Detection and Diagnosis of Important Soil-Borne Pathogens

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Abstract

The agricultural industry has serious economic concerns globally because soil-borne plant diseases can result in catastrophic losses in crop yields, both in terms of quantity and quality. If a suitable and precise management approach is to be optimized, early, quick, and reliable pathogen identification is crucial. Historically, the most popular techniques for diagnosing plant diseases have relied on labour-intensive, time-consuming colony-based morphological approaches. For precise disease diagnosis and detection, technologies based on nucleic acids are now often utilized. Innovative molecular tools for pathogen detection and differentiation have been made possible by current developments in standard and variable PCR methods, including nested, quantitative, magnetic capture hybridization (MCH); multiplex, biological, post, and isothermal amplification; development of DNA and RNA-based probes; and next-generation sequencing (NGS). These nucleic acid-based detection techniques are used to identify symptomatic and asymptomatic infections caused by culturable and non-culturable fungal pathogens. Even though molecular diagnostic methods have made

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significant strides recently, there is still more to be done regarding their development and use in plant diseases. Molecular methods that are more consistent, efficient, and user-friendly than conventional methods are needed for soil-borne pathogen diagnosis. These approaches have high significance because of their unique specificity in separating related species at various taxonomic levels. Scientists are currently working on the problem of creating efficient tools for plant disease molecular diagnostics. This chapter talks about current developments in the creation and application of molecular methods to detect several soil-borne plant diseases.

Keywords

Soil-borne plant pathogens · Diagnosis · Molecular identification · Polymerase chain reaction · Next-generation sequencing

5.1 Introduction

Healthy crops are crucial for food quality and life in sustainable farming. In reality, a problem is "detected" by objectively observing the symptoms it causes, but a problem is "diagnosed" by identifying the particular pathological condition causing it. The foundation for a healthy crop, aid in risk management, and ensure safety during agricultural production are diagnosing or quickly identifying plant pests and diseases.

A rising concern is that the biggest threat to international agriculture is soil-borne diseases (Singh et al. 2018; Kashyap et al. 2021). Agriculture today makes up around half of the land that is livable worldwide. In previous centuries, as the human population grew, the area covered by crops progressively rose. Rice, wheat, and maize were cultivated on an estimated 540 million hectares worldwide, according to McDonald and Stukenbrock (2016), and plant diseases can significantly lower crop yield. Similar to this, diseases and pests connected with maize, rice, wheat, potatoes, greengram and soybean generate yearly losses of between 17 and 30 per cent worldwide (Manzar et al. 2022a; Kashyap et al. 2022a; Manzar et al. 2021; Reznikov et al. 2018; Savary et al. 2019).

Food security and safety are provided through systematic crop disease control in agriculture, which is essential for the expanding world population (FAO 2018; Sarrocco and Vannacci 2018). Most of the harm is caused by the unintentional spread of invasive alien disease species into new locations due to international commerce and transportation (Ghelardini et al. 2017). In the Great Irish famine (caused to the late potato blight (*Phytophthora infestans*)) of Britain and Ireland (between 1845 and 1849), nearly one million people lost their lives (Cox and Large 1960). Recently, *Pyricularia graminis* f. sp. *tritici*, a blast disease that originated in South America, damaged more than 15,000 acres of wheat in Bangladesh (Callaway 2016). *Xylella fastidiosa*, a xylem-inhabiting plant pathogen, severely damaged olive trees in Italy since 2013. According to genetic research, Italian strains were

comparable to Central American isolates (Marcelletti and Scortichini 2016; Giampetruzzi et al. 2017). The planting material (white pine blister) exchange transferred the pathogen *Cronartium ribicola* from Europe to Northern America. At the same time, the subspecies Americana of the Dutch elm disease fungus *Ophiostoma novo-ulmi* arrived with rock elm logs in Europe from North America (Ghelardini et al. 2017).

Climate change influences plant-pathogen interactions, viz. the spread of diseases in agriculture can be attributed to increased temperatures, climatic extremities, and changes in yearly precipitation patterns. Most of the cultivating soil is also planted with monocultures or even just one genotype, creating a genetically homogeneous environment that makes it simple for host-specific crop diseases to spread (Schmidhuber and Tubiello 2007). Developing quick, effective, affordable tools for early pathogen identification and control is essential. Early disease identification is important since using chemicals or biological agents to cure a significant disease incidence with increased severity and incidence harms the environment and natural ecology (Padaria et al. 2016; Sharma et al. 2017). Using the resistant germplasm as the first line of defence is the most efficient strategy to combat plant diseases (Sharma et al. 2010). However, integrated disease management systems rely heavily on the availability of quick, accurate, and focused disease detection technologies without resistant strains (Tarafdar et al. 2018).

Plant pathogen detection and identification, such as commencing sampling and reaction inhibition, provide several challenges. The development of sensitive and targeted molecular techniques has transformed the identification of soil-borne pathogens in recent years. All practising plant pathologists will soon be exposed to the quick and exciting changes in diagnosis. The immunological and nucleic acid-based tests, in particular, are currently accessible for various bacteria. While conventional methods like baiting, culturing, and microscopic observations are still in use and serve as the backbone of plant pathologists, molecular techniques are readily accessible. The most important approaches for novel molecular methods to identify soil-borne diseases and their utility in agriculture are covered in this chapter. In addition to its other benefits, such as facilitating a quicker diagnosis without the need of a diagnostic laboratory, on-site diagnosis can aid in early illness assessment in domains depending on their relevance.

5.2 Major Plant Pathogens Causing Soil-Borne Diseases

Numerous soil-borne plant diseases have been identified, and high disease-suppression soils have been found. Even in the presence of pathogen inoculum and favourable conditions for the development of illness, disease growth is restricted in these soils. Even while the fundamental processes at work in these soils aren't fully understood and are known to differ according to the pathosystem, it is assumed that the suppressive effect is complex in nature, coming from a combination of general and specific suppression.

Numerous crop species are adversely affected by the diversity of soil-borne diseases, including root, vascular, and seed rot, which can be caused by fungi, bacteria, phytoplasmas, viruses, protozoa, and nematodes. Frequent severe crop destruction results in significant annual economic losses. It might be challenging to see soil-borne bacteria with the naked eye. They are tiny, relying on the biotic and abiotic components of the soil to exist, and complete their life cycle in the soil. The principal soil-borne fungi—*Phytophthora*, *Rhizoctonia*, *Fusarium*, *Pythium*, *Verticillium*, and *Armillaria*—infect roots, resulting in root rot, wilt, yellowing, stunting, and dieback, which eventually cause the death of the plants. *Armillaria* and *Rhizoctonia* induce root rot, *Verticillium* and *Fusarium* cause wilt, and *Phytophthora* causes late blight (*Armillaria* is a honey mushroom that produces brackets or flowers at the base of a tree). Compared to fungus, bacteria are a less common kind of soil-borne disease. *Erwinia*, *Rhizomonas*, *Ralstonia* and *Streptomyces* are a few soil-borne bacterial pathogens that cause the diseases soft rot, corky root, bacterial wilt and scab (Kashyap et al. 2022a). Viral infections seldom spread through the soil because they need live plant tissue, although they have been seen to move on fungus or nematodes and enter through water. Soil-dwelling creatures called plant pathogenic nematodes mostly affect roots. They cause branching and swelling by feeding on the roots' terminals.

5.3 Traditional Methods for Soil-Borne Pathogen Detection

Isolation and cultivating, reinoculation, microscopic techniques, and biochemical testing in the laboratory are conventional/traditional ways of diagnosing soil-borne pathogens. These procedures have tremendous utility since they are reasonably priced and not technically difficult. They need a high level of competence in interpretation and analysis, are time-consuming, and are usually slow. Additionally, taxonomy and fungal plant pathology knowledge and skills are required. Conidia, sclerotia, or mycelia, and symptoms that develop after infection, have historically served as the foundation for diagnosing or identifying a fungal condition. This disease diagnosis is usually cumbersome and impractical when quick results are sought (Sharma et al. 2015). They are not suitable for quick diagnosis or large-scale sample analysis, and producers must rely on specialist diagnostic facilities because they are not easily accessible.

Furthermore, correct identification needs the assistance of trained and experienced people because eye inspection is usually inadequate. Making timely disease management decisions may be more challenging due to the chance that the pathogen would remain dormant in plant tissue (Tarafdar et al. 2013; Tarafdar et al. 2013). It can be difficult to differentiate between many plant diseases due to their physical resemblance. Examples are the *Macrophomina phaseolina* and the *Phoma* species (Somai et al. 2002). A thorough understanding of taxonomy is required for determination. Identifying various populations of the same pathogen with diverse features, such as toxin production, fungicide resistance, or variations in virulence, can sometimes be getting difficult. For a high number of samples, this approach proved

inadequate. Additionally, quarantining pathogens to lower the danger of illness and the spread of the inoculum necessitates using exact, quick detection techniques.

5.4 Immunological/Serological Detection of Soil-Borne Pathogens

Immunological methods' underlying notion of antigen-antibody interaction has many drawbacks, including low test sensitivity and affinity and the possibility of contamination. Due to advancements over the past 10 years, it is now possible to detect and quantify several hazardous species using immunological approaches, including nematodes and mycoplasmas. For more than 20 years, immunological techniques have been researched. Furthermore, fungus's high inconsistency and phenotypic serological flexibility have rendered plant disease detection ineffective (Luchi et al. 2020; Meng and Doyle 2002). Applying and developing cutting-edge and efficient diagnostic procedures to prevent fungal plant disease is essential. As a result, molecular approaches that make it easier to identify and quantify pathogens are being used to diagnose soil-borne infections. The drawbacks of traditional and serological diagnostic techniques can be overcome by molecular testing.

Beginning in the 1970s, the use of antibodies in serological detection systems for the rapid and precise diagnosis of diseases accelerated with the advent of monoclonal antibody technology. Soil-borne bacteria can be discovered if bacterial antigens are used to generate antibodies. These methods were used as laborious analytical instruments. This requires using specific antibodies to find the matching antigens in test samples. Each antibody has a distinct antigen-specific binding site. Monoclonal antibodies, which may be produced indefinitely and are highly specific when utilized in immunological testing, allow for identification at the genus, species, and isolate levels (Hardham et al. 1994).

Serological diagnostic methods provide several advantages. Antibodies may take weeks to produce, but if properly kept, they are frequently stable for a long period and produce effects quickly. They have not yet been fully utilized in diagnosing plant diseases other than viruses and bacteria, although they offer a wide range of applications for the general and accurate detection of unique epitopes of numerous soil-borne microorganisms. Tests for antibodies have significantly improved. They can now distinguish between strains within a species, are nanogram sensitive, and take less time to conduct in lab and field settings. Second, diagnosis depends only on a structural element of the organism, such as the coat protein, which offers very little information about the virus.

Thirdly, serology is only useful when an antigen that can be used to create an antiserum is accessible or when the antiserum is ready. Finally, serology is worthless for identifying as-yet-unidentified soil-borne diseases. The capacity to recognize IgM or rising antibody titres determines how a serological diagnostic is organized. Serological methods are used to diagnose the majority of prevalent bacterial illnesses that are transmitted through soil. The antibody-antigen combination may be used in various ways due to its endurance. The enzyme-linked immune sorbent assay

(ELISA), which comes in various formats and offers numerous endpoint detection choices, is the most significant. The ELISA can measure a pathogen's presence and offer proof of it.

5.5 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a different method that uses the studied antibody colour change to determine the presence of soil-borne pathogens. The target epitopes (antigens) from viruses, bacteria, and fungi are accurately bound using this method by antibodies bound to an enzyme. The interaction between the substrate and the immobilized enzyme causes colour changes, which may be used to identify substances. Specific monoclonal and recombinant antibodies easily available on the market can greatly improve ELISA performance. Specific monoclonal antibodies have been used in ELISA to achieve lower detection limits in the region of 105–106 CFU/mL. For the on-site detection of plant diseases, tissue print-ELISA and lateral flow devices have been developed. Although it cannot be used to diagnose infections early on before symptoms appear because the sensitivity for bacteria is so low (105–106 CFU/mL), it may be used to confirm plant illnesses once visible signs appear. The ELISA tests can be classified as a direct, indirect, sandwich, or competitive ELISAs depending on the antigen-antibody combination.

5.5.1 Direct ELISA

A target protein (or a target antibody) placed on the surface of microplate wells is treated with an enzyme-labelled target antibody (or a specific antigen to the target antibody). The activity of the microplate well-bound enzyme is evaluated after washing.

5.5.2 Indirect ELISA

The primary antibody is treated with a target protein immobilized on the surface of microplate wells before being incubated with a secondary antibody against it. After washing, the activity of the microplate well-bound enzyme is measured. Even though indirect ELISA requires more steps than direct ELISA, the primary antibody does not need to be labelled because labelled secondary antibodies are commercially available.

5.5.3 Sandwich ELISA

A second antibody that is also specific to the target protein but has been enzyme-labelled is used to treat a target protein-specific antibody placed on the surface of

microplate wells. The activity of the microplate well-bound enzyme is evaluated after washing.

The enzyme-labelled antibody (green) and the immobilized antibody must identify various target protein epitopes (orange). Sandwich ELISA is more selective than direct ELISA because it combines antibodies to two different epitopes on the target protein. Sandwich ELISA is beneficial when extreme accuracy is needed.

5.5.4 Competitive ELISA

An antibody that is specific for the target protein and has been immobilized on the surface of microplate wells is used to treat samples that contain the protein and a known amount of the target protein. The activity of the microplate well-bound enzyme is measured after the procedure. The sample will seem lighter when there are less antibody-bound enzyme-labelled antigens present. When it is low, on the other hand, more enzyme-labelled antigen is bound to antibodies, which results in a deeper colour. When the target antigen in a sandwich ELISA test is a small molecule like dioxin, histamine, or a pesticide, two antibodies cannot attach to it simultaneously. Competitive ELISA may be used to measure low molecular weight targets.

5.5.5 Phage Display

Phage display-based antibody engineering has the potential to revolutionize the production of antibodies by making the process faster and more affordable than current monoclonal antibody techniques (Mitchell et al. 1997; Wilson and Finlay 1998; Aujame et al. 1997). To produce foreign proteins (antibodies) as fusions to phage coat proteins, cloning sites that have been introduced to filamentous phage vectors are used in this technique. Before being chosen for certain proteins with particular binding capabilities, *Escherichia coli* cells are transformed with phage libraries and cultured in culture. The technique has been used for diagnosing plant diseases and general plant biology. For example, *Ralstonia solanacearum* Race 3 and Black Currant Reversion Associated Virus have been detected using phage display to create particular antibody fragments that can be used in ELISA (Griep et al. 1998). Due to the ability to manufacture specific antibodies in large quantities without the need of expensive hybridoma technology or test animals, antibodies will soon be available at a greatly reduced cost.

5.6 Lateral Flow Devices

The lateral flow device is one of the most extensively used diagnostic tools available to farmers today (LFD). These devices are simple to use and swiftly generate results—typically in less than 10 min. The LFDs that can be purchased commercially to identify viral infections in plants are the most beneficial. As little as 3 ng mL^{-1} of

antigen may be detected by an LFD-based test for *Rhizoctonia solani*, which is equivalent to the sensitivity of conventional ELISA methods (Thornton 2008). In contrast to the plant viruses and bacterial pathogens that are typically the objectives of commercial LFD-based tests and for which specific antibodies are frequently available, this work focused on a soil-borne plant pathogenic fungus. The development of species-specific antibodies against fungi has proven to be more difficult; however as was already said, some targets have achieved success.

5.7 Biochemical Methods for Soil-Borne Pathogen Detection

Biochemical traits specific to each creature can be used to identify it. On one end of the scale, certain qualities are shared by large populations while, on the other, some are exclusive to individual populations within the species. In order to determine the taxonomic rank at which an organism is categorized, it is essential to characterize the pathogen. Embracing gel electrophoresis for soluble protein analysis are bacteria and fungi. It is crucial to standardize these procedures since gene expression is a characteristic of all of them and may be affected by environmental factors.

Similar to this, Pernezny et al. (1995) used substrate to pinpoint *Xanthomonas campestris* as the bacterial species in charge of a serious outbreak of bacterial spots in Florida lettuce crops; the pathovar presence was determined to be vitians by its fatty acid composition. In some situations, the creation of unusual metabolites by an organism can be utilized to identify it.

For instance, identifying *Aspergillus flavus* strains capable of making aflatoxin was aided by synthesizing volatile C15H24 compounds, including alpha-gurjunene, trans-caryophyllene, and cadinene. Non-toxic strains did not create these chemicals (Zeringue et al. 1993). When identifying bacterial plant pathogens using fatty acid profiles (FAME Analysis), the bacterium is often grown in pure cultures first. Wet cells are methylated and saponified to around 40 mg. By using an ether-hexane combination to extract the fatty acid methyl esters (FAME), gas chromatography is used to examine the results.

Because the fatty acid profiles of the field-collected strains most closely mirrored that of this pathovar, *Xanthomonas campestris* pv. *vitians* was discovered to be the pathogen that produced an outbreak of a bacterial spot on lettuce (Pernezny et al. 1995). The four species of the *Erwinia herbicola* group and the five species of the *Erwinia amylovora* group could be distinguished in more detailed research by Wells et al. (1994). When electrophoresizing, soluble proteins from plant diseases usually produce intricate patterns that can be used for identification. Proper staining methods may be able to disclose a particular protein dye, which, for example, may include enzyme activity, rather than utilizing a broad protein stain like Coomassie Blue. 4250 Australian isolates of *Rhizoctonia solani* were divided into 10 groups, termed zymograms, by MacNish et al. (1994), who stained for pectic enzymes.

5.8 Molecular Methods for Soil-Borne Pathogen Detection

Many experts agree that nucleic acid (NA)-based methods are among the best for finding soil-borne plant infections. More contemporary methods, including immunological methods, DNA/RNA probe technologies, and polymerase chain reaction (PCR) amplification of nucleic acid sequences, are increasingly being used to identify plant diseases (Manzar et al. 2022a). These techniques have a number of benefits over traditional diagnostic techniques, including the fact that they are more accurate, faster, and easier to use without specialized taxonomic expertise. More significantly, these techniques make it possible to identify bacteria that cannot be grown. Furthermore, molecular identification techniques aid in the discovery of new diseases with unidentified aetiologies. These instruments might be employed to accurately gauge the biomass of infections and confirm their presence (Biswas et al. 2012a, 2012b; Sharma et al. 2012b).

5.9 Nucleic Acid-Based Detection Techniques for Soil-Borne Pathogens

Most NA-based detection techniques, particularly those that employ PCR, are rapid, specialized, and sensitive. This provides a more robust diagnosis. While molecular testing verifies the diagnosis for other diseases or determines whether litigation is feasible, traditional procedures are helpful for different conditions. It is challenging to separate pathogens taxonomically because many plant pathologists cannot swiftly differentiate important disease taxa like *Pythium* or *Phytophthora* by visual inspection. To help create a genome database, various bacteria, even nonsporulating ones, can be awarded species I.D.s as sequencing expertise increases.

Diagnosticians and other applied plant pathologists are mainly situated to increase the genetic library for plant diseases due to their exposure to various conditions on diverse hosts. Sequencing the ITS or mitochondrial genes may be helpful since it provides a DNA fingerprint for many plant illnesses. Many of these diseases must be cultivated before being detected. The study of this area may easily recognize these sequences. Massive sequencing technology advancements have profoundly influenced genomic research and considerably increased the throughput of cost-effective sequences. The pyrosequencing method of DNA sequencing is built on the sequencing-by-synthesis methodology. The management of fungal plant diseases currently does not make extensive use of pyrosequencing technologies.

5.10 Polymerase Chain Reaction (PCR)

For developing monoclonal antibodies and using the polymerase chain reaction to amplify nucleic acid sequences, J.F. Kohler, C. Milstein, and K. Mullis were awarded two Nobel Prizes in 1984 and 1993 (PCR). A thermostable DNA polymerase catalyses an exponential amplification of a target DNA strand in the polymerase

chain reaction (PCR), the mainstay of NA-based disease detection. This valuable and inexpensive molecular method can duplicate or amplify tiny fragments of DNA or RNA. By connecting two synthetic oligonucleotides, or "primers", to the target genomic sequence and extending them using a Taq polymerase, this in vitro amplification technique amplifies a single copy of the nucleic acid target (a thermostable DNA polymerase). Because of the DNA hybridization and replication fidelity, PCR was initially used to detect illnesses caused by bacteria and viruses. These days, both plant illnesses and diseases transmitted through the soil are frequently identified using it. Due to its exceptional sensitivity, advanced PCR methods, such as reverse-transcription PCR (RT-PCR), have also been used in addition to traditional PCR technology for the identification of plant pathogens. The many PCR types used in pathogen detection are described in the section below.

5.11 Random Amplified Polymorphic DNA (RAPD)

The Random Amplified Polymorphic DNA (RAPD) technique is a simple, rapid, and inexpensive way to amplify a tiny amount of total genomic DNA at low annealing temperatures. It uses short synthesized oligonucleotides of random sequences as primers. A somewhat unique profile pattern is visible when the ensuing PCR product is resolved. As a result, RAPD markers have established themselves as useful tools for studying the genetics of fungal populations (Nasir and Hoppe 1991). This marker makes it possible to detect even the smallest DNA changes in the organism. For molecular taxonomy, genomic mapping, and evolutionary studies, several fungal species have been identified using RAPD (Nasir and Hoppe 1991). By examining DNA products created by RAPD, it has been possible to learn about the variation and segregation of genetic traits among strains.

5.12 Restriction Fragment Length Polymorphism (RFLP)

The phylogenetic separation, description, and categorization of soil-borne illnesses is made possible by nuclear ribosomal DNA (rDNA) amplified using restriction fragment length polymorphism (RFLP) (RFLP). Restriction fragment length polymorphisms in DNA encoding specific genes can be used to identify the species of a pathogen. This method of identifying a species depends on having a good database on the variability in fragment length polymorphisms that may be found among isolates of individual species because conspecific isolates may differ in the presence or absence of specific restriction sites, changing the RFLP banding.

As an illustration, Camele et al. (2005) employed thorough RFLP of PCR-amplified rDNA to identify and separate 10 *Phytophthora* species infecting different crops, enabling selective identification of these *Phytophthora* spp. The restriction patterns of 27 other *Phytophthora* species were identified and used to amplify and further digest the amplicons generated by PCR using *Phytophthora*-specific primers (Drenth et al. 2006, 2006). Following analysis of the ITS region

using PCR-RFLP, several anastomosis groups were discovered in *Rhizoctonia solani* isolates (Pannecoucque and Hofte 2009). The ability to discriminate between pathogenic and non-pathogenic *Pythium myriotolum* strains was also made feasible (Gómez-Alpízar et al. 2011). Sharma et al. have identified the genetic diversity in populations of *M. phaseolina*, a PCR-amplified rDNA-targeting microbe isolated from chickpea (2012a).

5.13 Amplified Fragment Length Polymorphism (AFLP)

A PCR-based tool and variation of the RFLP, the amplified fragment length polymorphism (AFLP) is used in genetic research, DNA fingerprinting, and the practice of genetic engineering. It has been used to distinguish between different species, although it is most usually employed to examine genotypic diversity in a population (Gargouri et al. 2006). Infections connected to recent disease outbreaks, such as sorghum ergot, can be traced back to their geographic source using the latter trait. Despite being an effective diagnostic tool, AFLP analysis takes a lot of time, requires complex technical skills, and is not suited for everyday use in diagnostic clinics.

5.13.1 Simple Sequence Repeats (SSR)

Simple sequence repeats (SSRs), often referred to as microsatellites or short tandem repeats (STRs), are repeating patterns made up of one to six nucleotides that are found in every eukaryotic genome. They are known for producing the best and most precise markers, which are frequently applied in soil-borne diseases to identify genetic changes between even among closely related species (Prospero et al. 2004). The distribution of these nucleotide units across the genome is essentially random, and their recurrence patterns may differ from person to person. To produce PCR products of various lengths, one can employ primers that surround such varied locations.

Microsatellites are a common genetic marker used for DNA fingerprinting due to their extraordinary versatility. The abundance of thousands of potentially polymorphic markers and a high degree of polymorphism in SSRs are advantages. SSR markers are a reliable solution for a broad range of applications, such as genome analysis and genetic mapping (Szabo and Kolmer 2007). Microsatellite markers exclusive to the *Phytophthora ramorum* pathogen were used in the additional study to discriminate between the A1 and A2 mating types of isolates from this disease that originated in two distinct countries.

5.13.2 Multiplex PCR

Using a single reaction mixture and many primer pairs, the multiplex PCR test enables the simultaneous amplification of numerous pathogens (Sint et al. 2012).

The generated amplicons can then be separated and shown using electrophoresis. The multiplex test requires the creation of primers, and specific sets of primers should have equivalent annealing temperatures for effective amplification. It makes it possible to accurately and simultaneously detect several DNA or RNA targets using a single procedure. It is advantageous in plant pathology because sensitive detection is necessary to produce pathogen-free plant material, and different soil-borne pathogens frequently infect a single host. Wheat (Sun et al. 2018), strawberries (Li et al. 2011), and turfgrass are a few examples of hosts where several infections can be found at the same time in a single multiplex PCR test (Asano et al. 2010).

5.13.3 Real-time PCR

Real-time PCR, which is based on the nucleic acids of bacteria, fungi, and viruses, is used to rapidly identify plant illnesses. The important component in managing plant diseases is detection and pathogen quantification (Le Floch et al. 2007; Lees et al. 2002). Real-time PCR has significantly improved pathogen identification and quantification, while quantification based on culture techniques is frequently considered inaccurate and unreliable (Tarafdar et al. 2018). Real-time PCR differs from end-point PCR in that each PCR cycle includes a measurement of the amplified PCR product. Since the exponential phase of the reaction is being monitored as it progresses, real-time PCR allows for accurate template quantification. Real-time PCR is gaining popularity for identifying and quantifying a variety of pathogenic fungus, oomycetes, bacteria, nematodes, viruses, and biocontrol agents that affect plants. A specific increase in fluorescence during PCR amplification can be used to identify pathogenic fungi.

5.13.4 Colony PCR

This efficient method for crude mycelium-based amplification utilizes the ITS1–5.8S-ITS2 section of the fungal ribosomal DNA cluster. PCR generally has a high success rate. This method ought to be widely applied to streamline molecular taxonomic studies and enable more in-depth, sequence-based analyses of fungal isolates. The data were directly obtained from fungal hyphae without any prior DNA extraction or other processing. It is possible to successfully amplify DNA from the fungus *Cladosporium*, *Geomyces*, *Fusarium*, and *Mortierella*. Yeasts discovered in the soil may always have their DNA enhanced. Mutualistic *Basidiomycota* and *Ascomycota* were also successfully amplified without the need for DNA extraction from cleaned mycorrhized root tips, and *Tuber melanosporum* fruiting bodies could be swiftly recognized using a direct PCR using species-specific primers (Walch et al. 2016; Bonito 2009).

5.13.5 Nested PCR

Nested PCR is an endpoint PCR variation that uses two sets of primer pairs for two rounds of PCR amplification to boost specificity and sensitivity. Nesting makes it easier to employ non-specific PCR primers for amplifying different pathogens in the first round of PCR, followed by the use of pathogen-specific primers in the second round. The main goals of the PCR modification were to improve sensitivity and specificity. Two primer sets are used to carry out two successive PCR reactions, treating the results of the first round of amplification with the same treatment in the second round (Ni et al. 2011; Grote et al. 2002; Kamolvarin et al. 1993).

5.13.6 Bio PCR

The bio-PCR test amplifies the endpoint PCR technique, which involves a pre-assay incubation step in a sick sample to increase the biomass of the causal agent. This method focuses solely on the target pathogens by cultivating the target pathogen in a growing medium that prevents the growth of non-target microorganisms to maximize detection.

5.14 DNA or RNA Probe-Based Assays

5.14.1 In Situ Hybridization

Using the in situ hybridization (ISH) technique, the mRNAs present in the fixed sample may be identified. The main goal of this test is to design an antisense small-scale RNA probe that will bind the target mRNA (interesting sequence). But it's also feasible to use cDNA probes and artificial oligonucleotide probes. Because they are detectable and straightforward to measure for, the radioactive isotopes 35S, 125I, and 32P are widely employed to label probes. Tyramide, bromodeoxyuridine, biotin, digoxigenin, alkaline phosphatase, and digoxigenin can all be used to label nonisotopic probes. Photographic, X-ray film autoradiography, liquid emulsion, and microscopic techniques are a few examples of signal detecting techniques.

5.14.2 Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) is a cutting-edge approach for the diagnosis of plant diseases that are still relatively new. The specificity of DNA sequences is combined with the sensitivity of fluorochrome-based detection methods (Hijri 2009; Cui et al. 2016). Using DNA or RNA probes that are fluorescently coloured either directly or indirectly, FISH assays may locate specific DNA or RNA sequences in cells or tissues (Shakoori 2017). Using wide-field epifluorescence or confocal laser

scanning microscopy, stained cells from the standard FISH methods are seen when fluorescently mono-labelled oligonucleotide probes hybridize the ribosomal RNA (rRNA) of microbial cells (Lukumbuzya et al. 2019). The rRNA sequences of plants that have been infected with a pathogen are specific to that pathogen. FISH can recognize this specific information provided by RNA (Fang and Ramasamy 2015). Southern tomato blight is brought on by the disease *Sclerotium rolfsii*, which can be found in soil. The FISH technique that used an oligonucleotide probe dyed with Cy3 and Cy5 successfully identify soil smears in DNA isolation with 0.06 pg L^{-1} of *S. rolfsii* (Milner et al. 2019). FISH's most vital points are repeatability, sensitivity, specificity, precision, and speed (Bozorg-Ghalati et al. 2019). In mixed-species specimens, it could also pinpoint the primary pathogens and offer details on resolution and morphology (Frickmann et al. 2017). A common pitfall that reduces test specificity is false-positive results using autofluorescence materials.

5.15 Isothermal Amplification Techniques

5.15.1 Loop-Mediated Isothermal Amplification (LAMP)

Due to its outstanding efficacy, specificity, ease of use, and speed, LAMP requires four primers, two long outside and two short inside, each recognizing six different sequences in the target DNA. DNA synthesis will begin when the target sequence hybridizes with the first inner primer, which comprises a sense and antisense DNA sequences. The single-stranded DNA produced by the outer primer serves as a template for the creation of a DNA molecule with a loop structure by the second inner and outer primers. The term "strand-displacement DNA synthesis" refers to this procedure. The constant cycle reaction causes products with repeated target DNA sequences of varying lengths to accumulate.

The reaction tube is incubated at 63–65 °C in a standard water bath or heat block in a laboratory setting to maintain a constant temperature. Unaided eyes can perceive the amplified product as a white precipitate or a yellow-green-coloured solution after adding SYBR green to the reaction tube. The primary benefit of LAMP is that it may be completed rapidly and at a constant temperature. Since it uses an expedient isothermal technique, it is ideal for plant pathogen identification at the point of care in the field.

It also has a high amplification efficiency and sensitivity since it can generate many PCR products from a small quantity of DNA input. Due to the assay requiring only a few pieces of essential equipment, this process is also affordable. The sensitivity of hybridization assays, such as LAMP-ELISA hybridization and LAMP paired with colorimetric gold nanoparticle hybridization probes, may be improved by using amplicons containing many inverted repeats produced by LAMP, according to specific reports. The electrochemical sensor, in conjunction with LAMP offered a reliable platform for pathogen detection due to its outstanding sensitivity, which allowed it to recognize as little as ten copies of pathogen genomic DNA. LAMP-biosensor technology has a significant potential for in-field testing,

detection, and identification of plant diseases (Tsugunori et al. 2000; Fukuta et al. 2003; Ghosh et al. 2016; Ghosh et al. 2017).

5.15.2 Rolling Circle Amplification

Rolling circle amplification is a widely used isothermal enzymatic assay that utilizes DNA or RNA to diagnose plant diseases. In addition to RCA, several techniques, like direct sequencing and RFLP, have effectively discovered and classified plant diseases with much less time and price than conventional methods. The main components required for this experiment are deoxynucleotide triphosphates, a circular template, a short DNA/RNA primer, and a homologous buffer. For 40 *Fusarium* strains, naked eye viewing of the RCA result has been made possible by adding fluorescent dye to the reactions (Davari et al. 2012). Ligating padlock probes with RCA has also been shown to detect fungal infections (Najafzadeh et al. 2011). The RCA test offers the advantages of simplicity, efficacy, and lack of temperature cycling apparatus (Dong et al. 2013; Goo and Kim 2016). Using this method, it is also possible to analyse gene expression, single nucleotide polymorphism, mRNA splicing, and post-translational modifications of protein molecules (Gao et al. 2019).

5.16 DNA-Based Point-of-Care Diagnostic Methods

Diagnostic tests that can be performed at the point of care (POC) and without costly equipment are desperately needed. Despite having several advantages over other technologies, PCR-based methods are much less effective for POC applications because they require energy to carry out the temperature modifications necessary for DNA amplification. The best way to overcome this constraint uses isothermal DNA amplification. For instance, POC detection of pathogen DNA utilizing isothermal amplification combined with lateral flow strips and portable fluorometers has been accomplished.

- POC—DNA Extraction methods: To successfully extract DNA from plant tissues, it is necessary to be able to properly remove a variety of contaminants that may otherwise interfere with the DNA amplification process. A rapid and efficient DNA extraction method using a lateral flow device (LFD) has been devised for POC testing and plant pathogen identification.
- In an extraction buffer, the sample is agitated with metal ball bearings before the lysate is transferred to the release pad of an LFD nitrocellulose membrane. The membrane is then added to the DNA amplification process using PCR or another isothermal amplification technique after being partly removed. It is possible to do the extraction outside since the isolated DNA is very stable on the membrane at ambient temperature.
- Another method uses a simple dipstick composed of cellulose, which can analyse plant samples in as little as 30 s. Plant tissues are macerated by giving them a

vigorous 8–10 s shake in a tube with extraction buffer and one or two ball bearings. Before entering the tube containing the amplification mix, the sample is first put in a cellulose dipstick tube and three times rinsed with wash buffer in a separate tube. The technique works on various domesticated species, including mature tree leaves and notoriously tricky tissues such as rice, tomato, and sorghum (mandarin, lime, and lemon). It is compatible with a variety of amplification methods, such as PCR, LAMP, and RPA, and it may be used to detect pathogen DNA and RNA in tissues that have been infected.

5.17 Recent Advances in Soil-Borne Pathogen Detection

5.17.1 Ancillary Ways of Pathogen Detection

Thermography, fluorescence imaging, hyperspectral imaging, and gas chromatography are a few techniques for indirectly identifying infections.

Thermography is a promising method for evaluating the heterogeneity in the infection of soil-borne diseases and can record changes in the surface temperature of plant leaves and canopies. Thermography uses thermographic cameras to record and analyse colour variations in emitted infrared light. Plant diseases affect how much water a plant losses when its stomata open and close (Hillnhütter et al. 2011). Thermographic imaging shows the disease that results may be observed, and without the effect of outside temperatures, the amount of water lost can be determined (Oerke et al. 2006).

Another cutting-edge technique is hyperspectral imaging, which may be used to indirectly detect plant illnesses and gather crucial information on the health of plants over a wide spectrum of wavelengths between 350 and 2500 nm. For the diagnosis of agricultural diseases and plant phenotyping, it is increasingly frequently utilized in large-scale agriculture. This method allows for quick processing of imaging data and is exceptionally accurate. Because they monitor variations in reflectance brought on by the biophysical and metabolic impacts of infection, hyperspectral methods are used to detect plant infections. Hyperspectral imaging methods have been used to identify and report infections caused by *Magnaporthe grisea* in rice, *Phytophthora infestans* in tomatoes, and *Venturia inaequalis* in apple trees (Delalieux et al. 2007; Zhang et al. 2003).

5.17.2 Gas Chromatography

Identifying the volatile chemical signature of diseased plants is another non-optical indirect way of plant disease identification. Plant pathogen infections may cause the emission of certain volatile organic compounds (VOCs) that are highly diagnostic of the sort of stress the plants are experiencing. When strawberries are infected with *Phytophthora cactorum*, a fungus that causes crown rot, *p*-ethyl guaiacol and *p*-ethyl phenol are released as identifiable VOCs from the damaged section of the plant/fruit.

The volatile signature of plants may be examined using gas chromatography (GC) technology to check for a particular VOC that may indicate the presence of a specific disease. Gas chromatography and mass spectrometry (GC-MS) are widely employed to detect unidentified molecules in volatile samples and improve compound separation and analysis effectiveness. Due to its high specificity, GC/GC-MS can offer more accurate details on plant disease than the optical imaging-based detection techniques listed above. The quantitative information collected from the VOC sample shows that illnesses can be identified at various stages (Kashyap et al. 2022a).

5.18 On-Site Direct Diagnosis of Plant Diseases

There are now several on-site direct diagnostic methods available. They are straightforward to understand and useful for farmers in making prompt decisions and early adoption of this technology for precise disease management strategies that might lessen the effect of plant illnesses. On-site testing can provide immediate response without shipping the sample to an off-site laboratory if it is done "field-side" in the farmer's presence. Utilizing a fluorogenic probe-based test, for instance, which entails magnetic bead-based nucleic acid extraction followed by qPCR using portable real-time PCR, *Spongospora subterranean*, a soil-borne disease of potatoes, may be quickly and easily diagnosed on-site. Compared to the laboratory-based method, the portable real-time PCR methodology can identify the pathogen with as little as 100 copies of *Spongospora subterranea* DNA, even when the pathogen colonization in the host is very low. The revolutionary portable real-time PCR may be used in place of laboratory-based methods to detect infections.

X-ray crystallography is now one of the most sophisticated techniques for diagnosing certain diseases using a particular protein released by the pathogen or host during contact. Using X-ray crystallography equipment at Diamond Light Source, researchers at the Iwate Biotechnology Research Centre (Japan) found the deadly rice blast disease *Magnaporthe oryzae*. The gene-for-gene paradigm was used for the first time to identify a pathogen at the molecular level using a crystallographic-based technique.

5.18.1 Immunofluorescence (IF)

A fluorescence microscopy-based optical method is applied to detect pathogen infections in root tissues. Plant samples are cut into tiny tissue slices and adhered to microscope slides for this operation. The specific antibody is detected by conjugating a fluorescent dye to observe the distribution of the target molecule across the sample. They are using IF, and the onion crop infection caused by *Botrytis cinerea* was found. Crown rot, a novel disease in Europe, may be found using IF and FISH together (Wullings et al. 1998). Similar to FISH, a flaw in other fluorescence-based methods such as photobleaching results in erroneously negative

consequences. The reduction in sensitivity brought on by photobleaching may be controlled, though, by reducing the amount of light exposure time and intensity, increasing the concentration of fluorophores, and choosing fluorophores that are more resistant to photobleaching.

5.18.2 Flow Cytometry (FCM)

It is a widely utilized laser-based optical technique for cell sorting, biomarker detection, and protein modification. FCM is a unique tool for detecting plant illnesses even though it has been used to count bacteria, distinguish between live and non-viable bacteria, describe bacterial DNA, and examine fungal spores. It has also been used to research antibiotic susceptibility and cell cycle dynamics.

5.18.3 Next-Generation Sequencing

Next-generation sequencing (NGS), high-throughput sequencing (HTS), and pyrosequencing are cutting-edge diagnostic techniques that revolutionize the detection of pathogens in various plant samples. As opposed to conventional molecular technologies, which require prior knowledge of the pathogens' sequence information, the NGS approach is unlimited, making it possible to identify any known and undiscovered pathogens in a single experiment. At its genomic core, phytopathogens are a collection of soil-dwelling bacteria, and the development of NGS technology has spawned novel methods for the detection and taxonomic identification of phytopathogens. The organism need not be cultivated or have its past sequencing data to apply this procedure, which takes some time but is essential for finding novel bacteria, viruses, and viroids (only around 10% of bacteria are culturable). NGS can quickly identify both known and unknown plant diseases. The primary steps in DNA-based NGS include DNA isolation and fragmentation, library preparation, massively parallel sequencing, bioinformatics analysis, variant/mutation annotation, and interpretation. Massive parallel signature sequencing, pyrosequencing, colony sequencing, and sequencing by oligonucleotide ligation detection (SOLID) are some of the most frequently employed advanced sequencing methods in HTS (Rajesh and Jaya 2017). Using RNA-sequencing, it may be possible to comprehend and study the dynamic nature of the transcriptome (RNA-Seq). The most popular NGS platform for RNA-Seq is the Illumina HiSeq platform, which has taken the NGS market by storm. The most recent release for the platform was a desktop sequencer named MiSeq (Kukurba and Montgomery 2015; Hariharan and Prasannath 2021).

When identifying early-stage infections in plants brought on by various fungal/oomycete diseases, symptoms in the host plant are typically necessary. Several of the abovementioned molecular and serological methods are often utilized to find these infections. But since it may target several different pathogen loci in a plant metagenome that is affected, next-generation sequencing (NGS) has the most potential as a diagnostic tool (Sharma et al. 2016). Finding significant eukaryotic plant

diseases with NGS has several possibilities. It may raise the fraction of NGS readings for targets with low abundance by concentrating specific nucleic acids in heterogeneous samples using targeted genome capture (TGC) oligonucleotide probes. Metagenomes and the Electronic Probe Diagnostic Nucleic Acid Analysis (EDNA) have the potential to simplify the detection of oomycete and fungal plant diseases significantly. EDNA is more reliable than electronic probes, which simply rely on matches between queries and metagenome data in diagnosing oomycete and fungal plant diseases.

By amplifying certain DNA regions, the PCR method may identify diseases like bacteria, viruses, and fungus. The drawback of the approach is that the search is quite selective since one base their study on which pathogen is most likely to be present based on certain symptoms. NGS eliminates the need for a prior decision because it can directly identify all possible pathogens. This rapidity is a significant asset in a sector where time is money. The same principle applies to cultivation: the longer something is developed, the longer it takes to battle disease. All parties in the supply chain benefit from rapid diagnostics since they may help producers, importers, and exporters save much money. The disadvantage of this strategy is the time and effort required to generate and assess a large number of sequences.

5.18.4 Disease Diagnostics Kits

Biotechnology has made it feasible to develop diagnostic tools which assist farmers worldwide in managing various diseases that affect their crops. Thanks to improved diagnostic techniques that take up less processing time, infections may be identified with greater precision. The fast identification of DNA or proteins particular to each disease, ailment, or condition is how these diagnostics function. A qualified person must use the tools and procedures. Diagnostic kits offer a large selection of ELISA kits for plant pathogen detections with good test performance characteristics for the precise, quick, simple, and high-throughput identification of the organisms that cause plant disease. Compared to conventional diagnostic procedures and PCR-based approaches, immunological techniques based on ELISA kits provide several benefits. A range of ELISA-based rapid test strips with obvious colour change indicators is now readily available due to the usage of lateral flow devices (LFD), which are designed for on-site, accurate, and quick diagnosis of plant diseases by untrained workers.

5.19 ELISA (Enzyme-Linked Immunosorbent Assay) Kits

The ability of an antibody to recognize a particular protein fragment or antigen linked to a plant pathogen is the basis for ELISA kits. The kits are simple to use and take around 5 min to measure sickness in the field. Additionally, they don't require specialized knowledge or pricey laboratory equipment. Several ELISA test kits are available; infections are already caused by pathogens such *Erwinia amylovora*,

Ralstonia solanacearum, *Phytophthora* sp., etc., in grains, root crops, ornamentals, fruits, and vegetables.

5.19.1 Direct Tissue Blotting

Additionally, this approach searches for plant pathogens using specific antibodies. Before introducing antibodies, samples of the diseased tissue are pressed onto specialized paper to be tested for protein content. The antibody-pathogen combination is then exposed to a dye-inducing reagent for reaction. The colour reaction shows a positive result and the presence of the pathogen in the affected tissue.

5.19.2 DNA/RNA Probes

An additional set of tools that may be used to identify plant diseases are nucleic acid (DNA/RNA) probes. These probes are nucleic acid fragments arranged like the DNA or RNA of the pathogen. Since the sequences complement one another, the probes may be utilized to identify specific diseases (Goodwin et al. 1989).

5.19.3 Squash Blot Method

The squash blot method uses a specific type of paper called a membrane to "squash" plant tissue from a plant that is suspected of having a disease. A probe that can interact with the DNA or RNA of the plant pathogen alleged to be present in the tissue is then applied to this membrane. The binding will occur when there exist complementary sequences. A colour reaction demonstrates the existence of the disease after adding several more chemicals to the membrane, which indicates that the probe and the pathogen DNA/RNA have formed a bond. Lack of a colour reaction means a bad result or the absence of sickness.

5.19.4 Use of Pocket Diagnostic Rapid Test Strips for Plant Diseases

Different lateral flow rapid test strips identify various plant pathogens. After breaking it up into small bits, place the sample in the container with the buffer and ball bearings. Shake the sample in the liquid for about a minute to break it up. While drawing liquid into the pipette, watch out for sample debris and air bubbles. To acquire reliable findings in less than 10 min, add 2 drops to the sample well of the testing device while maintaining levelness.

5.19.5 PCRD-Nucleic Acid Detection

The traditional method for confirming the presence of nucleic acid following DNA amplification in PCR is DNA agarose gel electrophoresis. PCRD offers a rapid and simple alternative to gel electrophoresis that may be finished in minutes without requiring expensive equipment, exposure to intercalating dye, or UV radiation. PCRD is a nucleic acid lateral flow immunoassay (NALFIA), which may be used in conjunction with PCR, loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), or helicase-dependent amplification (HDA). The PCRD format may be used by large throughput laboratories and small field-based laboratories.

5.19.6 Diagnostic Kits' Advantages

Quick tests that may be performed in the field in a matter of minutes allow for making judgements on the spot, which is favourable for yield since it enables the implementation of management measures earlier than if a sample were sent to the lab. A rapid test can lower the cost per sample since fewer samples must be sent to the lab, reducing the cost per sample.

5.20 Conclusions

It is now feasible to quickly and precisely identify the major genera and species of disease-causing organisms by combining contemporary, sophisticated immunological, and nucleic acid-based methods. Due to their high sensitivity and accuracy, monoclonal antibodies and PCR-based techniques can potentially displace current technologies. Thanks to NA-based methodologies, often regarded as fast pathogen detection tests, an increasing range of strategies are now accessible for addressing disease challenges that are of relevance in applied plant pathology programmes. Molecular processes may be put to use right now to advance our lab's technical capabilities and get ready for any threats. Given that these techniques are a bit challenging and time-consuming for data analysis, they must be carried out by qualified specialists.

Additionally, since the majority of these approaches do not give real-time detection, early warning systems and in-field testing are less suitable for them. Any pathogen detection methodology's limitations must be understood for optimal implementation, and NA-based procedures are no exception. Utilizing the right parameters is crucial when using NA-based tools to assure accuracy. Understanding the reliability of customary laboratory techniques and the need to accumulate several lines of evidence is also necessary for critically using such technologies. Modern, cutting-edge techniques have reproducible sensitivity and are frequently noticeably quicker than traditional techniques. Prompt assessment of fungal resistance levels may also help in the creation of successful resistance management techniques.

However, there is still a glaring knowledge deficit in this field of research since no single technique can satisfy the growing need for speedier, more efficient, reproducible, and sensitive results.

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