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Alternative Molecular Approaches to PCR for Plant Pathogen Detection and Identification

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ABSTRACT: Plant diseases, caused by a wide range of pathogens including fungi, bacteria, viruses, nematodes, and protists, continue to pose significant threats to global agriculture by reducing crop yields and impacting food security. Human activities such as large-scale monoculture farming and international trade have accelerated the spread and emergence of plant pathogens. Accurate and early detection is critical for managing these diseases effectively. This review presents an overview of traditional and advanced diagnostic methods used in plant pathology, including visual inspection, pathogen culturing, staining techniques, and various forms of microscopy. It further explores modern approaches such as serological assays (e.g., ELISA), nucleic acid-based methods (e.g., PCR, blotting techniques), DNA microarrays, and VOC analysis. The integration of these techniques enables rapid, sensitive, and specific detection, supporting timely intervention and better disease control strategies to safeguard crop health.

Keywords: Plant pathogens, diagnostic techniques, ELISA, PCR, microscopy, VOC analysis, hybridization, crop disease detection.

INTRODUCTION

Plant pathogenic microorganisms are responsible for significant yield reductions in many economically vital crops, leading to both financial and social challenges. Human-induced factors such as large-scale monoculture farming and global trade have accelerated the spread of plant diseases and facilitated the emergence of new ones. Therefore, early detection and accurate identification of pathogens are essential to reduce agricultural losses. Plants are vital to life on Earth—they provide food, raw materials, and oxygen. Our survival is closely linked to plant health. However, plants are also susceptible to various diseases. At present, plant diseases pose a serious threat to agriculture, with pathogens and pests accounting for up to 40% of yield losses in major crops each year (FAO, 2019; Savary *et al.*, 2019; Baldi and La Porta 2020). Given that the world's population is expected to reach 9.7 billion people by 2050 and that rising global food consumption is a direct result of this, the socioecological impact of plant diseases is equally as significant as its economic impact (FAO, 2017). Given that agriculture now occupies 50% of the available land, the ideal technique appears to be to boost the yields that can be obtained; this tactic is known as agricultural intensification (McDonald and Stukenbrock 2016; Baldi and La Porta 2020). These diseases are caused by a wide array of pathogens, including fungi, bacteria, viruses, nematodes, and protists. When a plant disease outbreak occurs, it becomes crucial to determine the specific pathogen responsible, as proper identification is key to containing its spread. A range of diagnostic tools exists for this purpose, such as serological tests (e.g., gel diffusion, precipitation tests, ELISA) and non-serological methods like PCR. Serological approaches are particularly useful due to their high sensitivity in detecting and quantifying pathogens. Plant pathologists utilize both field-based and laboratory-based diagnostic procedures to pinpoint the cause of a plant disease. Among these, serological methods play an essential role in the detection and identification of plant viruses. Conventional virus diagnosis involves the use of indicator plants, host range testing, symptom analysis, characterization of virus morphology (such as particle size and shape), and understanding of vector relationships. While individual diagnostic assays may offer insight into virus identity, a combination of methods—ideally those that are accurate, specific, cost-effective, and sensitive—is often necessary for reliable diagnosis. Advancements in molecular biology, immunology, and biochemistry have enabled the development of modern techniques that are faster, more precise, and less labor-intensive. In the last

30 years, two major innovations have revolutionized molecular plant pathogen detection: the development of antibody-based diagnostics (particularly monoclonal antibodies) and enzyme-linked immunosorbent assays (ELISA). Currently, numerous immunodiagnostic and molecular diagnostic methods are employed in plant virology, generally categorized into two types: protein-based techniques—such as precipitation or agglutination tests and ELISA—and nucleic acid-based techniques, which include PCR and its variants.

Rank	Viruses	Bacteria	Fungi	Oomycetes
1	Tobacco mosaic virus (TMV)	*Pseudomonas syringae* pathovars	*Magnaporthe oryzae*	*Phytophthora infestans*
2	Tomato spotted wilt virus (TSWV)	*Ralstonia solanacearum*	*Botrytis cinerea*	*Hyaloperonospora arabidopsidis*
3	Tomato yellow leaf curl virus (TYLCV)	*Agrobacterium tumefaciens*	*Puccinia* spp.	*Phytophthora ramorum*
4	Cucumber mosaic virus (CMV)	*Xanthomonas oryzae* pv. *oryzae*	*Fusarium graminearum*	*Phytophthora sojae*
5	Potato virus Y (PVY)	*Xanthomonas campestris* pathovars	*Fusarium oxysporum*	*Phytophthora capsici*
6	Cauliflower mosaic virus (CaMV)	*Xanthomonas axonopodis* pv. *manihotis*	*Blumeria graminis*	*Plasmopara viticola*
7	African cassava mosaic virus (ACMV)	*Erwinia amylovora*	*Mycosphaerella graminicola*	*Phytophthora cinnamomi*
8	Plum pox virus (PPV)	*Xylella fastidiosa*	*Colletotrichum* spp.	*Phytophthora parasitica*
9	Brome mosaic virus (BMV)	*Dickeya* (*dadantii* and *solani*)	*Ustilago maydis*	*Pythium ultimum*
10	Potato virus X (PVX)	*Pectobacterium carotovorum* (and *P. atrosepticum*)	*Melampsora lini*	*Albugo candida*

Fig. 1.

Fig. 1 summary of the ten most significant plant pathogens across viruses, bacteria, fungi, and oomycetes, based on findings from Scholthof *et al.* (2011); Dean *et al*. (2012); Mansfield *et al*. (2012); Kamoun *et al*. (2015).

— Visual Observation

— Laboratory Techniques

1. Pathogen culture Observation
2. Microscopic Observation
3. Staining Methods
4. Serological Methods

Late Blight of Potato

Alternaria Leaf Spot

Citrus Canker

(A) VISUAL OBSERVATION: SIGNS AND SYMPTOMS:

- Identification of disease by visual observation on the whole plant or any infected part of the plant.
- Initial detection and identification of plant diseases typically begin with visual inspection.

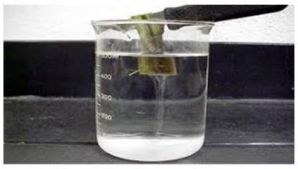
(B) Laboratory Techniques

- Fungal Culture observation
- Bacterial Culture observation
- Ooze test

Colonies of *Alternaria alternata* appear black to dark olive or gray and have a suede-like to woolly texture.

Fungal Culture Observation

BACTERIAL CULTURE OBSERVATION



Culture of *Ralstonia solanacearum*

Ooze test

MICROSCOPY

Microscopy is a specialized field focused on using microscopes to observe structures and regions that are invisible to the unaided human eye—those beyond the eye's natural resolution limit. Various types of microscopes are employed for the detection and identification of microscopic entities, including:

1. Stereoscopic Microscope
2. Fluorescence Microscope
3. Light Microscope
4. Scanning Electron Microscope (SEM)
5. Transmission Electron Microscope (TEM)

Microscopy, Staining, Serological, and Hybridization-Based Techniques for Plant Pathogen Detection. Plant diseases, caused by a wide range of pathogens including bacteria, fungi, viruses, nematodes, and protists, lead to major yield losses and economic

setbacks globally. Human activities like large-scale monoculture and global trade further contribute to the spread of these diseases. Early and accurate detection of pathogens is vital to prevent agricultural damage, especially as plant health is critical to our food, materials, and breathable air.

Traditionally, disease diagnosis involves visual inspection, host range testing, symptom analysis, and vector identification. However, advances in molecular biology and immunology have revolutionized the detection landscape with faster, more accurate tools.

Microscopy Techniques. Microscopy allows for the visualization of structures invisible to the naked eye. Different types are employed in plant pathology:

1. **Stereoscopic Microscope** – Ideal for surface-level examination.
2. **Fluorescence Microscope** – Uses fluorescent dyes to detect pathogens.
3. **Light Microscope** – Essential for viewing stained tissue samples.
4. **Scanning Electron Microscope (SEM)** – Offers 3D views of surface structures.
5. **Transmission Electron Microscope (TEM)** – Enables viewing of internal ultrastructures.

(a) Light Microscopy. Semi-thin resin-embedded sections of infected tissues are stained and examined for phytoplasmas.

(b) Transmission Electron Microscopy (TEM). Since phytoplasmas lack defined shapes, high-resolution TEM is used for visualizing them within host tissues.

(c) Immuno-Electron Microscopy (IEM). Uses immunogold labelling to detect antigens in plant and insect tissues, highlighting phytoplasma antigens on the cell surface.

Staining Methods

Staining enhances the visibility of cells and structures under microscopes.

- **Direct Staining**: Stains the organism, leaving the background clear.
- **Negative Staining**: Stains the background while leaving the organism unaltered.

Types of Staining:

- **Simple Stain**: Uses one dye, stains all cells uniformly.
- **Differential Stain**: Uses multiple stains to distinguish between cell types.
- **Special Stains**: Highlight specific structures like spores or capsules.

Gram Staining. Differentiates bacteria into Gram-positive (thick peptidoglycan layer, purple) and Gram-negative (thin wall, red after counterstain). It's a critical first step in bacterial identification.

Serological Techniques

Serology relies on specific antigen-antibody interactions to detect pathogens.

- **Antigen**: A foreign molecule that triggers an immune response.
- **Antibody**: A protein that binds specifically to its corresponding antigen.

Assays that use particular antibodies to identify plant pathogens are known as immunological or serological assays. Numerous useful antigenic compounds are produced by microorganisms. In order to identify (Alvarez, 2004). According to Lopez *et al*. (2003); Fang and Ramasamy (2015), the antibodies utilized in these tests attach to particular epitopes on these antigens. The antibodies' attachment to the antigen can be identified by employing particular antibodies conjugated with, for example, an enzyme, a fluorophore, or a nanoparticle. This makes it possible to infer the infections' existence indirectly (Fang and Ramasamy 2015).

Direct Serological Tests

- **Precipitation Test**: Antigen and antibody form visible precipitates.
- **Micro-precipitation Test**: Conducted at a micro-scale for economical use of antiserum.
- **Agglutination Test**: Causes clumping of antigen particles.
- **Gel Diffusion Test**: Antigen and antibody diffuse through agar to form precipitation lines.

Indirect Serological Tests

- **ELISA (Enzyme-Linked Immunosorbent Assay)**: Highly sensitive plate-based assay developed by Clark and Adams (1976) for detecting plant viruses. It was initially created in the 1970s and has since grown to be a well-recognized technique for detecting microbial infections across the world. Due to its speed and suitability for automation and high-throughput screens, it is frequently employed (Alvarez, 2004; Posthuma-Trumpie *et al*., 2009; Martinelli *et al*., 2015). Direct, indirect, sandwich, and competitive ELISA assays are among the various ELISA formats that have been developed; they all operate on the same principle, which is the use of particular antibodies conjugated with an enzyme that can turn a colorless substrate into a colored product (Alhajj and Farhana 2023). The amount of the target antigen—and thus the target pathogen—in the sample determines how much color is formed (Kumar *et al*., 2008; Atmar, 2014).

- **Immunosorbent Electron Microscopy (ISEM)**: Combines microscopy with serology for virus detection.
- **Lateral Flow Assay**: Quick, paper-based strip test providing results in minutes.
- **Immunofluorescence**: Fluorescent dye-tagged antibodies bind to antigens, allowing detection under a fluorescence microscope.

Hybridization-Based Detection

Hybridization methods identify specific nucleic acid sequences by using labeled probes.

Blotting Techniques:

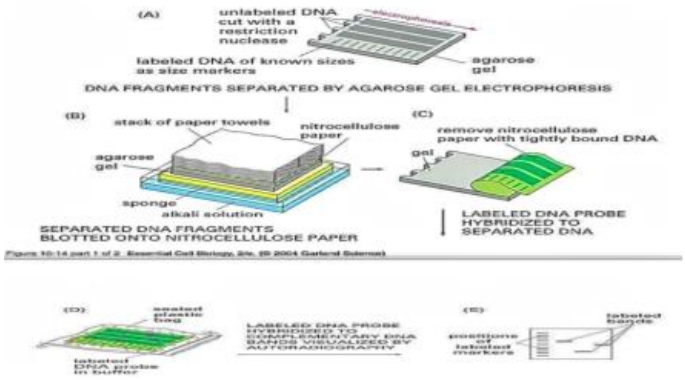
1. Southern Blot – Detects specific DNA sequences (developed by Edwin Southern).
2. Northern Blot – Targets RNA molecules.
3. Western Blot – Identifies proteins.

These methods often follow gel electrophoresis and are pivotal in studies related to gene expression, pathogen evolution, and diagnostics.

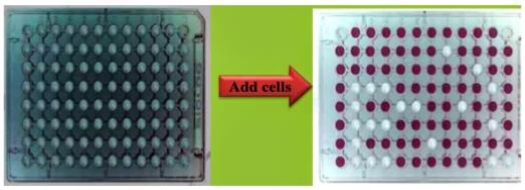
(1) Southern Blotting

- Developed by Professor Sir Edwin Southern in 1975, this technique revolutionized DNA analysis.

• He was awarded the Lasker Prize for Clinical Medical Research for his contribution to identifying specific DNA sequences.
• The method involves three main steps: DNA separation, transfer to a membrane, and hybridization with a labelled probe.
• It is specifically used to detect targeted DNA sequences within complex DNA samples.
• The process combines agarose gel electrophoresis (for separating DNA fragments by size) with probe hybridization (for identifying specific sequences).
• Hybridization is the central principle of this technique.
• Applications include gene mapping, evolutionary studies, DNA fingerprinting, and gene discovery.



(2) Northern Blotting
• Northern blotting is a molecular technique used to identify specific RNA sequences.
• It was introduced by James Alwine and George Stark at Stanford University in 1979.
• In this method, RNA is separated by electrophoresis, transferred onto a membrane, and then hybridized with a complementary probe.
Applications:
• Analyzing gene expression through mRNA levels
• Determining the size of mRNA transcripts
• Investigating RNA stability and degradation
• Measuring RNA half-life
(3) Western Blotting
• Western blotting is an immunological technique established in 1981 by W. Neal Burnette in Seattle, USA.
• It identifies specific proteins from a complex mixture using antibodies that bind to the target protein.
• SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is typically used before the blotting process to separate proteins by size.
Applications:
• Confirmatory diagnosis for HIV infection
• Accurate detection of Bovine Spongiform Encephalopathy (BSE)
• Diagnosis of Lyme disease
DNA Microarray / DNA Chip / Biochip
• A DNA microarray consists of tiny DNA spots fixed to a solid surface, enabling simultaneous analysis of thousands of sequences.
• Invented by Patrick O. Brown in 1981.
Principle: Based on the binding of complementary DNA sequences.
Process:
• Unknown DNA is fragmented using restriction enzymes and labeled with fluorescent markers.
• These fragments are then hybridized with specific probes on the chip.
• Only fragments with complementary sequences bind, while unbound fragments are washed away.
• Laser scanning detects fluorescence from bound sequences, and a computer records the hybridization patterns for identification.
BIOCHEMICAL METHODS
• Techniques include **BIOLOG** and **volatile compound analysis**.
• These methods utilize advanced redox chemistry to simultaneously test and identify both aerobic Gram-negative and Gram-positive bacteria within a single assay panel.



96 well contains different carbon sources and other tests. If the cells are metabolically active, they reduce the redox dye and a purple color is formed in all the positive well.
VOC Emissions and Plant Disease. The release of volatile organic compounds (VOCs) by plants is influenced by pathogenic infections. These diseases are typically triggered by a wide range of pathogens, including fungi, bacteria, mollicutes, parasitic plants, viruses, viroids, nematodes, and protozoa.
Methods for Assessing Plant VOC Emissions. Measuring plant VOC emissions generally involves the following three steps:
1. **VOCs Collection** – Capturing the volatile compounds released by the plant.
2. **Separation of VOC Mixture** – Distinguishing the various compounds within the VOC blend.
3. **Identification and Quantification** – Detecting and measuring the individual VOCs present in the sample.

DISCUSSION

Plant diseases, caused by a variety of pathogens such as fungi, bacteria, viruses, and nematodes, are a major threat to global agriculture, leading to significant crop losses each year. These losses, estimated to affect up to 40% of major crops, are intensified by human activities like monoculture farming and global trade. With the world population expected to reach 9.7 billion by 2050, the need for early and accurate detection of plant diseases is more critical than ever to ensure food security. Diagnosis usually begins with visual inspection, where symptoms such as leaf spots, wilting, or mold are observed. However, more precise identification is often carried out in laboratories using advanced tools and techniques. Laboratory methods include culturing pathogens on media to observe their growth, and microscopy, which allows experts to view pathogens too small to be seen with the naked eye. Various staining techniques are also used to improve visibility under the microscope. Serological methods, such as ELISA, rely on antibodies that bind to specific antigens of the pathogen and are widely used for their sensitivity and speed. Additionally, molecular techniques like PCR and DNA hybridization detect the genetic material of pathogens with great accuracy. Methods such as Southern, Northern, and Western blotting further help identify DNA, RNA, and proteins respectively. More recently, DNA microarrays and VOC (volatile organic compound) analysis have been developed to detect multiple pathogens at once or to analyze disease-specific plant emissions. Together, these traditional and modern approaches provide a robust system for identifying plant diseases, helping to protect global agriculture and maintain food production in a changing world.

CONCLUSIONS

The early and accurate detection of plant pathogens is fundamental to mitigating the impact of plant diseases on global agriculture. As the global demand for food continues to rise alongside population growth, ensuring plant health has become increasingly vital. Traditional diagnostic methods, such as visual inspection and culture-based identification, remain useful as preliminary tools, but they often lack the sensitivity and specificity required for rapid and large-scale disease management. Advances in microscopy, staining techniques, serological assays like ELISA, and molecular methods such as PCR and nucleic acid hybridization have significantly enhanced diagnostic capabilities. Furthermore, emerging technologies like DNA microarrays and VOC analysis offer high-throughput, sensitive, and specific approaches for pathogen detection and monitoring. The integration of these techniques into plant disease diagnostics allows for more timely interventions, improved disease surveillance, and better-informed management strategies. Collectively, these diagnostic advancements contribute not only to reducing yield losses but also to safeguarding global food security and agricultural sustainability in the face of evolving plant health challenges.

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