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REVIEW

New Approaches to Plant Pathogen Detection and Disease Diagnosis

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Abstract

Detecting plant pathogens and diagnosing diseases are critical components of successful pest management. These key areas have undergone significant advancements driven by breakthroughs in molecular biology and remote sensing technologies within the realm of precision agriculture. Notably, nucleic acid amplification techniques, with recent emphasis on sequencing procedures, particularly next-generation sequencing, have enabled improved DNA or RNA amplification detection protocols that now enable previously unthinkable strategies aimed at dissecting plant microbiota, including the disease-causing components. Simultaneously, the domain of remote sensing has seen the emergence of cutting-edge imaging sensor technologies and the integration of powerful computational tools, such as machine learning. These innovations enable spectral analysis of foliar symptoms and specific pathogen-induced alterations, making imaging spectroscopy and thermal imaging fundamental tools for large-scale disease surveillance and monitoring. These technologies contribute significantly to understanding the temporal and spatial dynamics of plant diseases.

Keywords: bioinformatics, biotechnology, disease control and pest management, epidemiology, microbe-genome sequencing, microbiome, modeling, pathogen detection

Although molecular biology or remote sensing technologies have made considerable progress, it is now possible to develop rapid, sensitive, specific, and precise detection and diagnostic protocols. However, some unresolved aspects remain, such as the need to demonstrate the viability and actual infective capacity of the organisms detected within plant microbiota when molecular tests are applied. Additionally, improving the accuracy of remote sensing detection models, particularly in disentangling biotic versus abiotic induced symptoms, remains a challenge. In this review, we provide a concise description of some of these new technologies, discuss their practical applications, and address some aspects that require further investigation.

The goal of sustainable agriculture is to fulfill the food needs of the ever-expanding global population while concurrently fostering sustainable economic development of agricultural areas. Plant diseases are one of the main factors limiting agricultural production and threaten the global food supply (Jeger et al. 2023; Ristaino et al. 2021; Savary et al. 2019). Throughout history, efforts have been made to combat pests and diseases to minimize the resulting losses from the damage they cause. Disease control measures encompass strategies including preventing a pathogen's entry into specific areas and eradicating or managing a pathogen when it is already reported in an area (Spadaro and Gullino 2019). Accurate detection and identification of a pathogen are essential because they can

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provide crucial information about its ecology, the potential threat it represents to crops, and information regarding the implementation of appropriate management practices to reduce any deleterious impacts (Koebnik et al. 2023).

The evolution of detection methods has been an iterative process, adapting in response to the technological capabilities in each era (Martinelli et al. 2015; Venbrux et al. 2023). The simple observation of visual symptoms, which is often the initial diagnostic strategy, is frequently insufficient to determine the causal agent of a disease. Even if symptoms are evident, diagnosis is not always straightforward. This challenge is not exclusive to plant diseases and occurs in diagnosis in other fields, as exemplified in human clinics by the recent COVID-19 pandemic, whose symptoms closely resemble those of common flu-like viruses or even mild colds (Czubak et al. 2021; Gardiner et al. 2012). There are numerous examples with plant diseases where misidentification is possible; for instance, tumors caused by *Agrobacterium* are very apparent but can sometimes be mistaken as plant genetic aberrations or, occasionally, as nematode-induced galls (Choi et al. 2019). Moreover, foliar discoloration caused by any plant pathogen can frequently be mistaken for a physiological nutrient deficiency. Different bacteria belonging to the genus *Xanthomonas* that infect citrus cause similar symptoms at the onset of the infectious processes. However, depending on the specific species of *Xanthomonas*, the infection may lead to citrus canker or citrus bacterial spot. The former is considered a very serious and quarantine-worthy disease in many countries, whereas the latter is a disease of less concern and is not usually regulated (Graham et al. 2004). The specific needs of a situation can dictate the diagnostic method to be used. Returning to the parallel between plant diseases and COVID-19, the first step taken to control the pandemic was the development of reliable diagnosis strategies to identify the pathogen, which ranged from immediate virus detection, using serological lateral flow devices, to less immediate but more sensitive and precise methods based on quantitative PCR (qPCR) (Rong et al. 2023).

In plant pathology, the situation parallels this reality, where detecting and identifying the pathogen are crucial steps in managing diseases, and the situation dictates the diagnostic method to use. For instance, to evaluate on-site disease incidence within a specific geographical area, a quick analysis of a large number of samples may be necessary, with an emphasis on speed rather than accuracy or sensitivity (Cambra et al. 2000; Hornero et al. 2020; Zarco-Tejada et al. 2018). Conversely, in cases where precise identification of a specific type of pathogen is required, such as one that necessitates molecular characterization, a very accurate diagnosis is essential. This approach may render the analysis of an extensive sample set unfeasible, thereby de-emphasizing the importance of speed. Unfortunately, achieving both speed and precision in a single method is not always possible, requiring different detection strategies for the two needs. Organizations such as the American Phytopathological Society, the European and Mediterranean Plant Protection Organization, and the International Seed Testing Association have outlined a set of criteria in terms of sensitivity, specificity, selectivity, repeatability, reproducibility, robustness, and accuracy that detection methodologies must meet to be validated (Cardwell et al. 2018; EPPO 2021a, b, 2022a, b; Groth-Helms et al. 2023; ISTA 2006) (Table 1). Multiple studies describe these validation processes conducted by various research groups for a variety of pathogens (Cellier et al. 2020; Junker et al. 2018; Sarniguet et al. 2013).

This article presents a comprehensive review of pathogen detection strategies and plant disease diagnosis. However, our intent transcends the mere compilation of articles on all available techniques, as some reviews have already covered this aspect (Martinelli et al. 2015; Venbrux et al. 2023). Instead, we focus on and discuss two major strategies that are currently yielding exceptional outcomes and that are poised to serve as the keystone of plant disease diagnosis in the future. The first approach describes the use of nucleic acids and the diverse technologies employed in their detection, involving either precise targets of specific pathogen genomes or the relatively recent use of entire nucleic acid content to identify pathogens within the plant's global microbiome. Within this strategy, we place special emphasis on next-generation sequencing (NGS) techniques, which have already demonstrated significant utility and undoubtedly are destined to underpin laboratory diagnosis in the future (Lebas et al. 2022). The second set of strategies that we address encompasses imaging spectroscopy and remote sensing techniques for disease detection. These approaches have undergone significant advances in recent decades, but significant diagnostic challenges remain in developing, refining, and applying them in the coming years (Cheshkova 2022; Singh et al. 2020).

Nucleic Acid-Based Detection and Diagnosis Methods

Recently, nucleic acid-based techniques have replaced many conventional detection approaches. Conventional methods often require the prior isolation of pathogens in culture media, as with bacteria, fungi, or oomycetes, and subsequently their identification. Alternatively, serological approaches may be utilized and have proven particularly useful, for example, in the case of viruses. Indeed, serological methods have not always been replaced by nucleic acid-based technologies, especially when antisera, antibodies, and specific tests are available, sufficiently accurate, and sometimes already commercialized. However, in the absence of such resources, the development of serological tests can be more challenging and time-consuming compared with molecular methods. Thus, methods such as the enzyme-linked immunosorbent assay remain relevant in certain situations, particularly for extensive or routine screenings (De Boer and López 2012; Fang and Ramasamy 2015; Kalimuthu et al. 2022; Venbrux et al. 2023) when speed is prioritized over factors such as sensitivity and precision. Furthermore, serological methods, including devices similar to those used in clinical settings, are still being developed (Byzova et al. 2018; Hodgetts et al. 2015; López-Soriano et al. 2017).

Nucleic acid-based techniques have proliferated primarily due to their advantages in terms of sensitivity and specificity. Regulatory agencies, such as the European and Mediterranean Plant Protection Organization, primarily include PCR-based methods, either conventional or real-time, in their guidelines, which are usually adopted by official diagnostic protocols (EPPO 2023). An essential aspect to consider in PCR protocols is the need to fine-tune the specificity of diagnostic reactions. A meticulous selection of sequences that unequivocally identify a pathogen is imperative. In this regard, genomic analyses have gained particular importance in recent years, as they are indispensable for a better knowledge of pathogens and the elements within their genome that distinguish them as that organism (Catara et al. 2021; Gardiner et al. 2012; Garita-Cambronero et al. 2017).

Although specificity, sensitivity, and speed in obtaining results are argued as positive factors of nucleic acid-based techniques, they also present a limitation: They detect microorganisms in any physiological state or just inert traces of DNA or RNA molecules from the deceased microorganisms (Cangelosi and Meschke 2014; Emerson et al. 2017). The dilemma of specifically detecting viable organisms may be particularly relevant in the case of reproductive or postharvest materials. For instance, detecting traces of nucleic acid from a virus, bacterium, or fungus in treated or disinfected fruits or seeds may not be significant because the pathogen will be unable to spread from the fruit or, in the case of a seed-transmitted pathogen, to produce a diseased plant (Narayanasamy 2011). Nowadays, the need to detect microorganisms solely in a viable state is a subject of intense debate, and various amplification strategies are briefly discussed and described in the next section (Hiddink et al. 2023).

TABLE 1
Comparison of the definitions of terminologies used in detection and diagnostics by The American Phytopathological Society, the European and Mediterranean Plant Protection Organization (EPPO), and the International Seed Testing Association (adapted from the EPPO document: https://upload.eppo.int/download/221odbcdc6308)

Organization	Equivalent terms used by the different organizations								
The American Phytopathological Society (Cardwell et al. 2018; Groth-Helms et al. 2023)	*Sensitivity* (*analytical*): Synonymous with "limit of detection"; smallest detectable amount of analyte that can be measured with a defined certainty. Analyte may include antibodies, antigens, nucleic acids, or live organisms.	*Sensitivity* (*diagnostic*): Proportion of known infected reference samples that test positive in the assay; infected plants that test negative are considered to have false-negative results. *Sensitivity* (*relative*): Proportion of reference samples defined as positive by one or a combination of test methods that also test positive in the assay being compared.	*Specificity* (*diagnostic*): Proportion of known uninfected reference plants that test negative in the assay; uninfected reference plants that test positive are considered to have false-positive results.		*Selectivity*: Capability to discriminate between the organism of interest and other organisms and components of the sample, such as host tissue. In binary analysis, selectivity is the equivalent of global accuracy taking into account all false reactions, both positive and negative.	*Repeatability*: Level of agreement between replicates of the same sample in the same exact conditions by the same operator, equipment, and reagents. For example, the test repeated by analyst A on instrument ABC using reagent lot XYZ on the same day (VIM 2008).	*Reproducibility*: Ability of a test method to provide consistent results for the same sample tested by the same method in different laboratories (VIM 2008).	*Robustness*: Assessment of an assay or material to produce expected results when subjected to testing outside its verified range of use (ICH 2005). Changes in variables such as temperature, humidity, and stability can be observed to verify whether the assay or material will maintain its validated characteristics when mishandling occurs or if there is a moderate risk that test conditions cannot be adequately controlled.	*Accuracy*: Assessment of nearness of a test value to the expected value. The expected value may be obtained from a known reference standard, reagent of known activity, or well-documented titer. This term may be used in other fields and regions to represent both trueness (ICH 2005) and bias (ISO/IEC 2008) or is an umbrella term broken down into specific categories of trueness and bias to evaluate systematic error (VIM 2008).
European and Mediterranean Plant Protection Organization (EPPO 2018, 2021a, b, 2022a, b)	*Analytical sensitivity*: Smallest amount of target that can be detected reliably.	*Diagnostic sensitivity*: Proportion of infected/infested samples testing positive compared with results from an alternative test or with the assigned values of samples.	*Diagnostic specificity*: Proportion of uninfected/uninfested samples testing negative compared with results from an alternative test or with the assigned values of samples.	*Analytical specificity* (comprises inclusivity and exclusivity): *Inclusivity*: Performance of a test with a range of target organisms covering genetic diversity, different geographical origin, and hosts. *Exclusivity*: Performance of a test with regard to cross-reaction with a range of nontargets.	*Selectivity*: Extent to which variations in the matrix affect the test performance (matrix effect).	*Repeatability*: Level of agreement between replicates of a sample tested under the same conditions.	*Reproducibility*: Ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions.	*Robustness of a test*: Extent to which altered test conditions affect the established test performance values (e.g., analytical sensitivity, analytical specificity).	*Accuracy*: Defined by the following formula: TP + TN/N, where TP = true positives, TN = true negatives, and N = total number of samples.
International Seed Testing Association (ISTA 2006)	*Limit of detection*: Lowest content that can be measured with reasonable statistical certainty.			*Cross reactivity*: Response (of method) to analogues, metabolites, or other nontarget components that may be present in the matrix(es).		*Repeatability*: Closeness of the agreement between the results of successive measurement of the same measure and carried out in the same conditions of measurement (IUPAC 2023).	*Reproducibility*: Precision under reproducibility conditions (i.e., conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment).	*Ruggedness test*: Intra-laboratory study to study the behavior of an analytical process when small changes in the environmental and/or operating conditions are made, akin to those likely to arise in different test environments.	

Approaches for the Detection and Identification of Pathogen Nucleic Acids in a Sample

Methods based on nucleic acid detection can be categorized into three major groups, which are sometimes interconnected. First, due to their current importance, is nucleic acid amplification techniques based on PCR or isothermal amplification of specific pathogen targets (Byzova et al. 2018; De Boer and López 2012). Second is a group of methodologies based on nucleic acid hybridization, which, although usually less sensitive than PCR techniques, yield excellent results for mass sampling, especially in the case of diseases caused by viruses (Melcher et al. 2014; Sánchez-Navarro et al. 2018). Third, there are sequencing techniques that are used after pathogen isolation for identification or, more recently, targeted massive sequencing techniques aimed at detecting a pathogen, a group of pathogens, or their presence within a plant's microbiota (Piombo et al. 2021).

Pathogen detection by PCR has become routine in most plant pathology diagnostic laboratories (Byzova et al. 2018; Hariharan and Prasannath 2020). The technology has evolved from conventional endpoint PCR, characterized by visualizing the PCR products at the end of the reaction. It includes multiple variations aimed at amplifying DNA or RNA or, for example, multitarget strategies to amplify different DNA or RNA sequences in the same sample. This is done for the simultaneous detection of multiple pathogens or different sequences of the same pathogen, enabling more precise detection and identification (Cesbron et al. 2020; Hariharan and Prasannath 2020; Pallás et al. 2018). Visualization of PCR products generated from the sample is most often achieved through gel electrophoresis of the PCR products, although there are other less widespread alternatives (Hariharan and Prasannath 2020; Nakano et al. 2017).

In recent years, conventional PCR has been replaced by real-time qPCR, which generally has higher sensitivity and relies on automated systems in which no further processing of PCR is required for visualization, with the consequent advantage of a lower risk of laboratory contamination. Furthermore, qPCR enables the quantification of pathogen concentrations in samples, offering accurate quantification methods. More recently, other PCR-automatized strategies with excellent sensitivity features have been developed that, although not yet widely used in diagnosis, are promising. One example is droplet digital PCR (ddPCR), where the sample is divided into thousands of water-in-oil droplets, each potentially holding zero or one copy of the template DNA/cDNA, which is then amplified. ddPCR is also a PCR-automatized system with excellent sensitivity and allows for the absolute quantification of nucleic acids. ddPCR, similar to conventional PCR and unlike qPCR, is an endpoint technique that does not require a standard curve for quantification. ddPCR has already been applied for the detection of various plant pathogens, including viruses, bacteria, fungi, or oomycetes (Lu et al. 2020; Morcia et al. 2020; Santander et al. 2019; Zhao et al. 2016). Today, PCR techniques, in one variant or another, have become the gold standard in plant pathology diagnostics, as has occurred in other fields, such as clinical diagnostics.

The other major group of amplification methods used in diagnosis is based on isothermal amplification (Van Ness et al. 2003). These methods are characterized by not involving different temperature cycles in the reactions; thus, they do not require the use of thermal cyclers. Moreover, it is often argued that they are more suitable for field analyses. The most used technique among these isothermal methods is loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000). LAMP is a highly effective and specific amplification technique to detect pathogens, and it has been widely applied in various biological fields due to its ease of use (Le and Vu 2017). LAMP has the advantage of not requiring complex sample preparation, and results are obtained in a shorter time than with other amplification methods and can be recorded in portable devices, making it more convenient for in-field application (Bühlmann et al. 2013; Gomez-Gutierrez and Goodwin 2022; Le and Vu 2017; Palacio-Bielsa et al. 2015; Panno et al. 2020). Other isothermal amplification methods include RPA, RCA, and NASBA. These methods, such as LAMP, do not rely on thermal cycling or gel electrophoresis to visualize the results, making them convenient for in-situ applications, despite their limited market share (Ivanov et al. 2021; Venbrux et al. 2023).

Regardless of the type, nearly all nucleic acid-based techniques require prior extraction protocols. In the case of plant material, this can be challenging due to the presence of inhibitors (Uchii et al. 2019). Furthermore, the nucleic acid must maintain sufficient integrity to be amplified, and sometimes meticulous care is necessary to prevent its degradation. Occasionally, to verify the quality of nucleic acid preparations, internal controls are included in the reactions. An internal control may involve amplifying sequences that are consistently present in the sample, such as those from the host plant's genome, or introducing synthetic molecules directly into the sample to act as artificial positive controls. The successful amplification of an internal control confirms the quality of the extracted nucleic acids (EPPO 2021b; Mittelberger et al. 2020).

Specificity of Nucleic Acid-Based Detection and Diagnosis Approaches

All diagnostic techniques must meet appropriate sensitivity and specificity requirements, among other needs, as stated above (Table 1). The sensitivity of a detection method is a relatively straightforward concept, as it corresponds to the minimum amount of the pathogen that can yield a positive result using that method. Specificity is defined as the ability of a method to detect a pathogen in a sample when it is present and to not detect it when the sample is uninfected. In other words, specificity measures the proportion of true negative results out of all the individuals who are disease-free. This implies the ability to differentiate the target pathogen from other closely related taxa that may have similar genetic traits and could be a component of the plant's microbiota. Therefore, selecting appropriate target DNA or RNA sequences in diagnostic strategies is essential to differentiate the pathogen from other nonpathogenic microorganisms present in the plant (Catara et al. 2021).

In a disease diagnosis protocol based on genomic-informed targets, it may be advisable to use sequences corresponding to genes that are somehow related to the pathogen's virulence. However, genes that play a role in pathogenicity often undergo selection and rapid evolution, which significantly increases the likelihood of false negatives in the tests (Boureau et al. 2013). Moreover, other targets, not associated or not yet linked with infectivity, can also distinguish between pathogens and non-pathogens, making them useful for disease diagnosis (Catara et al. 2021). In any case, and regardless of the design of the amplification protocol, the selection of the target sequence in the pathogen must be especially meticulous and the result of an exhaustive analysis. Over the past few years, many comparative genomics studies have been conducted to identify unique amplification targets that differentiate pathogens from non-pathogens and to design specific PCR protocols for disease diagnosis (Catara et al. 2021; Garita-Cambronero et al. 2016; Larrea-Sarmiento et al. 2018; Yasuhara-Bell et al. 2023).

As mentioned earlier, another intriguing aspect of specificity worth discussing is whether it is necessary to precisely detect the pathogens only when they retain their virulence features and not when they are epidemiologically irrelevant (i.e., living versus dead organisms). Molecular techniques initially lacked this capability, as they primarily rely on identifying nucleic acid fragments that may exhibit high stability and remain in the environment for an extended period, allowing them to be detectable. To address this issue, techniques such as PCR or nucleic acid sequence-based amplification

for amplifying messenger RNA, which have much lower stability and a shorter half-life, have been proposed (Golmohammadi et al. 2012; Scuderi et al. 2010; Wong et al. 2020). However, these technologies have not yet yielded the desired results for routine use, precisely due to the low stability and usually low concentration of these molecules, which limit the sensitivity level of techniques aimed at amplifying them.

A second group of strategies aimed at the exclusive amplification of living microorganisms involves the use of DNA intercalating agents, such as ethidium monoazide or propidium monoazide (PMA) (Hu et al. 2013; Nakano et al. 2017). These strategies involve rendering the nucleic acids from damaged microorganisms non-amplifiable by covalent binding with ethidium monoazide or PMA upon photoactivation (Nocker et al. 2006; Nogva et al. 2003). The approach relies on the integrity of a microorganism's outer membranes, assuming those that are degraded and allow the entry of ethidium monoazide, PMA, or its new improved version PMAxx correspond to nonviable organisms (Fig. 1). The methodologies have been assayed in phytopathology primarily focusing on plant-pathogenic bacteria, although there are some examples with fungal pathogens and even nematodes (Christoforou et al. 2014; Hu et al. 2013; Santander et al. 2019; Sert Çelik et al. 2020; Wang and Turechek 2020). However, similar to RNA amplification techniques, the so-called viability PCR based on intercalating agents is not entirely flawless, as amplification suppression may not be complete for all dead cells in the sample, occasionally leading to false-positive results, particularly when the PCR target presents at high concentrations (Nogva et al. 2003; Seinige et al. 2014; Wang and Turechek 2020).

NGS Nucleic Acid Detection

In less than 25 years since the beginning of the "omics era" with the first genome sequence of a free-living plant pathogen, the bacterium *Xylella fastidiosa* (Simpson et al. 2000), a revolution in NGS, and its application in understanding the molecular basis of pathogen and host biology has occurred. This revolution has been driven by studies on comparative genome or transcriptomic analysis (Adams et al. 2021; Liu et al. 2023).

Many studies conducted to date have provided a plethora of genomic data, primarily used to identify specific targets to deploy detection protocols, most of which are based on nucleic acid amplification as mentioned above (Ben Khedher et al. 2022).

Similar to other nucleic acid-based techniques for plant pathogen detection, for NGS, it is necessary to fine-tune all the steps concerning sample collection, nucleic acid purification, and the inclusion of positive, negative, and process controls. It should also undergo validation following established procedures and conditions used when proposing any new detection protocol. Fortunately, the scientific community has started to set the minimum required parameters to obtain high-quality and reproducible NGS detection protocols. As these aspects are out of the scope of this article, the interested reader can access this material from other sources (EPPO 2022c; Lebas et al. 2022; Massart et al. 2022).

Shotgun or amplicon-based metagenomics, the principles and characteristics of which have been recently reviewed (Piombo et al. 2021), can potentially be used to perform the sequencing, detection, and, to some extent, relative quantification of all the microorganisms present in a biological sample simultaneously. This capability opens the possibility of using it as a prescreening tool, providing a snapshot of the whole system, studying not only a specific host–pathogen interaction but also all the other organisms associated with the pathosystem under study and population changes caused by external forces. The information could be fundamental for developing broad-spectrum protocols to boost the screening tools for phytosanitary surveillance, similar to the approach being deployed for microbial surveillance in regard to human health (Dubois et al. 2022).

Metabarcoding, as an amplicon-based approach, is currently the more widely accessible approach to apply NGS to diagnose and surveil plant-pathogenic prokaryotes, fungi, and oomycetes. Despite its low technical complexity, a main drawback of metabarcoding is selecting the genomic target for taxonomic discrimination.

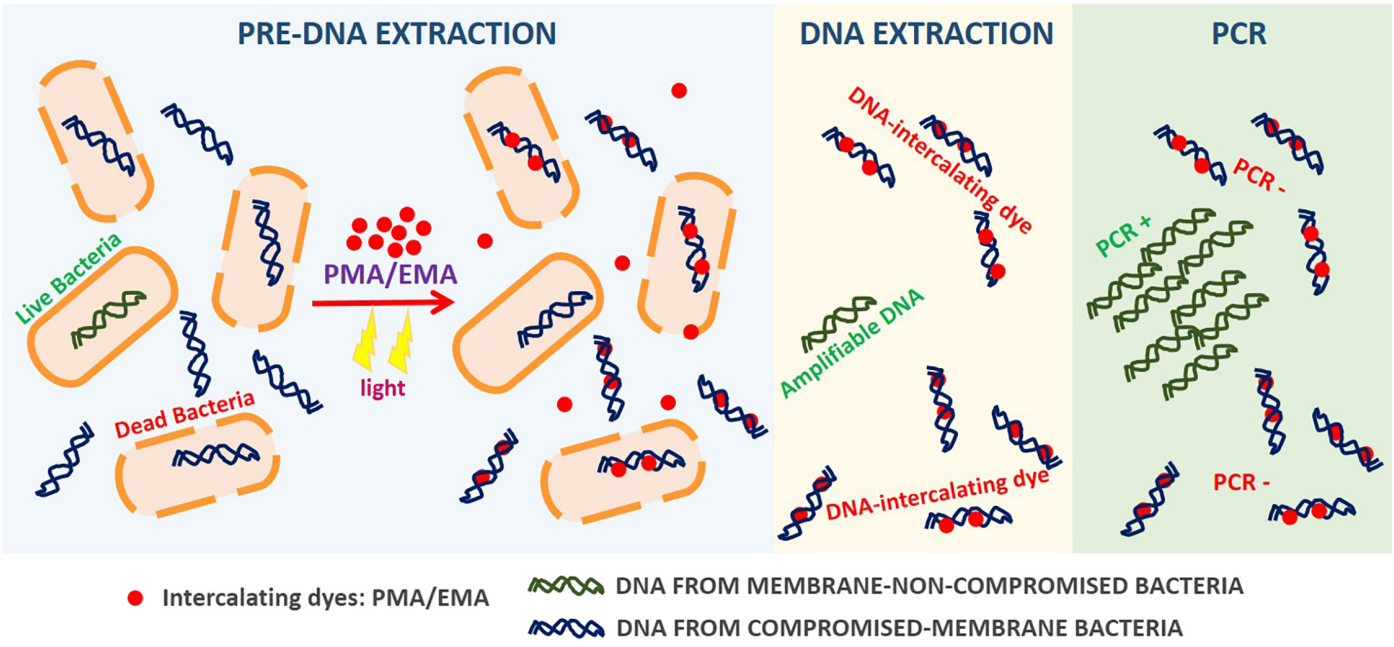

FIGURE 1
Schematic representation of a viability PCR assay for a population of live and dead bacteria. Intercalating agents propidium monoazide (PMA) and ethidium monoazide (EMA) covalently bind to free DNA or DNA from damaged cells. After extraction, free DNA or DNA from damaged cells are not amplified by PCR.

Partial 16S rDNA amplicons have been widely used in prokaryotes despite their low discriminative power at species or intraspecific levels and the possibility of amplifying genetic material from plant organelles (Giangacomo et al. 2021; Muhamad Rizal et al. 2020). Recent research in this field has highlighted the importance of exploring new genomic targets, such as other single-copy housekeeping genes, for example, the gene for the B subunit of the DNA gyrase (Barret et al. 2015). This gene outperformed 16S rDNA in discriminating the amplicons obtained up to a species or subspecies level with a low amount of host amplicon contamination (Newberry et al. 2023). Taxonomic assignment improvement is also needed for typing eukaryotic plant pathogens, such as oomycetes and fungi, where 18S rDNAs and partial regions of the internal transcribed spacer are the most used targets for metabarcoding. The primers used in the PCR amplification step can bias the results and should not be used to infer the absence of any particular species if used as a screening tool. However, these targets seem to be helpful when primers are adapted to study the diversity or presence of a particular genus (Chen et al. 2022; Makiola et al. 2019; Reich et al. 2023; Rossmann et al. 2021). To address this limitation, the use of a full-length internal transcribed spacer region or the addition of another target gene, such as the translation elongation factor 1-alpha, are strategies proposed mainly for gaining taxonomic accuracy and discrimination power, which is feasible by applying long-read third-generation sequencers such as PacBio and Oxford Nanopore platforms (Jacky et al. 2021).

When exploring and utilizing new sets of barcoding genes, it is essential to have curated and high-quality reference databases for the selected genomic targets. Thanks to the current availability of open databases with massive general data and the development of bioinformatic pipelines devoted to creating reference taxonomical databases, this multitarget approach could become more implanted into the identification and surveillance of plant pathogens (Dubois et al. 2022; Makiola et al. 2019). Another factor to consider when using metabarcoding is its dependence on a pre-amplification step of the target by PCR, which, in this case, can be seen as a double-edged sword. On one side, the approach renders metabarcoding a highly sensitive detection tool, but on the other side, it runs a significant likelihood of introducing external contamination, which could be amplified with each PCR cycle, potentially yielding false or low-quality data. Proper handling of materials during sampling and nucleic acid extraction, along with the use of environmental controls taken at the sampling site and in the laboratory, assists in discriminating the actual set of organisms present in the analyzed sample (Jacky et al. 2021).

Whole-genome metagenomic sequencing is another approach to determine the presence of all the DNA associated with plant-pathogenic organisms, including those that are unknown or not culturable, and it does not require previous genetic knowledge of the pathogen causing the disease. In contrast to metabarcoding, shotgun metagenomics avoids PCR-associated biases and obtains information from longer DNA regions, which provide more reliable taxonomic assignments. However, it also provides much more genomic information about the pathogenic and other metabolic characteristics of the organisms in the sample (Venbrux et al. 2023). Nevertheless, shotgun metagenomics is less accessible for general diagnostics and plant disease surveillance due to the associated drawbacks of specialized sample processing, sequencing depth, computational resources, and the need for more specialized bioinformatic knowledge, as discussed below (Piombo et al. 2021). Despite not being widely used, a few examples demonstrate the feasibility of the technique for detecting plant-pathogenic fungi, oomycetes, and bacteria (Venbrux et al. 2023). Shotgun metagenomics has been more widely used for detecting viral pathogens, enabling early and accurate nontarget detection, which is helpful in phytosanitary surveillance and certification programs for propagating disease-free materials, as well as for surveillance frameworks using other types of samples, for example, sewage water (Duarte et al. 2023; Roux et al. 2021; van de Vossenberg et al. 2020).

Current portable real-time third-generation sequencers, such as those using Oxford Nanopore technology, are making whole shotgun metagenomics increasingly accessible, affordable, and less time-consuming. This allows for viral RNA/DNA sequencing in as little as 1 h and the completion of the entire metagenomics analyses pipeline in up to 24 h (Sun et al. 2022). Despite being successfully applied in several pathosystems related to fruit trees and herbaceous and ornamental plants (Lee et al. 2022; Sun et al. 2022), Oxford Nanopore technology still has a series of obstacles that preclude its broader application in plant pathogen surveillance. The obstacles include the lower read accuracy when compared with other sequencing platforms, especially second-generation sequencers, and the lack of a user-friendly bioinformatics platform. Current bioinformatic tools for the platform often underperform in terms of accuracy and require users with proficiency in coding and command of a Linux-based environment. Addressing these technical limitations will likely have a significant impact on future detection, identification, and characterization of pathogens threatening agriculture, similar to its application in clinical and public health (Gauthier et al. 2023).

Current advancements in data analyses, machine learning algorithms, and artificial intelligence may, in the near future, integrate knowledge generated by multiple scientific disciplines and deploy dynamic models for disease surveillance and outbreak predictions to give a rapid response at a landscape scale. In this context, historical and current NGS data archives can be used in bioinformatic predicting tools to identify novel strains of pathogen lineages, understand their evolution, and track their movement in real time. These capabilities can allow for more accurate fine-tuning of current model parametrization and better constraint of the chains of transmission. Initiatives in this direction are already in progress, exemplified by Nextstrain, which has been used to understand the epidemiology and improve management responses to detection of pathogens such as tomato brown rugose fruit virus based on the pathogen evolutionary information obtained from NGS projects (van de Vossenberg et al. 2020).

One aspect to discuss regarding detection methodologies based on the comprehensive analysis of the plant microbiota is the compelling need to identify whether any of its components are genuinely harmful to the plant (i.e., pathogens), simply resident microflora that exert no detrimental effects (Mannaa and Seo 2021), or even phytobiome communities involved in beneficial interactions with the plant that improve the health and growth of the host, conferring tolerance to biotic and abiotic stresses (Ali et al. 2023a, b). Is it essential for diagnosis to determine all the viruses, bacteria, fungi, oomycetes, or viroids in a sample? Further studies are needed to deepen our understanding of the plant microbiota and its impact on plant health. Metagenomic analyses can contribute to addressing this by identifying genes in the samples involved in the infective processes of microorganisms. Although NGS technologies are already being implemented in diagnostic laboratories, it is crucial for regulatory organisms to have a clear understanding of those microbiota components that, either individually or in combination with others, are capable of causing a disease or syndrome. This understanding helps prevent unnecessary measures based on detecting a microorganism whose potential harmful effects are unknown, similar to what was discussed earlier regarding nonviable microorganisms that do not pose any epidemiological risk (Mannaa and Seo 2021; Trivedi et al. 2020).

Spectral-Based Detection of Pathogen-Induced Symptoms

Visual monitoring is a widely used method for plant disease detection. When integrated into a prognosis system alongside regional

weather and other epidemiological parameters, it may become a valuable tool for predicting the spread of diseases in specific geographic areas (Ul Haq and Ijaz 2020). Methods for visual monitoring are now based on a firmer scientific understanding and can be applied in a more informed and nuanced manner to ensure appropriate methodology to maximize accuracy and reliability (Bock et al. 2022). The development and use of ordinal disease scales and standard diagrams are well-established examples (Chiang et al. 2014; Del Ponte et al. 2017).

However, visual inspection is typically an expensive, laborious, and time-consuming methodology (Habib et al. 2022). Furthermore, as mentioned earlier, disease diagnosis based on the host plant's symptoms is not always accurate. Numerous diseases have symptoms similar to physiological abnormalities induced by external factors, and some infections can remain asymptomatic or exhibit only mild, weakly identifiable symptoms in the initial stages of development (Habib et al. 2022). Moreover, visual detection frequently results in disease detection occurring when the optimal window for implementing effective control measures has already passed (Steiner et al. 2008). Diseases, as well as abiotic stress, often exhibit temporal and spatial heterogeneity within a cropped field. Differences in the physical environment, including factors such as soil conditions and microclimate, can interact with crop development and the life cycles of pathogens, resulting in heterogeneity of disease incidence and severity across the field (Oerke 2020). As a result, assessing site-specific disease management on a large scale requires a detailed recording of spatial distribution and disease progression. This, in turn, requires extensive georeferenced monitoring of crop diseases to ensure precise timing and application of control measures (Nutter et al. 2011). Consequently, there is a need for accurate and time-efficient methods for disease monitoring, encompassing detection, identification, and quantification (Oerke 2020).

Detection of Pathogen-Induced Symptoms with Imaging Spectroscopy and Spectral Analysis

Pathogens that colonize and parasitize plants induce changes in the metabolism and alter the biochemical and physical status of plant tissues, resulting in visible disease symptoms (Oerke 2020). These visible symptoms become apparent after a pathogen-specific latency period that is influenced by environmental factors, with durations ranging from days to months. The observable effects provide a physical foundation for their remote monitoring using sensing techniques (Zhang et al. 2019).

Symptoms on susceptible crops can include (i) lesions and necrotic tissues, which may vary in color and shape depending on the specific host and pathogen involved, and they can occur in localized areas or be uniformly distributed throughout the canopy (Cao et al. 2013; Moshou et al. 2004); (ii) degradation of pigment systems (pathogen infection can commonly lead to the deterioration of chloroplasts and other organelles, resulting in alterations in pigment content, including chlorophyll, carotenoids, and anthocyanins) (Grisham et al. 2010; Zhang et al. 2012); and (iii) wilting, which results from the loss of plant rigidity due to dehydration. With some diseases, particularly those affecting the roots or vascular system, water flow may be restricted within the plants, leading to dehydration throughout the entire plant (Calderón et al. 2013).

Most imaging spectroscopy studies have focused primarily on foliar pathogens in annual crops, where disease symptoms are characterized mainly by the first two types of symptoms (i.e., necrotic tissues or distinct color changes in the aboveground parts of the plant). However, imaging spectroscopy is still poorly developed for the detection of diseases caused by soilborne plant pathogens, mainly fungi, oomycetes, and nematodes, which parasitize plant roots, disrupting the xylem vessels and reducing nutrient and water uptake with a reduction in leaf transpiration rate, which leads to a decline characterized by leaf chlorosis and defoliation (Hillnhütter et al. 2010). The symptoms often become visible in the later stages of the disease (Oerke 2020).

Spectral Imaging Methods and Indicators of Biotic-Induced Stress

Remote sensing techniques based on spectral analyses have successfully detected biotic-induced symptoms of disease even at the early (pre-visual) stages of infection (Zarco-Tejada et al. 2018, 2021). Imaging spectroscopy and thermal imaging measure the reflected and emitted radiation by plants across the electromagnetic spectrum in several narrow spectral bands, particularly in the visible (400 to 700 nm), near-infrared (700 to 1,300 nm), shortwave infrared (1,300 to 2,500 nm), and thermal infrared (8 to 14 μm) spectral regions. It can also detect the emission of solar-induced fluorescence in the 650- to 800-nm spectral region, a signal widely considered a proxy for plant photosynthesis (Mohammed et al. 2019). Spectral indicators obtained by these remote sensing techniques, in the form of vegetation indices, spectral-based plant traits, fluorescence emission, and canopy temperature, are proposed for the detection of subtle physiological changes occurring in vegetation at both early and advanced stages of pathogen infection (Hernández-Clemente et al. 2019). Recent studies have demonstrated that hyperspectral and thermal imagery obtained by aerial platforms can detect physiological changes and symptoms associated with diseases, such as holm oak (*Quercus ilex*) decline induced by *Phytophthora cinnamomi* (Hornero et al. 2021); physiological alterations in olive (*Olea europaea*) caused by *Xylella fastidiosa* infection (Zarco-Tejada et al. 2018); wilt of olive caused by *Verticillium dahliae* (Calderón et al. 2013); Aphanomyces root rot in lentil (*Lens culinaris*) caused by *Aphanomyces euteiches* (Marzougui et al. 2019); Rhizoctonia crown and root rot of sugar beet (*Beta vulgaris*) induced by *Rhizoctonia solani* (Reynolds et al. 2012); Cercospora leaf spot of sugar beet caused by *Cercospora beticola*, *Erysiphe betae*, and *Uromyces betae* (Mahlein et al. 2010); late blight and early blight in potato (*Solanum tuberosum*) caused by *Phytophthora infestans* and *Alternaria solani*, respectively (Gold et al. 2020); South American leaf blight in rubber trees (*Hevea brasiliensis*) caused by *Pseudocercospora ulei* (Sterling and Di Rienzo 2022); and yellow rust in wheat (*Triticum aestivum*) caused by *Puccinia striiformis* f. sp. *tritici* (Devadas et al. 2009; Ren et al. 2021), among others.

The detection of biotic-induced symptoms using imaging spectroscopy, based on the sensitivity of band ratios and normalized indices, relies on their sensitivity to photosynthetic and non-photosynthetic plant pigments such as chlorophyll *a*+*b*, carotenoids, anthocyanins, and xanthophylls, as well as changes occurring to specific spectral bands due to structural changes in the leaf and canopy at advanced stages of the disease progression. These plant pigments absorb radiation in the 400- to 700-nm spectral region. Thus, reflectance indicators calculated in this region are sensitive to changes in the photosynthetic dynamics of infected vegetation. The near-infrared and shortwave infrared regions have also been demonstrated sensitive for disease monitoring because this region tracks the absorption due to plant water, dry matter, and nutrients that are affected under biotic stress (Camino et al. 2022). The fundamental basis underlying the spectral detection of symptoms induced by pathogen infection is based on the photoprotective role of xanthophylls, protection from damage by anthocyanins (Lev-Yadun and Gould 2008), and damage of the photosynthetic apparatus under infection. These molecules accumulate in infected vegetation and are produced during the degradation of chlorophyll into phaeophytin (Barnes et al. 1992; De La Fuente et al. 2013; Peñuelas et al. 1995). Overall, changes in the photosynthesis and stomatal

regulation (Zeng et al. 2010) caused by plant−pathogen interactions (Berger et al. 2007) lead to reductions in fluorescence emission (Calderón et al. 2013; Tung et al. 2013) and transpiration rates (Chaerle et al. 2004), producing phenolic plant defense compounds (Barón et al. 2016).

Several ratios and normalized indices derived from spectral data have been proposed since the late 1970s. The indices are calculated from spectral reflectance data measured by non-imaging and imaging spectrometers covering the visible, near-infrared, and shortwave infrared spectral regions. The indices are calculated after the data are calibrated and converted into spectral reflectance to be comparable across dates and changing laboratory or ambient light and atmospheric conditions. This physical quantity represents, for each wavelength, the reflected radiation measured from the leaf or the vegetation canopy under study. The normalized difference vegetation index has been widely used for vegetation monitoring (Rouse et al. 1974) because it is sensitive to vegetation growth and canopy density. The photochemical reflectance index (PRI) (Gamon et al. 1992) has been used for tracking the dynamics of the xanthophyll pigments pool, thus being proposed for the detection of biotic-induced symptoms due to the sensitivity to the light-use efficiency and photosynthetic performance (Calderón et al. 2013). Several PRI variants, such as the normalized PRI (Zarco-Tejada et al. 2013a) and other modified PRIs, have been proposed to track both biotic and abiotic stresses (Camino et al. 2021; Hernández-Clemente et al. 2011; Poblete et al. 2020). Other indices sensitive to plant pigments have proven useful for disease monitoring, such as the normalized phaeophytinization index (Barnes et al. 1992; Peñuelas et al. 1995). Additionally, there are indices sensitive to chlorophyll *a+b*, such as the Vogelmann index (Vogelmann 1993) and the transformed chlorophyll absorption ratio index (Haboudane et al. 2002), which are normalized by the soil adjusted vegetation index (Rondeaux et al. 1996). Other water-sensitive indices, such as the water index (Peñuelas et al. 1993) and the normalized difference water index (Gao 1996), have been used to monitor symptoms caused by fire blight in apple (*Malus domestica*) induced by *Erwinia amylovora* (Skoneczny et al. 2020), Southern corn (*Zea mays*) rust caused by *Puccinia polysora* (Meng et al. 2020), and Fusarium head blight caused by *Fusarium* on wheat (Huang et al. 2021). Other specific indices, such as the healthy index, were developed to monitor sugar beet diseases by multiple iterations and selection of spectral reflectance bands (Mahlein et al. 2013). A list of the most widely used vegetation indices proposed for vegetation stress detection is presented in Table 2.

Although vegetation indices and spectral transforms are sensitive to physiological changes in infected plants and can be used to detect disease incidence and severity, they still have limitations. Spectral indices are affected by multiple factors, including the soil background, sun angle effects, and vegetation shadows, as well as by multiple biochemical constituents absorbing radiation in overlapping spectral regions. The inversion of radiative transfer models enables the simultaneous retrieval of the leaf biochemistry and the canopy structural traits (Jacquemoud 1993; Jacquemoud et al. 1996, 2009; Ustin et al. 2009). Unlike single ratios and normalized indices, which are simultaneously sensitive to several traits, the plant traits estimated by the inversion of radiative transfer models reveal a more comprehensive status of the physiology of vegetation undergoing pathogen infection (Zarco-Tejada et al. 2018). In addition, quantifying traits by physically based simulations improves transferability to other pathosystems and geographic locations because the retrieval methods are not empirically based. One of the most widely used radiative transfer models is PRO4SAIL, a linked leaf model PROSPECT (Féret et al. 2017; Jacquemoud and Baret 1990) with a canopy simulation model SAIL/4SAIL (Verhoef 1984; Verhoef et al. 2007). This linked leaf-canopy simulation approach has been successfully used to estimate leaf biochemical constituents and canopy structural parameters from vegetation, which are then used as inputs in machine-learning models for disease incidence and severity detection (Poblete et al. 2021, 2023; Zarco-Tejada et al. 2018). Recent significant progress was achieved by developing a modeling framework to quantify the overall status of the physiological condition of infected vegetation. The approach focused on the quantification of (i) a pool of narrow-band spectral traits, (ii) solar-induced fluorescence and fluorescence efficiency, (iii) spectral-based leaf and canopy traits, and (iv) transpiration indicators of water stress (Zarco-Tejada et al. 2018). This multilayered functional plant-trait scheme has been successfully applied to the vascular pathogens *X. fastidiosa* (Zarco-Tejada et al. 2018, 2021) and *V. dahliae* (Poblete et al. 2021, 2023) using airborne imaging spectroscopy data collected from infected crops in Europe. These indicators were inputs for a multi-step modeling approach to detect disease-induced symptoms (Poblete et al. 2023), linking mechanistic and machine-learning algorithms.

Machine-Learning Models for Disease Incidence and Severity Assessment

Machine-learning algorithms for disease incidence and severity assessment are proposed with inputs such as spectral-based indices, leaf biochemical and canopy structural parameters estimated by model inversion techniques, solar-induced fluorescence, and canopy temperature (Poblete et al. 2023; Zarco-Tejada et al. 2018). To enhance the detection of infected vegetation, modeling schemes based on multistage classification methods have been implemented, enabling quantification of the trait's contribution to the overall model performance (Poblete et al. 2021).

The traditional approach for detecting infected vegetation has been based on empirical methods such as regression analysis, which typically involves a single input. For example, the PRI alone could detect yellow rust in winter wheat (Huang et al. 2007). In another study, a single thermal indicator between canopy temperature depression and partial least squares regression was used to detect Dothistroma needle blight in Scots pine (Smigaj et al. 2019). The detection was most accurate when the thermal imagery was obtained during periods of the greatest solar radiation and maximum photosynthetic activity. Studies by Huang et al. (2007) and Zhang et al. (2012) demonstrated that the physiological reflectance index was the only index sensitive to the detection of yellow rust, whereas other indices, such as the PRI, the normalized pigment chlorophyll ratio index, and the anthocyanin reflectance index, despite being sensitive to the detection of infection, were also sensitive to abiotic stresses such as water stress, leading to errors in the detection of biotic-induced symptoms. These confounding effects highlight the crucial aspect of distinguishing among symptoms caused by various pathogens. Gold et al. (2020) used hyperspectral data and partial least squares discriminant analysis to distinguish between fungal infections in potatoes due to *Phytophthora infestans* and *Alternaria solani*, two pathogens that cause similar necrotic leaf symptoms. Partial least squares discriminant analysis was also used to discriminate between oak wilt, caused by the fungus *Bretziella fagacearum*, and bur oak blight, caused by the fungus *Tubakia iowensis* (Fallon et al. 2020). Both pathogens produce similar symptoms that can be mistaken for oak wilt. To overcome the limitations of empirical approaches based on single indicators of infection, machine-learning algorithms coupled with radiative transfer models have made progress in understanding the intrinsic and complex relationships between physiology and remote sensing-derived plant traits to discriminate between infections. In a study conducted by Poblete et al. (2021), a multistage classification algorithm enabled the differentiation between two vascular pathogens, *X. fastidiosa* and *V. dahliae*. The results revealed that it was possible to distinguish between the two sources of infection through a multistage machine-learning classification algorithm. Specifically, the key spectral traits

TABLE 2
Indices derived from hyperspectral and thermal data related to plant physiological condition

Hyperspectral indices	Equation	Reference(s)
Structural indices		
Normalized difference vegetation index	$NDVI = (R_{800} - R_{670})/(R_{800} + R_{670})$	Rouse et al. 1974
Renormalized difference vegetation index	$RDVI = (R_{800} - R_{670})/\sqrt{(R_{800} + R_{670})}$	Roujean and Breon 1995
Optimized soil-adjusted vegetation index	$OSAVI = ((1 + 0.16) \cdot (R_{800} - R_{670})/(R_{800} + R_{670} + 0.16))$	Rondeaux et al. 1996
Modified soil-adjusted vegetation index	$MSAVI = \frac{2 \cdot R_{800} + 1 - \sqrt{(2 \cdot R_{800} + 1)^2 - 8(R_{800} - R_{670})}}{2}$	Qi et al. 1994
Triangular vegetation index	$TVI = 0.5 \cdot [120 \cdot (R_{750} - R_{550}) - 200 \cdot (R_{670} - R_{550})]$	Broge and Leblanc 2001
Modified triangular vegetation index 1	$MTVI1 = 1.2[1.2(R_{800} - R_{550}) - 2.5(R_{670} - R_{550})]$	Haboudane et al. 2004
Modified triangular vegetation index 2	$MTVI2 = \frac{1.5[1.2(R_{800} - R_{550}) - 2.5(R_{670} - R_{550})]}{\sqrt{(2R_{800} + 1)^2 - (6R_{800} - 5\sqrt{R_{670}}) - 0.5}}$	Haboudane et al. 2004
Modified chlorophyll absorption index	$MCARI = [(R_{700} - R_{670}) - 0.2(R_{700} - R_{550})] \cdot (R_{700}/R_{670})$	Haboudane et al. 2004
Modified chlorophyll absorption index 1	$MCARI1 = 1.2[2.5(R_{800} - R_{670}) - 1.3(R_{800} - R_{550})]$	Haboudane et al. 2004
Modified chlorophyll absorption index 2	$MCARI2 = \frac{1.5[2.5(R_{800} - R_{670}) - 1.3(R_{800} - R_{550})]}{\sqrt{(2R_{800} + 1)^2 - (6R_{800} - 5\sqrt{R_{670}}) - 0.5}}$	Haboudane et al. 2004
Simple ratio	$SR = R_{800}/R_{670}$	Jordan 1969
Modified simple ratio	$MSR = \frac{R_{800}/R_{670} - 1}{(R_{800}/R_{670})^{0.5} + 1}$	Chen 1996
Enhanced vegetation index	$EVI = 2.5 \cdot (R_{800} - R_{670})/(R_{800} + 6 \cdot R_{670} - 7.5 \cdot R_{800} + 1)$	Liu and Huete 1995
Pigment indices		
Vogelmann indices	$VOG1 = R_{740}/R_{720}$	Vogelmann 1993
	$VOG2 = (R_{734} - R_{747})/(R_{715} + R_{726})$	Vogelmann 1993
	$VOG3 = (R_{734} - R_{747})/(R_{715} + R_{720})$	Vogelmann 1993
Gitelson and Merzlyak indices	$GM1 = R_{750}/R_{550}$	Gitelson and Merzlyak 1996
	$GM2 = R_{750}/R_{700}$	Gitelson and Merzlyak 1996
Transformed chlorophyll absorption in reflectance index	$TCARI = 3 \cdot [(R_{700} - R_{670}) - 0.2 \cdot (R_{700} - R_{550}) \cdot (R_{700}/R_{670})]$	Haboudane et al. 2002
Transformed chlorophyll absorption in reflectance index/optimized soil-adjusted vegetation index	$\frac{TCARI}{OSAVI} = \frac{3 \cdot [(R_{700} - R_{670}) - 0.2 \cdot (R_{700} - R_{550}) \cdot (R_{700}/R_{670})]}{((1 + 0.16) \cdot (R_{800} - R_{670})/(R_{800} + R_{670} + 0.16))}$	Haboudane et al. 2002
Chlorophyll index red edge	$CI = R_{750}/R_{710}$	Haboudane et al. 2002
Simple ratio pigment index	$SRPI = R_{430}/R_{680}$	Barnes et al. 1992; Peñuelas et al. 1995
Normalized phaeophytinization index	$NPQI = (R_{415} - R_{435})/(R_{415} + R_{435})$	Barnes et al. 1992; Peñuelas et al. 1995
Normalized pigments index	$NPCI = (R_{680} - R_{430})/(R_{680} + R_{430})$	Peñuelas et al. 1995
Carter indices	$CTRI1 = R_{695}/R_{420}$	Carter 1994
	$CAR = R_{695}/R_{760}$	Carter et al. 1996
Reflectance band ratio indices	$DCabCxc = R_{672}/(R_{550} \cdot 3R_{708})$	Datt 1998
	$DNIRCabCxc = R_{860}/(R_{550} \cdot R_{708})$	Datt 1998
Structure-intensive pigment index	$SIPI = (R_{800} - R_{445})/(R_{800} + R_{680})$	Peñuelas et al. 1995
Carotenoid reflectance indices	$CRI_{550} = (1/R_{510}) - (1/R_{550})$	Gitelson et al. 2003, 2006
	$CRI_{700} = (1/R_{510}) - (1/R_{700})$	Gitelson et al. 2003, 2006
	$CRI_{550\_515} = (1/R_{515}) - (1/R_{550})$	Gitelson et al. 2006
	$CRI_{700\_515} = (1/R_{515}) - (1/R_{700})$	Gitelson et al. 2006
	$RNIR \cdot CRI_{550} = (1/R_{510}) - (1/R_{550}) \cdot R_{770}$	Gitelson et al. 2003, 2006
	$RNIR \cdot CRI_{700} = (1/R_{510}) - (1/R_{700}) \cdot R_{770}$	Gitelson et al. 2003, 2006

(*Continued on next page*)

required to differentiate *V. dahliae*-infected trees from those affected by *X. fastidiosa* included the blue region, the structural parameter leaf inclination distribution function, and the carotenoid pigment content C_{x+c}. Conversely, to discriminate between *V. dahliae* and *X. fastidiosa* infections, the normalized PRI, the blue index BF_1, the fluorescence curvature index CUR, and the chlorophyll index CRI_{700M} were identified as essential factors for effectively distinguishing between these infections. The potential of using spectral

TABLE 2
(*Continued from previous page*)

Hyperspectral indices	Equation	Reference(s)
Plant senescencing reflectance index	$PSRI = (R_{680} - R_{500})/R_{750}$	Merzlyak et al. 1999
Pigment specific simple ratio chlorophyll a	$PSSRa = R_{800}/R_{675}$	Blackburn 1998
Pigment specific simple ratio chlorophyll b	$PSSRb = R_{800}/R_{650}$	Blackburn 1998
Pigment specific simple ratio carotenoid	$PSRRc = R_{800}/R_{500}$	Blackburn 1998
Pigment specific normalized difference	$PSNDc = (R_{800} - R_{470})/(R_{800} + R_{470})$	Blackburn 1998
Xanthophyll indices		
Photochemical reflectance index (570)	$PRI_{570} = (R_{570} - R_{531})/(R_{570} + R_{531})$	Gamon et al. 1992
Photochemical reflectance index (515)	$PRI_{515} = (R_{515} - R_{531})/(R_{515} + R_{531})$	Hernández-Clemente et al. 2011
Photochemical reflectance index (512)	$PRI_{m1} = (R_{512} - R_{531})/(R_{512} + R_{531})$	Hernández-Clemente et al. 2011
Photochemical reflectance index (600)	$PRI_{m2} = (R_{600} - R_{531})/(R_{600} + R_{531})$	Gamon et al. 1992
Photochemical reflectance index (670)	$PRI_{m3} = (R_{670} - R_{531})/(R_{670} + R_{531})$	Gamon et al. 1992
Photochemical reflectance index (670 and 570)	$PRI_{m4} = (R_{570} - R_{531} - R_{670})/(R_{570} + R_{531} + R_{670})$	Hernández-Clemente et al. 2011
Normalized photochemical reflectance index	$PRI_n = PRI_{570}/[RDVI \cdot (R_{700}/R_{670})]$	Zarco-Tejada et al. 2013a, b
Carotenoid/chlorophyll ratio index	$PRI \cdot CI = (R_{570} - R_{530})/(R_{570} + R_{530}) \cdot ((R_{760}/R_{700}) - 1)$	Garrity et al. 2011
R/G/B indices		
Redness index	$R = R_{700}/R_{670}$	Gitelson et al. 2000
Greenness index	$G = R_{570}/R_{670}$	Calderón et al. 2013
Blue index	$B = R_{450}/R_{490}$	Calderón et al. 2013
Blue/green indices	$BGI1 = R_{400}/R_{550}$	Zarco-Tejada et al. 2005
	$BGI2 = R_{450}/R_{550}$	Zarco-Tejada et al. 2005
Blue/red indices	$BRI1 = R_{400}/R_{690}$	Zarco-Tejada et al. 2012
	$BRI2 = R_{450}/R_{690}$	Zarco-Tejada et al. 2012
BF1	$BF1 = R_{400}/R_{410}$	Zarco-Tejada et al. 2018
BF2	$BF2 = R_{400}/R_{420}$	Zarco-Tejada et al. 2018
BF3	$BF3 = R_{400}/R_{430}$	Zarco-Tejada et al. 2018
BF4	$BF4 = R_{400}/R_{440}$	Zarco-Tejada et al. 2018
BF5	$BF5 = R_{400}/R_{450}$	Zarco-Tejada et al. 2018
Red/green indices	$RGI = R_{690}/R_{550}$	Zarco-Tejada et al. 2005
Ratio analysis of reflectance spectra	$RARS = R_{746}/R_{513}$	Chappelle et al. 1992
Lichtenthaler index	$LIC1 = (R_{800} - R_{680})/(R_{800} + R_{680})$	Lichtenthaler 1996
	$LIC2 = R_{440}/R_{690}$	Lichtenthaler 1996
	$LIC3 = R_{440}/R_{740}$	Lichtenthaler 1996
Chlorophyll fluorescence		
Reflectance curvature index	$CUR = (R_{675} \cdot R_{690})/R_{683}^2$	Plascyk 1975; Zarco-Tejada et al. 2000
Fraunhofer line depth (FLD) principle	$FLD = \frac{E_{out} \cdot L_{in} - E_{in} \cdot L_{out}}{E_{out} - E_{in}}$	Mohammed et al. 2019
Plant disease index		
Healthy index	$HI = \frac{(R_{534} - R_{698})}{R_{534} + R_{698}} - \frac{1}{2} \cdot R_{704}$	Mahlein et al. 2012
Thermal index		
Crop water stress index (CWSI)	$CWSI = \frac{(T_c - T_a) - (T_c - T_a)_{LL}}{(T_c - T_a)_{UL} - (T_c - T_a)_{LL}}$ LL, UL = lower and upper limits, respectively	Idso et al. 1981

features and plant traits to monitor pre-visual symptoms of disease infection has been explored for other pathogens; promising results were obtained in the pre-visual detection of rice leaf blast infection (Tian et al. 2021). The authors demonstrated the feasibility of identifying infections at their early stages by combining two to four spectral features.

Based on a multistage classification process, machine-learning models have been proposed to distinguish biotic and abiotic stressors (water and nutrient stress versus symptoms of vascular diseases) and multiple pathogens that trigger similar symptoms in plants (e.g., *V. dahliae* versus *X. fastidiosa*). Differentiating among types of stress is achieved by assessing the plant pigments' dynamics quantified by imaging spectroscopy, such as chlorophyll *a+b*, carotenoids, anthocyanins, and xanthophylls. Through screening analyses of spectral traits, these plant pigments show divergent trends as a function of pathogen-induced stress versus water or nutrient deficiency levels (Zarco-Tejada et al. 2021). Methods consist, first, of a feature-weighted random forest algorithm (Liu and Zhao 2017) to identify the plant traits that are most important for distinguishing between different types of stress. This is done by calculating the importance of each trait using the permutation of the out-of-bag method (Thomas et al. 2021). The plant traits used in this stage, such as non-collinear spectral indices, fluorescence, and thermal indicators, are assessed by the variance inflation factor (James et al. 2013). Second, a reclassification is performed to reduce uncertainty and disentangle abiotic-induced stress symptoms through unsupervised spectral clustering (Liu and Han 2014). A schematic representation of the multistage process using airborne hyperspectral and thermal imagery is presented (Fig. 2).

Although machine-learning models are accurate at detecting and diagnosing plant diseases, achieving overall accuracies exceeding 90%, these models are species- and pathogen-specific. Future research is focused on developing global models to detect pathogen-induced symptoms at early stages of infection and that distinguish between biotic and abiotic stresses.

Final Considerations on the Advantages and Disadvantages of Different Pathogen Detection and Disease Diagnostic Strategies

All routine plant pathogen detection methodologies should be capable of quickly and economically diagnosing a large number of plant samples with appropriate quality characteristics. Recent advances in biochemistry, molecular biology, and remote sensing have notably enhanced detection and diagnosis, improving their sensitivity, accuracy, and efficiency and even facilitating quick and straightforward detection directly in the field. However, certain methods can be labor-intensive or necessitate the use of complex equipment and highly trained personnel, which may not be available under field conditions or regions with scarce resources (Trippa et al. 2024). Beyond the characteristics emphasized throughout the article, an essential aspect that pathogen detection and disease diagnostic techniques must address is the economic factor. It is vital for these technologies to be not only accurate and reliable but also cost-effective from a practical economic standpoint, considering their impact on overall agricultural production costs. The financial cost of a detection method is relatively variable and includes the required materials, equipment and licensing, and labor costs. Each diagnostic method presents notable strengths and weaknesses, including those related to economic considerations. The pros and cons of prevalent pathogen detection and disease diagnostic approaches are summarized (Table 3) (Shoaib et al. 2023; Trippa et al. 2024; Venbrux et al. 2023). Unfortunately, the most economical methods, which, for example, require simpler protocols, are not usually the most effective in diagnosing or detecting pathogens. This is the case with isolation and culturing of fungi, oomycetes, or bacteria or with some nucleic acid hybridization methodologies, which, although simple and inexpensive, do not always meet speed and/or sensitivity requirements. It is evident that the cost of molecular biology techniques has been evolving; initially high prices have tended to decline as the methods become more common, with more vendors, and with competition from emerging alternatives. Consequently, sensitive PCR techniques and other amplification methods, including LAMP, RPA, RCA, and NASBA, have become more accessible and have low or moderate costs as the diversity of available strategies grow and new technologies are introduced. In other cases, such as with ddPCR, the cost of diagnostics is mainly determined by the expense of the novel equipment required. However, as with the previous examples, it is expected that the cost of ddPCR will decrease in the future. On the other hand, there are massive sequencing techniques that provide large quantities of information but at a high cost, not only due to the sequencing itself but also because of the need for subsequent bioinformatic analysis, which requires experts and often increases the cost. Similarly, remote sensing techniques can entail the processing of the data obtained by experts. Moreover, the cost associated with these techniques can significantly differ per hectare,

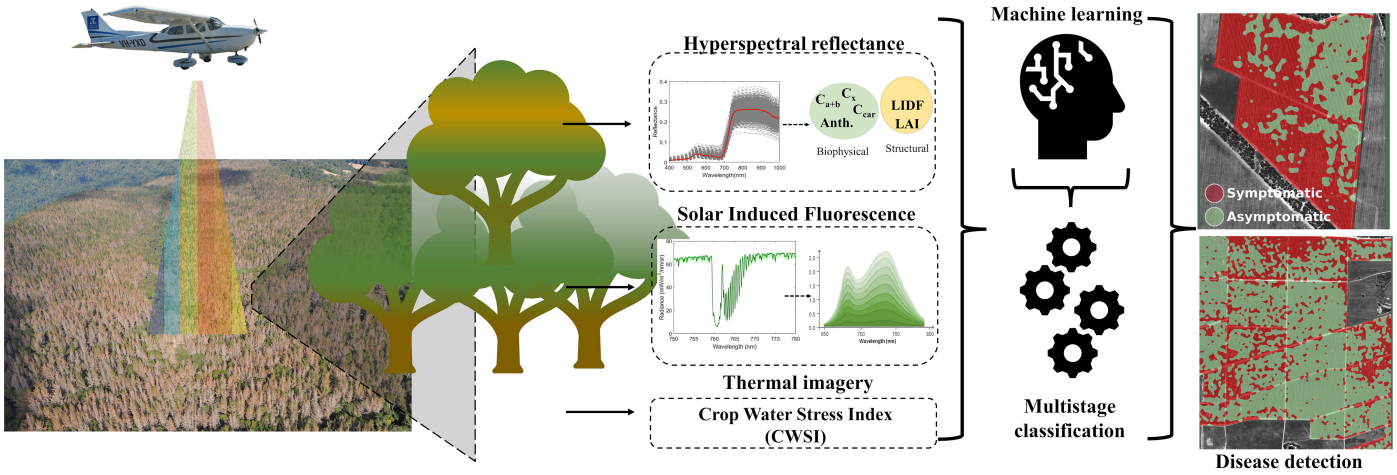

FIGURE 2
Graphical representation of the use of airborne hyperspectral and thermal imagery to detect infected trees using multistage machine-learning approaches.

TABLE 3
Comparison of various types of pathogen detection and disease diagnosis methods (Shoaib et al. 2023; Trippa et al. 2024; Venbrux et al. 2023)

Method[a]	Specificity	Sensitivity	Advantages	Disadvantages	Cost[b]
Cultivation based	Moderate	Moderate	Ease of use Quantification allowed Biochemical and phenotypical characterization No high-tech equipment required Discrimination between viable and nonviable organisms	Time-consuming Moderate throughput potential No multiplex capacity No in situ False negatives	Low-moderate
Serological					
ELISA	Good	Moderate	Ease of use High throughput Quantification capacity	Low sensitivity Moderate specificity No multiplex capacity	Low-moderate
PCR					
Conventional PCR	Good	Good	Moderate difficulty Rapid Detection of uncultivable pathogens	Moderate throughput No multiplex capacity No quantification capacity Specialized staff and lab infrastructure Contamination risk (amplicon processing) No in situ	Low-moderate
Multiplex PCR	Good	Moderate	Moderate difficulty Rapid Detection of uncultivable pathogens Multiplex capacity	Moderate throughput No quantification capacity Specialized staff and lab infrastructure Risk of contamination (amplicons processing)	Low-moderate
Real-time qPCR	Very good	Very good	Medium difficulty Rapid High throughput Multiplex capacity Quantification capacity Detection of uncultivable pathogens No PCR products further processing	Specialized staff and lab infrastructure No in situ	Moderate
ddPCR	Very good	Excellent	Rapid Good throughput Multiplex capacity Quantification capacity Detection of uncultivable pathogens No PCR products further processing	Medium difficulty No in situ Specialized staff and lab infrastructure	High
Isothermal amplification					
LAMP	Very good	Good	Ease of use Very rapid Possible in situ	Moderate throughput No multiplex capacity No quantification capacity	Low
RPA	Good	Good	Ease of use Very rapid Multiplex capacity Quantification capacity Possible in situ	Moderate throughput	Moderate
RCA	Good	Good	Ease of use Extremely rapid Good throughput Multiplex capacity Quantification capacity Possible in situ	Low purity annular template makes it difficult to control connection efficiency The template needs to be a single chain ring structure	Moderate

(*Continued on next page*)

[a] ELISA, enzyme-linked immunosorbent assay; ddPCR, digital droplet PCR; LAMP, loop-mediated isothermal amplification; RPA, recombinase polymerase amplification; RCA, rolling-circle amplification; NASBA, nucleic acid sequence-based amplification; and NGS, next-generation sequencing.
[b] The cost of each method was categorized as low, moderate, or high to facilitate comparison among them.

influenced by factors such as the quality of spectra produced and the equipment needed for data collection or the use of aerial or ground vehicles, including drones and aircraft, with costs directly linked to the size of the area under survey. With any approach, the number of samples or the area to be surveyed is a critical factor in determining costs.

Concluding Remarks

The availability and advancement of new nucleic acid analysis and remote sensing technologies in the context of precision agriculture have resulted in significant strides in improving a fundamental aspect of disease control: the detection of plant pathogens and diagnosis of plant diseases. Both technologies have already demonstrated their utility in several ways, ranging from the development of detection protocols to their direct application in measuring disease progression. Although this improvement can be described as remarkable in recent years, the work cannot be considered complete; it is ongoing, with several challenges and considerations that remain.

The first consideration is how to distinguish live, viable pathogens from those that are not active or not alive and thus noninfectious.

TABLE 3
(*Continued from previous page*)

Method[a]	Specificity	Sensitivity	Advantages	Disadvantages	Cost[b]
NASBA	Very good	Very good	Rapid High throughput Multiplex capacity Quantification capacity Possible in situ High selectivity to RNA molecules, free from background DNA interference No additional cDNA processing required	Medium difficulty Reaction components are complex and many enzymes required Not suitable for all kind of pathogens	Moderate
Hybridization	Moderate	Moderate	Rapid Ease of use No high-tech equipment required Possible in situ Multiplex capacity	Moderate throughput potential False negatives	Moderate
NGS					
Metabarcoding (microbiota)	Good	Good	Excellent throughput Multiplex capacity Relative quantification capacity Detection of uncultivable pathogens	Complex use Specialized staff and lab infrastructure or use external services Contamination risk due to need PCR	High
Metagenomics (microbiome)	Excellent	Good	Excellent throughput Multiplex capacity Detection of uncultivable pathogens	Very complex use Specialized staff and lab infrastructure or use external services	High
Spectral imaging coupled with machine learning	Low	Low	Identification of disease foci at early stage (plants exhibiting stress even before showing visible disease symptoms) Potential of performing real-time detection by continuous monitoring of the crops The integration of drone technology with advanced machine learning-based segmentation techniques holds the potential to deliver precise, high-throughput quantitative assessments of plant disease severity Able to detect biotic and abiotic stresses Automatization reduces the requirement for manual intervention, thereby increasing accuracy and precision	Possible false-negative and false-positive results Requires high computational capacity for data analyses Data interpretation to detect biotic stress in the plant can be complex and may require development of specific algorithms, usually involving machine learning or neural networks Expensive, especially for high-resolution hyperspectral images Requires large number of labeled features and may not be suitable for new disease symptoms not previously identified Additional research is needed to increase the availability of datasets for public use, to improve the training of predictive models and model validation for performance analysis Additional research is needed to scrutinize the potential benefits and disadvantages of these techniques regarding estimation of yield losses and resource use	High

Detecting pathogens only when they pose an actual threat is critical to avoid unnecessary interventions, such as control measures or treatments, which can have important economic and environmental repercussions. Technologies should evolve to provide this level of specificity, helping to refine disease management strategies. Second, the comprehensive analysis of microbial populations within plants requires analysis. Analyzing the entire spectrum of plant-associated microorganisms, including both pathogenic and nonpathogenic entities, can yield valuable insights into plant health and factors driving disease development. To achieve this, data processing methods need further refinement to distinguish between different microorganisms, their roles within plant ecosystems, and their interaction with the environment. This will contribute to developing a more holistic understanding of plant−microbe interactions. Third, advancements in remote sensing protocols are crucial for enhancing precision and clearly distinguishing between biotic and abiotic plant health stressors. Greater accuracy will facilitate early detection of diseases and their specific causes, enabling timely interventions and control measures. Both molecular methods and spectral imaging have their strengths and weaknesses. Molecular methods offer a high degree of certainty, which spectral imaging lacks, but they are limited by smaller sample size capabilities. Spectral imaging, on the other hand, can cover large areas quickly, though it still requires advancements to enhance precision and clearly differentiate between biotic and abiotic plant health stressors. One advantage of spectral imaging lies in its ability to capture the space-time dynamics of diseases, aiding in the understanding of their epidemiology and improving management. As technology advances and offers greater capacity to discriminate between similar symptoms caused by biotic or abiotic factors, the application of spectral image analysis will play an increasingly important role in assessing the phytosanitary status of large areas encompassing numerous host plants. Nevertheless, both techniques will continue to be used synergistically, preventing disease spread and optimizing the application of control measures. In the face of a growing global population and the need for sustainable agriculture, it is imperative for plant pathologists to address these challenges to achieve more effective disease control. Nucleic acid analysis and remote sensing technologies will contribute to developing a more resilient and efficient agriculture sector capable of addressing the food production-limiting issues that the planet's population faces. The ongoing collaboration among different disciplines of technology, plant pathology, and agriculture is a promising path toward a more sustainable and food-secure future.

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