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Novel Genomic Approaches for Detection and Identification of Plant Pathogens

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Genomic approaches can be used to dramatically for shortening the response times to outbreaks and inform disease management in novel ways. However, the use of approaches requires expertise in working with big, complex data sets and an understanding of their pitfalls and limitations to infer well-supported conclusions. Evolutionary framework to guide the use of genomic approaches in epidemiology and diagnostics of plant pathogens.
Novel genomic approaches including multiplex PCR, real time PCR, Nested PCR, Loop mediated isothermal amplification (LAMP), Lateral flow assay (LAF), nano particle biosensor specific for phytopathogenic fungi (Giachero *et al.*, 2022).

Polymerase chain reaction

Polymerase Chain Reaction (PCR) is a technique used in molecular biology to create several copies of a certain DNA segment. This technique was developed in 1983 by Kary Mullis, an American biochemist. PCR has made it possible to generate millions of copies of a small segment of DNA. This tool is commonly used in the molecular biology and biotechnology labs.

Principle of PCR

The PCR technique is based on the enzymatic replication of DNA. In PCR, a short segment of DNA is amplified using primer mediated enzymes. DNA polymerase synthesises new strands of DNA complementary to the template DNA. The DNA polymerase can add a nucleotide to the pre-existing 3'-OH group only. Therefore, a primer is required. Thus, more nucleotides are added to the 3' prime end of the DNA polymerase.

Multiplex polymerase chain reaction (Multiplex PCR)

Multiplex PCR refers to the use of polymerase chain reaction to amplify several different DNA sequences simultaneously. This process amplifies DNA in samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler. The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR.
Multiplex-PCR was first described in 1988 as a method to detect deletions in the dystrophin gene. It has also been used with the steroid sulfatase gene. In 2008, multiplex-PCR was used for analysis of microsatellites and SNPs. In 2020, RT-PCR multiplex assays were designed that combined multiple gene targets from the Center for Diseases and Control in a single reaction to increase molecular testing accessibility and throughput for Plant disease diagnostics.
Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting

multiple sequences at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. Alternatively, if amplicon sizes overlap, the different amplicons may be differentiated and visualised using primers that have been dyed with different colour fluorescent dyes. Commercial multiplexing kits for PCR are available and used by many forensic laboratories to amplify degraded DNA samples.

Fig: Comparison between PCR and Multiplex PCR

Real time PCR

Fig: Real time PCR

DNA amplification is detected in real-time with the help of a fluorescent reporter. The signal strength of the fluorescent reporter is directly proportional to the number of amplified DNA molecules.

Real-time detection of PCR products is enabled by the inclusion of a fluorescent reporter molecule in each reaction well that yields increased fluorescence with an increasing amount of product DNA. The fluorescence chemistries employed for this purpose include DNA-binding dyes and fluorescently labelled sequence-specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence signal as amplification occurs. The measured fluorescence is proportional to the total amount of amplicon; the change in fluorescence over time is used to calculate the amount of amplicon produced in each cycle.

The main advantage of real-time PCR over PCR is that real-time PCR allows you to determine the initial number of copies of template DNA (the amplification target sequence) with accuracy and high sensitivity over a wide dynamic range. Real-time PCR results can either be qualitative (the presence or absence of a sequence) or quantitative (copy number). Quantitative real-time PCR is thus also known as qPCR analysis.

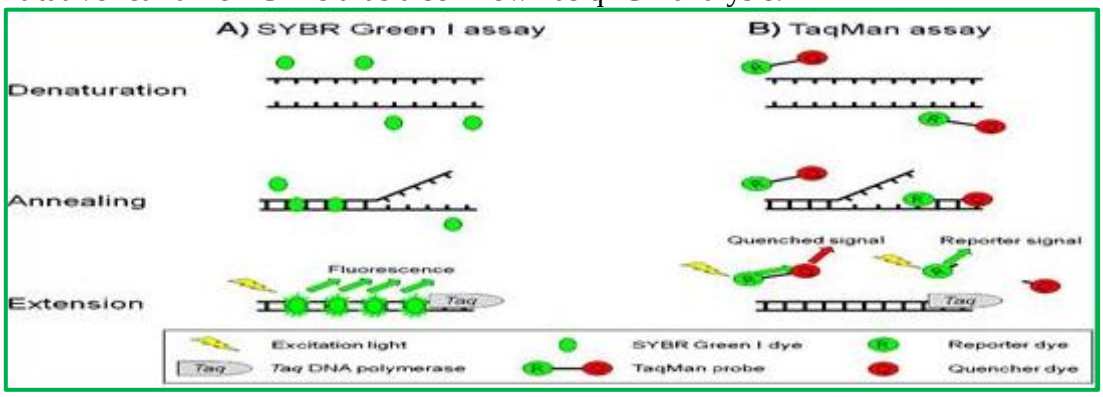

Fig: Types of dyes used in real time PCR

In contrast, PCR is at best semi-quantitative. Additionally, real-time qPCR data can be evaluated without gel electrophoresis, resulting in reduced bench time and increased throughput. Finally, because real-time qPCR reactions are run and data are evaluated in a unified, closed-tube qPCR system, opportunities for contamination are reduced and the need for post-amplification manipulation is eliminated in qPCR analysis.

Nested PCR

Nested polymerase chain reaction (**nested PCR**) is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites. Polymerase chain reaction itself is the process used to amplify DNA samples, via a temperature- mediated DNA polymerase. The products can be used for sequencing or analysis, and this process is a key part of many genetics research laboratories, along with uses in DNA fingerprinting for forensics and other human genetic cases.

Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product. This allows amplification for a low number of runs in the first round, limiting non-specific products. The second nested primer set should only amplify the intended product from the first round of amplification and not non-specific product. This allows running more total cycles while minimizing non-specific products. This is useful for rare templates or PCR with high background. The target DNA undergoes the first run of polymerase chain reaction with the first set of primers, shown in green. The selection of alternative and similar primer binding sites gives a selection of products, only one containing the intended sequence.

The product from the first reaction undergoes a second run with the second set of primers, shown in red. It is very unlikely that any of the unwanted PCR products contain binding sites for both the new primers, ensuring the product from the second PCR has little contamination from unwanted products.

Nested PCR is a variant of PCR that employs pair of two set of primers for two rounds of amplification to improve efficiency. Nesting also makes it easier to utilise generic primers to amplify several pathogens in the first round of PCR, then pathogen- specific primers in the second round. Nested PCR was used to detect *Phytophthora* species (*P. citrophthora* and *P. nicotianae*) from roots of citrus and infected soil, with non-specific primers (Ph2–ITS4) in the first round and specific primers (Pn5B–Pn6 for *P. nicotianae* and Pc2B–Pc7 for *P. citrophthora*) in the second round. One more species of *Phytophthora* i.e., *P. inundata* was detected with nested PCR using 10 specific primers based on ITS (Internal Transcribed Spacers) 1, 2 and 5.8S gene of DNA, HSP90 (Heat Shock Protein) gene, triosephosphate isomerase/glyceraldehyde- 3-phosphate dehydrogenase fusion protein (TIG), and 60S ribosomal protein L10 gene (RPL) (Brien *et al.*, 2009).

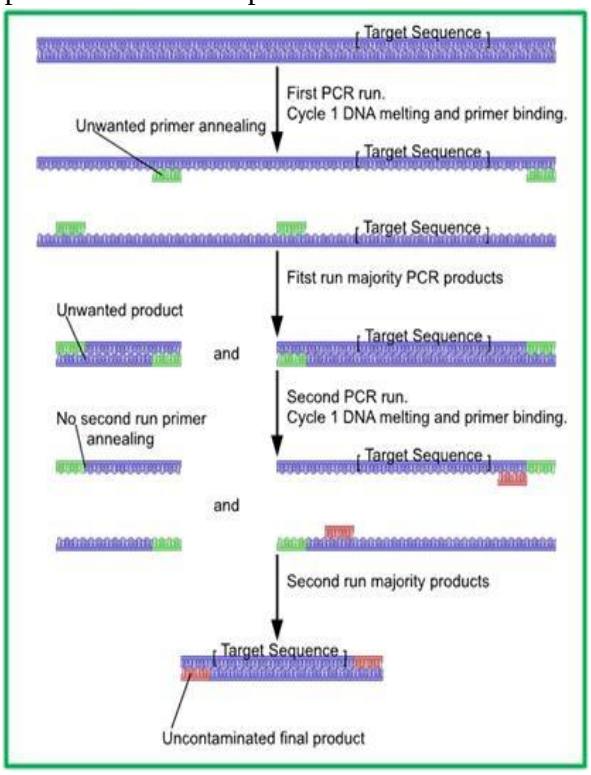

Fig: Nested PCR

Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (**LAMP**) is a single-tube technique for the amplification of DNA and a low-cost alternative to detect certain diseases. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) combines LAMP with a reverse transcription step to allow the detection of RNA. LAMP is an isothermal nucleic acid amplification technique. In contrast to the polymerase chain reaction (PCR) technology, in which the reaction is carried out with a series of alternating temperature steps or cycles, isothermal amplification is carried out at a constant temperature, and does not require a thermal cycler.

In LAMP, the target sequence is amplified at a constant temperature of 60- 65 ºC (140-149 ºF) using either two or three sets of primers and a polymerase with high strand displacement activity in addition to a replication activity. Typically, 4 different primers are used to amplify 6 distinct regions on the target gene, which increases specificity. An additional pair of "loop primers" can further accelerate the reaction. The amount of DNA produced in LAMP is considerably higher than PCR- based amplification. Primer design could be performed using several programs, such as PrimerExplorer, MorphoCatcher, and NEB LAMP Primer Design Tool. For screening of conservative and species-specific nucleotide polymorphisms in the most of diagnostics applications a combination of PrimerExplorer and MorphoCatcher is very useful, because allows to localize the species-specific nucleotides at 3'-ends of primers for enhancing the specificity of reaction (Gaikwad *et al.*, 2022).

The amplification product can be detected via photometry, measuring the turbidity caused by magnesium pyrophosphate precipitate in solution as a byproduct of amplification. This allows easy visualization by the naked eye or via simple photometric detection approaches for small volumes. The reaction can be followed in real-time either by measuring the turbidity or by fluorescence using intercalating dyes such as SYTO 9. Dyes, such as SYBR green, can be used to create a visible color change that can be seen with the naked eye without the need for expensive equipment, or for a response that can more accurately be measured by instrumentation. Dye molecules intercalate or directly label the DNA, and in turn can be correlated with the number of copies initially present. Hence, LAMP can also be quantitative.

In-tube detection of LAMP DNA amplification is possible using manganese loaded calcein which starts fluorescing upon complexation of manganese by pyrophosphate during in vitro DNA Synthesis. Another method for visual detection of the LAMP amplicons by the unaided eye was based on their ability to hybridize with complementary gold nanoparticle- bound (AuNP) single-stranded DNA (ssDNA) and thus prevent the normal red to purple-blue color change that would otherwise occur during salt-induced aggregation of the gold particles. So, a LAMP method combined with amplicon detection by AuNP can have advantages over other methods in terms of reduced assay time, amplicon confirmation by hybridization and use of simpler equipment (*i.e.*, no need for a thermocycler, electrophoresis equipment or a UV trans-illuminator) (Ristaino *et al.*, 2019).

LAMP assay has been developed to identify the citrus anthracnose pathogen *C. gloeosporioides*, citrus black spot pathogen *Phyllosticta citricarpa*, and *Pseudocercospora angolensis* causing Pseudocercospora leaf and fruit spot. The fungal pathogen *C. gloeosporioides* was detected in two hours with a DNA concentration of 10 fg/µl − 1 using LAMP technique Similarly, LAMP is proven to be faster (completed in less than 40 min) and

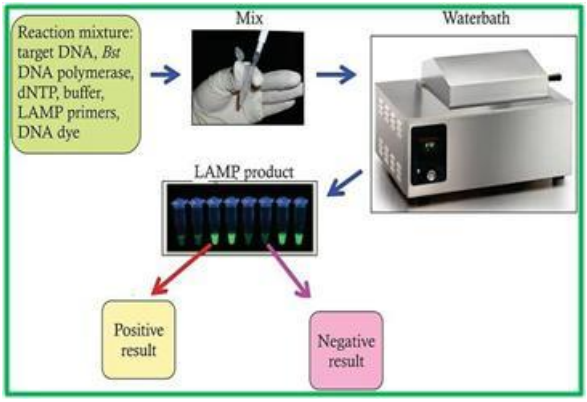

Fig: LAMP

much cheaper than other PCR-based methods for detecting *Phyllosticta citricarpa*. The LAMP detection for citrus black spot pathogen is sufficiently simple to be used during import inspections. LAMP was more sensitive (1000-fold) and faster (2.5 times) as compared to PCR for detecting *Pseudocercospora angolensis*.

Lateral Flow assay

A **lateral flow assay** (**LFD**), is an assay also known as a **lateral flow device (LFD)**, **lateral flow immunochromatographic assay**, or **rapid test**. It is a simple device intended to detect the presence of a target substance in a liquid sample without the need for specialized and costly equipment. LFTs are widely used in medical diagnostics in the home, at the point of care, and in the laboratory. For instance, the home pregnancy test is an LFT that detects a specific hormone. These tests are simple and economical and generally show results in around five to thirty minutes.Many lab- based applications increase the sensitivity of simple LFTs by employing additional dedicated equipment. Because the target substance is often a biological antigen, many lateral flow tests are rapid antigen tests (RAT or ART) (Berka *et al.*, 2020).

LFTs operate on the same principles of affinity chromatography as the enzyme- linked immunosorbent assays (ELISA). In essence, these tests run the liquid sample along the surface of a pad with reactive molecules that show a visual positive or negative result. The pads are based on a series of capillary beds, such as pieces of porous paper, microstructured polymer, or sintered polymer. Each of these pads has the capacity to transport fluid (e.g., urine, blood, saliva) spontaneously.

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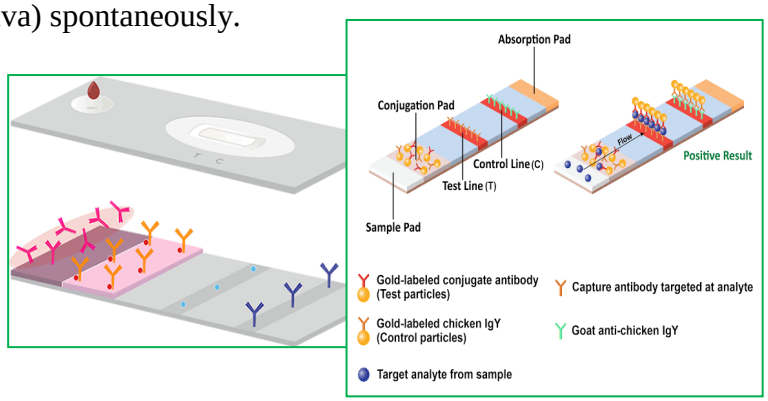

Fig: LAF assay

The sample pad acts as a sponge and holds an excess of sample fluid. Once soaked, the fluid flows to the second conjugate pad in which the manufacturer has stored freeze dried bio-active particles called conjugates (see below) in a salt–sugar matrix. The conjugate pad contains all the reagents required for an optimized chemical reaction between the target molecule (e.g., an antigen) and its chemical partner (e.g., antibody) that has been immobilized on the particle's surface. This marks target particles as they pass through the pad and continue across to the test and control lines. The test line shows a signal, often a color as in pregnancy tests. The control line contains affinity ligands which show whether the sample has flowed through and the bio-molecules in the conjugate pad are active. After passing these reaction zones, the fluid enters the final porous material, the wick, that simply acts as a waste container (Coomber *et al.*, 2023).

Conclusion

The molecular techniques compiled are precise, effective, lab-based and necessitate the use of sophisticated tools. On the other hand, knowledge in biotechnology, bioinformatics, and mycology are essential to avoid misconception of the results of molecular studies. Molecular

approaches to plant fungal disease diagnosis should become point-of-care testing by combining molecular techniques with other new technology breakthroughs. However, scientists/ researchers have hurdles in developing viable techniques for the molecular diagnosis of agricultural diseases. Furthermore, bioinformatics databases are utilised to store and retrieve nucleotide sequences of plant pathogen, all of which aid in the diagnosis and delimitation of molecular tools. Fungal diseases in plant are spreading rapidly and posing a threat to the global economy. As a result, it is vital to promptly and properly detect and diagnose phytopathogenic fungus.

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