated control programs that maximize protection of plants from disease and consumers from harmful toxins and chemical residues. Thus, it must be the prime focus of the plant pathologist to explore the molecular diagnostic tools to early detect the pathogens and disease and protect the crops, plants and the whole agriculture sector.

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