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Next-generation methods for early disease detection in crops

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Abstract

Plant pathogens are commonly identified in the field by the typical disease symptoms that they can cause. The efficient early detection and identification of pathogens are essential procedures to adopt effective management practices that reduce or prevent their spread in order to mitigate the negative impacts of the disease. In this review, the traditional and innovative methods for early detection of the plant pathogens highlighting their major advantages and limitations are presented and discussed. Traditional techniques of diagnosis used for plant pathogen identification are focused typically on the DNA, RNA (when molecular methods), and proteins or peptides (when serological methods) of the pathogens. Serological methods based on mainly enzyme-linked immunosorbent assay (ELISA) are the most common method used for pathogen detection due to their high-throughput potential and low cost. This technique is not particularly reliable and sufficiently sensitive for many pathogens detection during the asymptomatic stage of infection. For non-cultivable pathogens in the laboratory, nucleic acid-based technology is the best choice for consistent pathogen detection or identification. Lateral flow systems are innovative tools that allow fast and accurate results even in field conditions, but they have sensitivity issues to be overcome. PCR assays performed on last-generation portable thermocyclers may provide rapid detection results *in situ*. The advent of portable instruments can speed pathogen detection, reduce commercial costs, and potentially revolutionize plant pathology. This review provides information on current methodologies and procedures for the effective detection of different plant pathogens.

Keywords: plant disease; plant pathogens; early detection; diagnosis; in-field portable devices; PCR; LAMP

ABBREVIATIONS

ArMV	*Arabis mosaic virus*
DMS	differential mobility spectrometer
ELISA	enzyme-linked immuno-sorbent assay
ELISA-DASI	double antibody sandwich indirect
FRET	Forster resonance energy transfer
GFLV	*Grapevine fanleaf virus*
HRM	High-resolution melting curve assay
HTS	High-throughput sequencing
IC-PCR	Immunocapture-PCR
KEDS	kiwifruit early decline syndrome
LAMP	loop-mediated isothermal amplification
LMF	lateral flow microarray
NASBA	nucleic acids sequence-based amplification
NGS	next generation sequencing
nPCR	nested-PCR
OQDS	olive quick decline syndrome
PCR	polymerase chain reaction
PS-MS	Paper spray mass spectrometry
qPCR	quantitative real-time PCR
RAD	rapid apple decline
RCA	Rolling-circle amplification
RF	radio frequency
RPA	recombinase polymerase amplification
RS	Raman spectroscopy
RT-PCR	reverse transcription-PCR
SI	Spectral imaging
ToBRFV	*Tomato brown rugose fruit virus*
VLAMP	visual LAMP

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VOCs	volatile organic compounds
WGS	whole-genome sequencing

1 INTRODUCTION

The major plant pathogens worldwide are viroids, viruses, bacterial, nematodes, phytoplasma, oomycetes, and fungi. In addition, other pathogen groups that can cause disease in plants are protozoa and algae, parasitic phanerogams and phytomizo arthropods.[1-3] Plant pathogens may induce symptoms in certain plant species or cultivars. In addition, symptoms of infection caused by some pathogens may be visible only at a certain developmental or vegetative stage of the host plant. Fully asymptomatic plants usually do not show yield losses, but can serve as an inoculum source to spread the pathogen to other susceptible plants or crops. The infectivity process typically requires that the pathogen reproduce and establish a parasitic relationship with a susceptible host plant. In view of this, a great number of operations are required to diagnose a poorly understood disease. Examination of typical symptoms by simple comparison can be insufficient or misleading for correct diagnosis if the symptoms are poorly understood or when performed by inexperienced observers. Information on cultivation operations that precede the onset of the disease is critical. The written records on disease symptoms, their evolution over time, and their distribution in plant organs are also critical for directing actions for the diagnosis. The place of the pathogen on the plant (such as systemic, local, root system, and others) and its incidence, distribution, diffusion, and how they are affected by local climate conditions are featured important for assisting pathogen diagnosis. The distribution of infected plants is particularly useful information for the diagnosis of diseases, including non-parasitic diseases, taking into consideration the cultivation environment (water, heat, light, or oxygen excesses or deficiencies), agricultural management (rotation, sowing, transplanting, soil cultivation, type and amount of fertilization, irrigation, pruning, chemical treatments).[4] Soil features can also provide crucial information for the early diagnosis of impending symptoms.[5-7] The rhizosphere as an ecosystem can also act as a first shield against plant pathogens.[8] Thus, new innovative methods in plant disease diagnosis are needed to detect an associated pathogen still in the asymptomatic phase.[9] Traditional methods used for disease diagnosis in crops normally require the following key steps:

(i) Comparison of the disease symptoms observed with previous disease descriptions and the absence of symptoms in healthy control plants;[2,10,11]

(ii) Indicator plants of symptoms and previous knowledge of the host range of the pathogen;[4,10]

(iii) Observations of pathogen structures using microscopic techniques;[4,10]

(iv) Pathogen isolation from symptomatic tissues and growth on a selective culture media[10,12] a step used for most fungal, oomycetes, and bacterial pathogens;[13,14]

(v) Carry out the Koch's postulates, with the exception of obligate cellular parasites, such as viroid, viruses and biotrophic fungi,[15,16] and with greater difficulty for microorganisms harder to isolate such as some from asymptomatic plants or those related to complex diseases such as rapid apple decline (RAD),[17] 'vine decline' kiwifruit syndrome,[7] or olive quick decline syndrome (OQDS).[18]

Sampling procedures of infected tissue or plants, such as sample collection, transportation, and storage can affect the integrity of nucleic acids, proteins, and pathogen structures before analysis with negative consequences on successful diagnoses (detection or/and identification), such as the occurrence of false-positive or false-negative results. Besides, sampling the correct plant tissue at the correct time is critical to ensure an accurate diagnosis.[19] For example, brown stain disorder caused by thermal and light stresses in hazelnut fruit can be confused with the more severe symptoms caused by *Colletotrichum acutatum* (Fig. 1). The correct choice of sampled tissues can allow the recovery of greater amounts of the pathogen and reduce the presence of inhibitory compounds in diagnostic tests.[20] The success of a correct diagnosis is also related to a good DNA, RNA, and protein extraction method and each protocol should be previously standardized for different plant tissues (such as root, leaf, bark, woody branch) or species, and matrices where a target pathogen may be present (soil, water, and air). The correct choice of procedures for pathogen detection is extremely important for a fast and accurate diagnosis (Fig. 2). Therefore, this review briefly describes lab-based methods (serological and nucleic-acid based), as well as portable systems for pathogen detection currently available on the market.

Figure 1. Controversial detection phases. Hazelnut, *Corylus avellana* L. cv Tonda Gentile Trilobata (Piedmont Region, Italy beginning of July), is extremely sensitive to brown stain disorder (BSD), where fruits fall while still wrapped in the cupola, with aborted seeds and a shell either empty or filled with a brown spongy tissue. BSD is caused by thermal and light stresses combined with nutritional imbalances. Innovative detection methods help diagnosis, but expert interpretation by humans with previous field experience is necessary. For instance, a search for the pathogen *Colletotrichum acutatum* would be successful in both (a) and (b). The physiological disease (a1-2) can host (or not) the pathogen, but only in (b1-2) *Colletotrichum* is the causal agent of the biotic syndrome.

2 LAB-BASED METHODS

There are several methods and tools available today to phytopathologists to correctly diagnose a disease. However, handling proteins or nucleic acids is a very delicate process that therefore requires qualified personnel and state-of-the-art equipment. The choice of the most suitable method must be made considering the characteristics of the analysis that will then be carried out.[21]

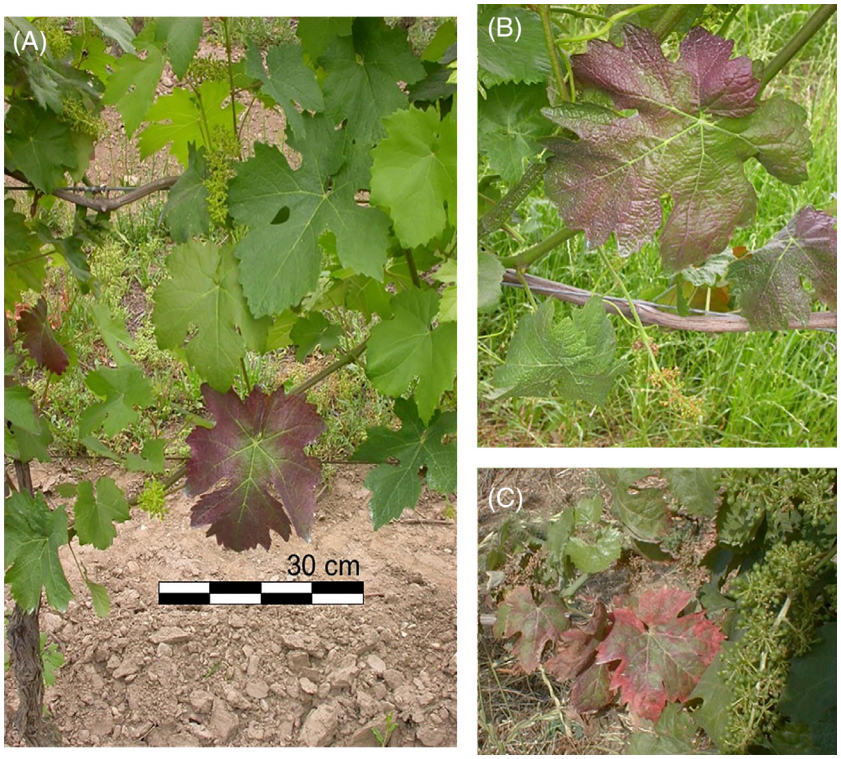

Figure 2. Controversial detection phases. The grapevine, *Vitis vinifera* L. cv Barbera (Piedmont Region, Italy, early June) can display very similar symptoms with different causes: (a) redness on the basal leaves due to the early season cold and (b) redness at the same time as other symptoms of Flavescence dorée (FD) due to *Candidatus* Phytoplasma vitis. The (a) symptom is not caused by the phytoplasma, but negative molecular detection can fail even in the presence of FD due to its erratic distribution in the plant. Visual inspection, based on operator's skills, must accompany the analytical method to determine whether or not phytoplasmic syndrome is present. Phytoplasmic syndrome is recognized by tracing three phytoplasmic symptoms at the same time in the same plant as in (c), where in addition to sectorial coloring, perinerval coloring and inferior folding of the leaf lamina are visible.

2.1 Serological methods

Serological diagnostic techniques based on specific mono or polyclonal antibodies produced from pre-defined antigens search for the presence of a certain pathogen within an infected tissue sample.[22] In the 1970s, the 'enzyme-linked immuno-sorbent assay' (ELISA) was developed to detect plant viruses present in aqueous solutions.[23] ELISAs are currently the most widely used immunodiagnostic techniques for their high-throughput potential and low cost.[24] Particularly, monoclonal antibodies increased the sensitivity and specificity of the test, making the ELISA the most versatile assay for pathogen diagnosis,[25] while polyclonal antibodies are safer for detection of variant pathogens or strains. Highly specific antibodies, that do not cross-react with closely-related pathogens or plant proteins, are crucial for a successful ELISA. Thus, the ELISA does not require great expertise in plant pathology and can allow simultaneous detection (multiplexing) of different pathogens in the same sample, where different pathogens are detected by antibodies conjugated with different enzymes. In turn, the sensitivity of ELISA is influenced by the pathogen type, antibody quality, sample freshness, correct plant tissue sampled at the right time, and pathogen titer. In general, most bacterial, fungal, and phytoplasma pathogens can be detected using serological techniques with antibodies that recognize and bind specifically to antigen epitopes.[26] However, the sensitivity of this method is usually considered relatively low ($\sim 10^5$ to 10^6 cfu mL) to detect plant pathogenic bacteria.[27] To improve the sensitivity of the ELISA, direct- and indirect-ELISA protocols have been developed.[28] The ELISA-DASI (double antibody sandwich indirect) uses coating with polyclonal antibodies to allow efficient trapping of target antigens that are then recognized by specific monoclonal antibodies. ELISA-DASI was developed for *Erwinia amylovora*[29] and more recently used for *Xylella fastidiosa* detection.[30] In addition, the immunocapture-polymerase chain reaction (IC-PCR) combination of serological and molecular assays has been established for the improved sensibility of pathogen detection, mainly viruses, and viroids.[25]

2.2 Nucleic acid-based methods

There are multiple techniques for the extraction of nucleic acids which vary in the purity of the extract, the concentration of the nucleic acid obtained, and the complexity of the extraction method. Recently, new diagnostic methods have been developed to detect pathogens infecting different plant species. These molecular methods are more recommended than serological methods because they have more sensitivity and accuracy, although at a higher cost.[31]

2.2.1 Methods based on molecular hybridization

Molecular hybridization technically refers to molecular hybridization on a solid support, where solid support means a nitrocellulose or nylon membrane. It is a diagnostic technique that allows identifying pathogens through the pairing of their genome with complementary nucleic acid molecules synthesized *in vitro*, called probes. The technique was later applied to plant viruses.[32] Pairing can occur between DNA / DNA, DNA / RNA, and RNA / RNA reported in increasing order of bond strength. The PCR product that will be

obtained will be a non-naturally occurring DNA in which all 5 nitrogenous bases are present. The obtained products, DNA or RNA, are subsequently purified and quantified. Dot blot and Tissue blot are among the simplest analyses that can be carried out in a normal diagnostic laboratory using the marked probes. In the dot blot, the target nucleic acid is immobilized on a membrane (solid support) by means of a spot (dot blot) of a few microliters of extracted nucleic acid. In the tissue blot a small pressure is applied to the membrane with a section of plant tissue.[33]

One method of reverse hybridization is the DNA array. Thousands of specific oligonucleotides are immobilized on the solid support (probe) and the nucleic acid of the sample to be identified is labelled. The successful probe/sample reaction is visualized through an optical scanner.[34] However, the high cost and low sensitivity associated with producing microarrays and reading the results limit their application relative to the potential for use.[35] Microarray technology can be used to simultaneously determine both host and pathogen genes as the disease progresses from infection to resistance or susceptibility at different stages of host development.[36]

2.2.2 *End-point PCR*

Almost 30 years after its discovery, polymerase chain reaction remains one of the most important and commonly used scientific advances in molecular biology. The PCR assays using specific or degenerate primers are widely used to detect plant viruses as well as other pathogens for being highly consistent and effective.[37] Similarly, retro-transcribed polymerase chain reaction (RT-PCR) assays are used mainly to detect RNA viruses, which requires an additional step using reverse transcriptase enzyme to convert the total RNA to cDNA before the regular PCR step.[37,38] The PCR and RT-PCR assays are sensitive, specific, and significantly more reliable than serological methods.[39]

PCR can amplify even a single copy of a single gene.[40] Despite its popularity in disease diagnostics, PCR has several inherent limitations. These limitations inspired the development of innovative PCR-based techniques such as nested-PCR (nPCR) and quantitative PCR or real-time-PCR (qPCR). The development of quantitative PCR (qPCR) techniques represented a significant advance over conventional PCR which relies on endpoint analysis. The qPCR allows real-time DNA quantification by monitoring the progression of the reaction during the amplification cycle, using a variety of fluorescent reporter chemicals (e.g., probes or dyes).[41] For example, one of the main disadvantages of classical PCR is the inability to quantify the initial number of sequence copies in the sample.[42] The quantity of product synthesized depends on the concentrations of primers and nucleotides in the reaction and differences in reaction conditions (temperature, time, kind of buffer, and concentration), not on the number of copies of the target DNA. This disadvantage has been overcome with qPCR, which allows the synthesis of new DNA strands to be monitored cycle by cycle by measuring a fluorescent signal that increases in proportion to the amount of double-stranded DNA synthesized. However, false positive and false negative results can reduce specificity and sensitivity, due to the high genetic similarity between species and the limited number of specific genes available to identify pathogens.[43] Another disadvantage is the possible generation of unwanted secondary amplicons.[42] With the introduction of nPCR, amplification sensitivity and specificity priming have increased. This is thanks to the introduction of two successive PCR reactions with different primer sets for a single target gene.[44] However, nPCR is not commonly used as a diagnostic method due to the long analysis times and two-step procedure which increases the risk of contamination.[45] Digital PCR (dPCR) is a technology that allows for the absolute quantification of DNA by splitting a sample into thousands of droplets, which are subsequently subjected to end-point PCR. Model density estimation does not need a standard curve.[46] Digital PCR produces more accurate results than qPCR with less variation between technical replicates.[47]

2.2.3 *Real-time PCR*

The real-time PCR can detect and quantify single or simultaneously multiple DNA fragments, by using different chemistry (i.e., SYBR Green, TaqMan). The quantification of the amplicon during amplification significantly reduced analysis times compared to conventional PCR, eliminating subsequent manipulations after amplification such as DNA electrophoresis.[48] In addition, TaqMan probes have allowed quantitative approaches to reach low detection limits of target DNA in complex mixtures.[38] The advantage of this system is the higher specificity conferred by the probe, which binds only to specific amplicons. In addition, the ability to use different fluorophores for different probes enables the detection of multiple target sequences in a single reaction, allowing multiplexing assays. Other types of fluorogenic probes include FRET (Forster resonance energy transfer), SunRise, molecular beacons, and Scorpions.[49] As a more simple alternative to fluorogenic probes, the accumulation of amplified products can be followed in real-time by using specific DNA dyes such as SYBR Green, with sensitivity comparable to that of ethidium bromide.[50] To date, real-time PCR methods alone or coupled with other methods are nowadays widely used in the field of pathogen diagnostics. In 2019, a method was developed to discriminate the *Tomato brown rugose fruit virus* (ToBRFV) from other viruses belonging to the *Tobamovirus* genus using immunocapture and RT-PCR.[51] Indeed, it was shown that real-time RT-PCR and end-point RT-PCR gave the same comparable results. Using direct crude extracts and leaf-disk crude extracts, the end-point RT-PCR was unable to provide a reliable result. This developed highly specific and sensitive real-time RT-PCR assay will be a particularly valuable tool for early ToBRFV diagnosis, optimizing procedures in terms of costs and time.[51] In the case of citrus fruit diseases, Huanglongbing, caused by the bacterium '*Candidatus Liberibacter asiaticus*', an early diagnosis is possible by the application of qPCR assays.[52] The High Resolution Melting (HRM) analysis has been successfully used for the identification of viral strains or the identification or differentiation of other pathogens, such as *Fusarium* species.[53,54] Recently, photonics-based qPCR uses a fully optical approach to achieve ultrafast temperature response with real-time temperature feedback using monolithic-scale reaction volumes. The system provides a micro-slot to act as a reaction vessel. A typical fluorescence qPCR system consists of two parts: (i) the PCR mix thermocycler to facilitate DNA amplification and (ii) measurement of the fluorescence emitted during reactions to confirm DNA amplification. The fiber optic system consists of a fiber optic microcavity, an ultrafast laser heating system, and a fluorescence excitation/sensing system.[55] Fiber optics offer numerous advantages for portable field and low power q-PCR, given their inherent multiplexing capability, small size, and the wide range of portable fiber integrated components developed for the telecommunications industry. With the introduction of multiplex PCR, it was possible to simultaneously identify two or more target DNA or cDNA, in the same reaction mixture. Mumford *et al.*[56] employed fluorescence to detect multiple viruses in real-time. However, despite the presence of these advantages over conventional PCR, often RT-PCR or qPCR showed a greater

complexity of the reaction and compatibility between the primers used. Furthermore, due to the complexity of pathogen taxonomy, it is often difficult to design specific primers for each target DNA and to distinguish the difference in DNA amplification of each size of the gene.[38]

2.2.4 Bio-PCR

The Bio-PCR technique includes a pre-assay incubation phase that allows the biomass of the pathogen to increase. The advantages of this technique are the increased sensitivity, the elimination of PCR inhibitors, and the detection of only viable cells, thus avoiding false positives due to the detection of DNA from dead cells. However, the reliability of the assay can be compromised by the action exerted, for example, by bacteria in the growth of fungal biomass suitable for detection. The disadvantages, therefore, are linked to the increase in the cost of the assay, the timing, and the possibility of pollution. Fungal pathogens *Alternaria radicina* and *A. dauci* that were transmitted by infected *Alternaria alternata* seeds, were detected using ITS-specific rDNA primers with the help of a deep-freeze-blotter method during the BIO-PCR test.[57]

2.2.5 Digital PCR

Digital PCR (dPCR) is a third-generation technology where the amplification of nucleic acids occurs by dividing the sample into several separate subcompartments, in each of which an independent amplification reaction takes place.[58] This method is used to directly quantify and clonally amplify the nucleic acid strands. The key difference between dPCR and traditional PCR lies in the method of measuring the quantities of nucleic acids.[59] A 'digital' measurement measures a certain variable quantitatively and discreetly, thanks to the fact that the sample is separated into a large number of parts and the reaction is carried out individually in each part. One of the limitations attributable to the dPCR is the control of the error mechanisms in the dilution phase. For best accuracy, dPCR methods should have mechanisms to control errors in the measured volumes and ensure there is no more than one target molecule in each compartment.[60,61] Poisson statistics can be used to determine the probabilities of more than a single target molecule being present in a compartment. Unlike classic qPCR, dPCR allows the absolute and reproducible quantification of target nucleic acids at the resolution of a single molecule. Digital PCR assay was developed for quantitative detection of *Bacillus subtilis*, a typical plant growth promoter present in rhizosphere samples.[62] Furthermore, reverse transcription has been coupled to dPCR for the absolute quantification of viruses and viroids.[63] A portable integrated digital PCR system has been developed for viral detection from urine samples.[64] This instrument and similar others may also be employed for plant pathogen detection.

2.2.6 Denaturing gradient gel electrophoresis PCR (PCR-DGGE) and temporal temperature gradient gel electrophoresis (PCR-TTGE)

Molecular methods based on direct analysis of environmental DNA without going through isolation in culture have been developed to study microbial communities. Among these, denaturing gradient gel electrophoresis (PCR-DGGE) and temporal temperature gradient gel electrophoresis (PCR-TTGE) have been widely used for the characterization of environmental and agri-food microbial ecosystems. These electrophoretic methods rely on the structural changes that DNA fragments take on during denaturation and are therefore useful for separating fragments.[65]

2.2.7 Isothermal amplification

Different variants of PCR methods are increasingly used for the diagnosis of plant diseases. To complete the first round of PCR, three different temperatures are used to denature double-stranded DNA (94–95 °C), anneal primer to the target DNA, and extend DNA synthesis. In general, the temperature ranges for a complete transition from double-stranded to single-stranded structure can range from approximately 0.5 °C to several degrees depending on the distribution of GC content along the DNA fragment.[66] This requires expensive instruments that can control temperature precisely. The development of PCR that amplifies at constant temperatures has provided a significant advantage, as it allows simpler, less-expensive instruments, but maintains optimal results.[67]

A good example of isothermal amplification is the recombinase polymerase amplification (RPA), an alternative to PCR, which can be performed in a single tube.[68] This method was developed by TwistDX Ltd (formerly ASM Scientific Ltd, Cambridge, England). The great advantage of RPA is the possibility to use a reverse transcriptase without producing cDNA. In this same sense, the RPA is isothermal and does not require thermocycling equipment. Interestingly, RPA reactions are performed rapidly in a holding tube and are easily adapted for rapid diagnosis tests at a low commercial cost per sample. In addition, this technique detects lower concentrations than some PCR or RT-LAMP tests.[69] A visual DNA diagnosis with integrated RPA and a AuNP probe for a simple and rapid and field deployed method was developed for the detection of *Tomato yellow leaf curl virus* (TYLCV) that maintain a high sensitivity and that can be applied directly to monitoring the disease in the field.[70] RPA integrated with lateral flow dipsticks (LFD) were used to detect *Phytophthora* root and stem rot and damping-off of soybean within 5 min.[71] *Phytophthora capsici* has been successfully monitored by Yu *et al.*[72] in tomato, pepper, pumpkin and cucumber using lateral flow strip-based RPA assay. Moreover, an RPA coupled with a lateral flow device (LFD) was proposed by Boluk *et al.*[73] to diagnose *Dickeya spp*. Another study investigated the potential effect of pre-formulation on test performance using an RPA test used on the *Phytophthora* genus. The lyophilized preformulated Phytophthora RPA assay was compared with a commercially available quantitative polymerase chain reaction (qPCR) assay and RPA kits using three qPCR platforms (Biorad CFX96, QuantStudio 6, and Applied Biosystems ViiA7) and an isothermal platform (Axxin T16-ISO RPA).[74] The responsible agent of blackleg and soft rot diseases of potato and several other plant species worldwide, without the need for DNA isolation. Recently, Clark *et al.*[75] has implemented an RPA method for a real-time detection of the Spinach Downy Mildew pathogen in leaves that can be performed outside of the laboratory setting and generate same-day results.

2.2.7.1. Loop-mediated isothermal amplification (LAMP). Loop-mediated isothermal amplification (LAMP) was developed in 2000 to improve the sensitivity and specificity of nucleic acid amplification. LAMP is performed isothermally for about 1 h and uses four primers.[76] The LAMP does not require expensive thermal cyclers or optimization of thermal cycles.[77] Fluorescence is the detection system most commonly used in LAMP diagnostic methods. While this is effective and fast, large-scale fluorescence instruments are not portable. They are expensive and there are technological barriers to the miniaturization of critical components like light sources, lasers, sensors, and cameras. LAMP uses a one-step amplification under isothermal conditions to amplify

a DNA or RNA sequence target using four primers: the forward outer F3, the backward outer B3, the forward inner FIP, and the backward inner BIP. The FIP and BIP contain two particular regions: F1c and F2 for FIP, and B1c and B2 for BIP, respectively, in which F1c and F2c are complementary to regions in the target sequences F1 and B1. There are two more primers, the loop forward (LF) and the loop backward (LB), that are used to accelerate the LAMP reaction. LAMP consists of FIP primers that anneal to the specific complementary sequence B3c and start DNA synthesis. At the opposite end of the same sequence, BIP amplifies in the same way. Next, the external primer F3 re-associates with the B3c region, and elongation begins. At this point, the ring formation phase begins: thanks to the activity of the *Bst* enzyme, the filament formed by FIP is released and creates the template for the next amplification cycle. Two continuous amplification cycles occur that generate a portion of ring DNA containing the target sequence and its end is used as a template for cyclic amplification. Cyclic amplification is similar to the manufacturing steps of the initial structure, but the loop acts as a single-stranded mold. For each single-stranded DNA template, through each cycle, two amplified products are produced in which one is identical to the template, and the other is twice the length of the template.[78] Primers for LAMP experiments are designed specifically for the target nucleotide sequence of each pathogen.[79] In addition to Primer Explorer and LAMP Designer, there are several commercial programs available and some extensible open-source resources such as eLAMP (Electronic LAMP) and LAVA (LAMP Assay Versatile Analysis). LAMP was used for multi-target, in-field detection of four pathogens that infect lettuce,[80] and *Magnaporthe oryzae* in turfgrass fields.[81] In addition, it has been also used to detect phytoplasma.[82] In 2020, a further innovation to LAMP was the introduction of electrochemical gene sensors on closed and portable electrochemical chips[83] (Fig. 3).

2.2.7.2. Visual LAMP. The DNA LAMP allows affordable, specific, very sensitive, and rapid diagnostic testing for plant pathogens in field conditions and does not require higher-skilled personnel. This method can be used to diagnose plant pathogens even in laboratories with limited resources. The LAMP amplifies and detects target DNA in a single step, by incubating the genomic DNA sample, six specifically designed primers, and *Bst* DNA polymerase mixed in the same test tube followed by incubating at 60 to 65 °C (based on the optimal annealing temperature). Thus, LAMP samples can be processed through inexpensive portable devices that are commercially available. Traditional lab PCR thermocyclers perform LAMP by using photometry to measure

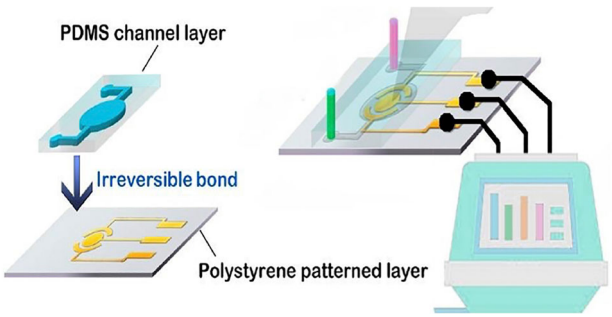

Figure 3. Schematic illustration of LAMP reaction electrochemical detection on a PGE chip with microfluidic channel. Principle of chip fabrication and electrochemical detection (from,[83] modified).

solution turbidity.[84] Unlike PCR-based molecular assays, LAMP can be performed using relatively simple instruments,[85] while the DNA polymerase is less sensitive to inhibitors than those used commonly for PCR.[86] In that same sense, the visual LAMP (VLAMP) combines isothermal DNA amplification with detection based on a colorimetric transition visible to the naked eye. This fast, inexpensive method has been recently modified to detect plant viruses, fungi, and bacteria.[87–89] An appropriate diagnosis requires high specificity and sensitivity methods, particularly for the early identification of a pathogen. Like PCR, LAMP can detect a single target in a complex matrix, or multiple targets from it. In addition, VLAMP is sufficiently sensitive to detect quarantine pathogens from small amounts of biological traces, such as frass samples[90] or DNA extracted from soil and water samples to monitor soil-borne pathogens in a greenhouse.[91] More recently, VLAMP detected plant pathogens in pre-symptomatic and infected plant tissue.[92] In reverse transcription LAMP (RT-LAMP), reverse transcriptase step is added to the LAMP mixture to convert RNA samples into cDNA for amplification.[63] A multiplex LAMP assay tackled the diagnostic challenge of detecting different pathogens simultaneously in a single sample present in the same tube. However, most plant pathology studies use a single assay to detect a single target pathogen, because multiplexing is more difficult with LAMP than with PCR.[93]

2.2.7.3. Nucleic acids sequence-based amplification (NASBA). NASBA, a primer-dependent continuous amplification, is used to directly amplify RNA by PCR using reverse transcriptase, RNase H, and T7 RNA.[94] Unlike conventional PCR, NASBA works at a single temperature, without thermal cycling. The products of NASBA are antisense to the target viral sequences. NASBA at 41 °C for 60 min has been combined with a real-time assay using molecular beacons.[38] Since it is more sensitive than conventional PCR, the reaction time can be reduced.[95] NASBA real-time analysis has been used to detect the *Strawberry vein banding virus*, *Apple stems pitting virus,* and other pathogens.[95-97]

2.2.8 Rolling-circle amplification (RCA) of circular DNA genomes

The RCA assay is a sequence-independent strategy that has been used for the efficient amplification of circular DNA viral and subviral genomes in DNA samples isolated from infected or symptomatic plants.[98] This assay uses the bacteriophage phi29 DNA polymerase enzyme, does not use target-specific primers, and does not require a thermocycler for the amplification reaction. The RCA allows the amplification of complete genomes, which after linearization of these with restriction enzymes, can be cloned into base plasmids (such as pBlueScript vector), and the cloned genome can be Sanger sequenced for identification.[99] For example, Basso *et al.*[100] using RCA assay from DNA samples, isolated from symptomatic plants, identified a novel and highly divergent ssDNA virus (Temperate fruit decay-associated virus, TFDaV) infecting apple, pear, and grapevine.[100] This assay has been shown to be highly efficient for the amplification of complete genomes from different plant species. Although it is simple and low cost, the identification method requires cloning and sequencing of the cloned DNA.

2.3 High-throughput sequencing (HTS)

High-throughput sequencing (HTS) offers additional benefits to Sanger sequencers in several molecular applications. HTS is used for metabarcoding of organisms through whole-genome

sequencing (WGS) and to detect specific target organisms through NGS (next-generation sequencing). DNA metabarcoding is used for transcriptomic and genomic studies, replacing more time-consuming and less cost-effective methods. It sequences DNA directly from environmental matrices (soil, water, plant tissues), without isolating single microorganisms from a mixed sample. This allows DNA metabarcoding to detect and identify a much greater diversity of microorganisms from environmental matrices and avoids the growth of undesirable organisms during isolation. The HTS is used in environmental science to study metagenomics. It can efficiently assess the biodiversity of microbial communities without prior knowledge of specific microorganisms present. Thus, HTS has a high potential for the detection of specific plant diseases and for the detection of invasive pathogens in environmental samples.[94] For example, metabarcoding has been successfully used to detect and identify oomycetes in soil samples before and after sample enrichment.[101] Another example, Illumina NGS technology (California, USA) uses a fundamentally different approach from the classic Sanger sequencing method, which makes it less expensive and less time-consuming for pathogen prospection and metagenomics.[102,103] The main feature of this technology is that it does not use primers for defined target pathogens, but rather for adapter sequences linked to unknown nucleic acids present in the sample to be sequenced, which makes it possible to explore metagenomics approaches.

3 PORTABLE DEVICES

Several portable instruments have been developed and commercialized in the last 10–15 years based on the analysis of transcripts, nucleic acids, proteins, and volatile compounds. The main purpose of these instruments is to simplify and speed up the analysis reducing the number of steps for data analysis without losing the effectiveness of the detection tests.

3.1 Lateral flow microarrays

Lateral flow microarrays (LFM) allow hybridization-based nucleic acid detection through an easily visualized colorimetric signal. Current lateral flow systems can provide consistent results in a few minutes. The instrument is able to detect target transcripts present in samples of infected plants. In addition, it is small, compact, and portable enough for uncomplicated use in field conditions.[104] The success of diagnosis from asymptomatic plant samples using LFM is based on the identification of strong host and pathogen biomarkers in a quick, sensitive, and inexpensive way. Therefore, LFM can detect small quantities of RNA and DNA from viruses, bacteria, phytoplasma, and fungi pathogens.

3.2 Portable LAMP and qPCR devices

Most portable devices use a LAMP-based detection system, that rapidly analyzes samples from putatively infected plants.[105] It supports both qPCR and LAMP analysis, detecting in real-time fluorescence originated from amplification of target sequences in infected samples using a fluorophore molecule that binds to the minor groove of amplified DNA. After a sample preparation step of 5 min, the extract is added to a reaction mix already loaded in the 8-24-48 chip supported by the device. Dedicated software analyzes the output using specified default parameters. The Genie® II (OptiGene, Horsham, England) is a compact, portable, robust, and lightweight device suitable for use both in the laboratory and in the field. This instrument is equipped with a rechargeable lithium-polymer battery that powers it for an entire working day. Interestingly, Genie® II runs an isothermal amplification, detecting target DNA by fluorescence that is visualized directly on a touchscreen. In addition, the instrument is equipped with two heating blocks, each of which can hold a single strip of eight microtubes with sealing caps that do not open after a run, preventing any sample contamination. These blocks can be individually checked or run together to process up to 16 samples per round. Using a portable extraction kit, samples can be extracted still in the field (2–3 min spent per sample) and then, 3 µL of crude extract containing the DNA template is added into a microtube containing the reagents for LAMP analysis. The instrument completes analyses in 30 min, significantly reducing the diagnosis time.[89,106] An efficient portable device allowing quantitative real-time PCR analysis is the bCUBE (Hyris, Milano, Italy), a miniaturized device that detects pathogens using isothermal analysis, custom thermal cycling protocols, and real-time PCR analyses.[107,108] The bCUBE device is 100 × 100 × 120 mm, weighs 1.15 kg, can be controlled remotely with a smartphone, and has standard certification in both Europe and North America. Curiously, up to 16 or 36 samples can be analyzed simultaneously using 10 to 25 µL samples in one run with output results in 45 min. Another portable instrument is the thermocycler Biomeme that turns a smartphone into a thermocycler for real-time PCR or isothermal analysis and it allows to obtain results in 30–60 min, depending on the test protocol. The Biomeme thermocycler also allows real-time multiplex detection of up to 27 samples in the same analysis. There are various kits on the market that adapt to the operator's requests, allowing them to be used by less experienced operators and therefore increasing accessibility to the tests.

3.3 Differential mobility spectrometer

The differential mobility spectrometer (DMS) is a portable instrument that uses radio frequency (RF) and non-linear ion mobility dependence at atmospheric pressure to detect marker metabolites at parts-per-trillion concentrations, a lower sensitivity than other detection methods. This allows rapid detection and identification of volatile compounds commonly not resolved by other techniques. This sensor is a modest and quantitative finder that needs insignificant sample preparation and gives rearranged 'yes/no' sensor yields. Interestingly, DMS can efficiently carry out the diagnosis of *Candidatus* Liberibacter asiaticus in citrus, before disease symptoms appear in an infected orchard.[109]

3.4 Paper spray ionization mass spectrometry

Paper spray mass spectrometry (PS-MS) is a representative ambient ionization method, which is based on applying a sample in solution to the front of a triangular piece of paper while a spray solvent is used to wet the paper from the rear and extract the analytes of interest.[110] Then, a high voltage is applied directly to the paper base resulting in an electrospray-like ionization event from the tip of the paper triangle close to the mass spectrometry inlet, when its heterocomposites are then analyzed in detail.[109] The PS-MS has been successfully used for the identification and differentiation of bacterial and fungi species, being very promising for the detection of several plant pathogens.[111] PS-MS has been a direct, fast, and low-cost for qualitative and quantitative identification of complex mixtures in different matrices. However, routine plant pathogen detection using PS-MS requires the availability of MS equipment and expertise for its handling. Most of the PS-MS methods reported in the literature demonstrate that the method

is sensitive enough to quantify low amounts of drugs of abuse in physiological settings. Common molecules have been quantified by PS-MS even below ng/mL.[112,113]

3.5 Canine olfactory detection

The use of animals as detectors is a growing field of study with a wide range of practical applications (Moser *et al.*,[114]). Canines have shown that they are capable of locating a range of organic and inorganic molecules.[115] The canine ability to determine direction depends on the sex of the animal.[116] Dogs have been proven to detect certain VOCs from human disease episodes,[114,117] including viruses.[118] Dogs were very specific, accurately distinguishing Citrus Huanglongbing (HLB) or avocado laurel wilt, or olive *Verticillium* wilt from other diseases,[119,120] and diagnosed disease weeks to years ahead of visual survey and molecular approaches with high accuracy. One limitation is the outbreak of infected hosts: with low prevalence and high incidence, dogs begin to warn at a high rate of targets, require more reward time, and significantly slow down search patterns.[119] On the other hand, dogs can also be taught to distinguish several targets, which could be beneficial for detecting the simultaneous appearance of multiple diseases or pests.[119] The 'Training and use of dog units in early detection of *Xylella fastidiosa*' project started in June 2021 in Salento (Italy) and was carried out by testing the ability of dog units to recognize *Xylella* both in artificial culture plates and in infected olive trees.

4 NEWLY DEVELOPED TECHNIQUES

These techniques are based on different principles and basically identify symptoms of infected plants (*i.e.* necrosis, change in leaf color, and leaf chlorosis). Their use for disease diagnosis should be accompanied by a field inspection to confirm the presence of an associated target pathogen in the symptomatic plant. Also, their use allows the ability to locate and monitor symptom-free plants and to intervene in a targeted and timely manner.

4.1 Hyperspectral imaging

Hyperspectral imaging combines conventional imaging with spectroscopic techniques that use reflectance data collected over a broad spectrum to reconstruct a spatial image of the analyzed matrix.[121] This technique is relatively expensive, has long data acquisition times, and the resulting data is complex for automated interpretation. Thus, these limitations still limit its use in field conditions.[122] The hyperspectral imaging was successfully used under laboratory and field conditions to detect the presence of target spots caused by *Corynespora cassicola* and bacterial spots caused by *Xanthomonas perforans*.[123] It is worth noting that these diseases initially show very similar symptoms in tomato plants. The spectral signatures of both diseases at three different stages of development showed significant differences using a multilayer perceptron neural network, achieving 99% accuracy under both fields (based on UAV) and laboratory conditions.[123] A recent review of Cheshkova[124] described the principal steps of the hyperspectral data analysis process and evaluated the possible applications of hyperspectral sensors and platforms on different scales for diseases diagnosis. Nagaraju and Chawla[25] also discussed some of the various existing deep learning models adopted to process image data to detect crop diseases highlighting the future scope for hyperspectral data analysis. Other modern techniques are available and are useful for an early and accurate disease detection.[125,126]

4.2 Raman spectroscopy

Raman spectroscopy (RS) allows non-destructive spectral acquisition in live biological systems such as tumor cells.[127] The spectra are generated by collecting photons dispersed in an inelastic way. The sample is hit directly by monochromatic electromagnetic radiation that induces an oscillating electric dipole responsible for the diffusion of the incident radiation. This technique gives results in a short time and requires no sample preparation. The RS was successfully used for early diagnosis of rice blast disease, which is usually diagnosed with the naked eye. The characteristic peaks of β-carotene, chlorophyll, and chitin were taken as initial variables to establish a partial least squares regression model, while the accuracy of the test classification was 94%.[128] For this, a leaf clip-on Raman sensor for early detection of nitrogen deficiency has been applied to several species. This handheld sensor reports measurements consistent with those obtained under laboratory conditions.[129] Raman spectroscopy was adopted to detect rot disease of maize caused by *Colletotrichum graminicola*,[130] rose rosette infection[131] and to investigate wheat and sorghum grains infected with ergot, black tip or mold.[132] RS combined with deep learning networks were recently used for the detection of Fusarium head blight (FHB)-infected wheat kernels.[133]

4.3 Smartphone-based fingerprinting of leaf volatiles

A recent portable smartphone-based instrument for plant disease diagnosis was developed by Li *et al.*[134] This device pumps air into a chamber where a detector containing a paper strip embedded with an array of chemical reagents reacts with a specific chemical group. It is able to distinguish uninfected from infected leaf samples in 15 min. The device detects and classifies 10 plant VOCs at parts-per-million concentrations. However, the uneven distribution of plant pathogens can cause false-negative results. The same group has developed a piezoelectric cantilever resonator, a real-time VOC-profiling sensor device on a stretchable substrate able to monitor plant host responses for early disease diagnosis and rapid stress identification of living plants with late blight caused by *Phytophthora infestans*.[135] As it has been a great challenge to eliminate the cross-sensitivity of various VOCs for sensors, they optimized this device proposing a virtual sensor array (VSA) which has successfully been applied to detect VOC biomarkers identifying the emissions from healthy plants and infected plants with an accuracy of 89%.[136]

4.4 Nanopore sequencing technology

MinION nanopore technology offers instant access to gigabases of long-read data. This portable instrument, developed by Oxford Nanopore Technologies (Oxford, England), is pocketable and powered *via* a USB port. In addition, it can sequence anything and can be used in the laboratory or in the field. Analytics in real-time provide instant access to actionable results. The same long-read RNA and DNA direct sequencing workflows are available across all products, providing truly scalable sequencing. This instrument is also characterized by high yields, up to 30 Gb data. So, it is suitable for food traceability using high-throughput targeted analysis. It is able to sequence individual DNA molecules as they are driven through biological nanopores by an applied electrical field. The MinION is a promising fast-detection technology for plant pathogen detection[137–139] because the instrument is smaller than a smartphone and provides output data within a few minutes. It has been explored for the sequencing of bacterial and virus pathogens,[132] detecting and genotype potato

viruses[103]; identifying *Xylella fastidiosa* subspecies and sequence in naturally infected olive samples[140] and in grapevine[141] and for the detection *Fusarium oxysporum* on *Zinnia hybrida* (zinnia) plants.[142]

4.5 MALDI-TOF MS

Matrix-assisted laser desorption ionization (MALDI-TOF MS) is one of the sophisticated technologies that has revolutionized a variety of fields including biological research, clinical settings, and microbiological diagnostics extending MS operations to large biological molecules like proteins.[143-145] MALDI-TOF MS has recently been introduced in microbiology laboratories as a rapid, accurate and cost-effective method for identifying microorganisms.[146] This technology constitutes a valid and interesting alternative to classical microbiology and molecular biology methods and it is applicable in various areas of clinical diagnostics and research.[17] From an organizational point of view, the use of MALDI-TOF MS considerably improves the times and the reference modality since the laboratory is able to communicate to the clinician the species of the bacterial isolate within a few minutes of the culture finding, with an advance of 24 h compared to the use of traditional biochemical tests. The application of this approach to detect plant pathogen causing fungal, bacterial, and phytoplasmal infections have been shown. For instance, MALDI-TOF MS identified the pathogenesis-related proteins from the infected tomato plant by *Fusarium oxysporum* f.*sp. radicis-lycopersici*[147] and *Ralstonia solanacearum*.[148] It was also applied to facilitate the identification of *Pantoea stewartii* subsp. *stewartii* that causes Stewart's wilt in sweet corn.[149]

Proteins that contribute to the rice plant resistance mechanism against bacterial pathogens such as *Xanthomonas oryzae* pv. *oryzae* have been also detected.[150] Intact spore MALDI-TOF MS has been used for a rapid identification of different species of *Puccinia* the causal agent of rust in wheat and other crop plants.[151] MALDI-TOF MS detected 48 proteins with differential expression and phosphorylation associated to phytoplasmal infection caused by Flavescence dorée in grapevine.[152] Moreover, this approach contributes to identifying bioactive compounds facilitating the study of the secretome of biocontrol agents such as *Bacillus* strains to control the wilt disease caused by *Fusarium oxysporum*.[153] This approach allowed the confirmation of the identity of antibacterial compounds such as the anti Erwinia

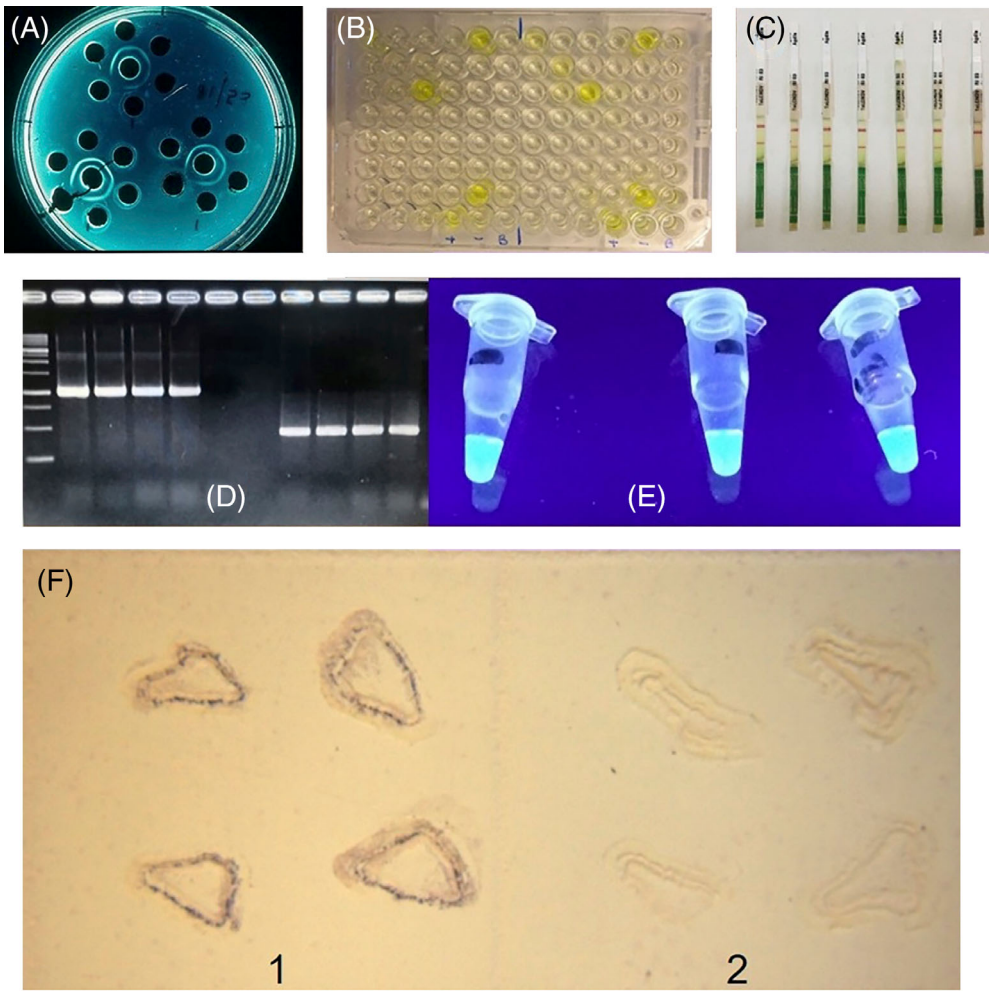

Figure 4. (A) Petri dish showing an Immunodiffusion in agar gel for the detection of *Tomato bushy stunt virus* (ToBSV)–White line are the results of interaction between the sap crude extract containing the viral particle and the specific antibody for ToBSV; (B) Detection of *Tomato brown rugose fruit virus* using Double antibody sandwich–Enzyme linked Immunosorbent Assay (DAS-ELISA); (C) Lateral flow device for the rapid detection of *Tomato leaf curl New Delhi virus*–Two lines positive sample, one line (control line) negative sample; (D) An example of PCR products visualized on 1.5% agarose gel staining with SYBR Safe; (E) Product of LAMP reaction under UV light, using calcein as fluorescent metal indicator; (F) Detection of *Citrus tristeza virus* using Direct Tissue Blot ImmunoAssay (DTBIA) where (1) is positive sample showing purple color on the phloem bundle line while (2) is the negative sample.

compounds isolated from the secretion of a rhizobacteria against the fire blight disease caused by *Erwinia amylovora* and the soft rot disease caused by *E. carotovora*.[11] MALDI-TOF MS has also been used to understand the role of the symbiotic endophytic bacterium, *Enterobacter cloacae*, in impairing resistance of banana plants against Black Sigatoka Pathogen, *Pseudocercospora fijiensis*.[154] The use of this technique for the detection of viral plant pathogens is still limited.[155]

5 CONCLUSIONS: PROS AND CONS OF EACH PATHOGEN DETECTION METHOD

In conclusion, techniques used to detect the complex nature of plant pathogens are heterogenous ranging from Immunodiffusion in agar gel and Double antibody sandwich–Enzyme-linked Immunosorbent Assay to portable devices, PCR, or LAMP-based techniques (Fig. 4). Plant pathogens present a specific dynamic in each plant species, in its different vegetative or developmental stages, with very variable symptoms and disease development, which require special attention for a successful diagnosis (Fig. 5). It should be noted that all methods used in diagnosis of plant diseases have important advantages and disadvantages (Table 1). The plant pathogen detection methods should have the ability to diagnose a substantial number of samples from plant disease in less time and in a cost-effective manner. Benefiting from rapid development in chemistry, plant molecular biology biotechnology, and nanotechnology, nucleic acid-based strategies have been considerably improved in terms of efficiency, accuracy, and sensitivity, thereby offering simple and rapid on-site detection solutions (Fig. 6). However, most of these detection techniques are either time-consuming or require sophisticated instruments that may not be available for field conditions or poor-resource areas. Therefore, integrating such technologies with high-performance visual methods could make detection easier and cheaper. The real-time PCR represents the most reliable tool for the specific and sensitive detection of plant pathogens. This method allows the detection of a small amount of pathogen DNA or RNA/cDNA (pg or fg) in plants even in the absence of visible symptoms. For these reasons, this method is largely used for phytosanitary inspection and even to intercept quarantine pathogens at the port of entry.[106] In the last few years, many efforts have been dedicated to the development of rapid methods for in-field analysis, such as LAMP assay. However, some steps of these protocols still need to be improved for a complete analysis under field conditions, such as the preparation of the amplification mixture, which usually needs laboratory support. On another hand, serological-based assays are known to be quick, simple, low-cost, and high-throughput, mainly for higher sample numbers. However, their effectiveness depends on the quality and availability of specific antibodies besides being quite time-consuming, but it can be a good test for preliminary screening of samples. Both molecular and serological methods provide a highly specific detection of pathogens, but are destructive and limited to the laboratory, require highly skilled manpower. However molecular methods can be used *in situ* for real-time detection of diseases before any symptoms are visible on the host. Furthermore, these methods can be also used to follow the progress of the disease or the effect of agrochemicals that are used to control the disease's spread. In recent years, spectral imaging represents a good alternative for untargeted plants disease detection mainly in large crops, thus not looking for specific pathogens, but rather capturing the overall symptoms or changes. This approach has a major benefit over destructive chemical methods that are focused on identifying specific pathogens responsible for the damage. The main benefit of spectral imaging strategies resides in their ability to non-destructively capture the spatially distributed spectral properties of plants, which can be used to localize disease foci at the first stages, often missed by destructive analyses. The other major advantage of spectral imaging is the ability to perform a real-time detection during acquisition using a model on a small

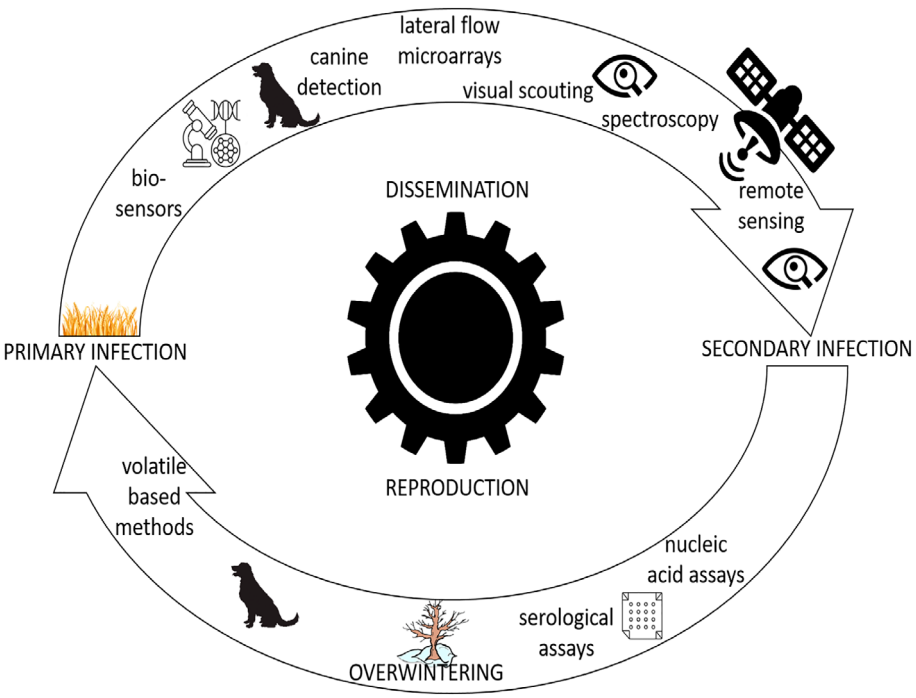

Figure 5. Application of different diagnostic techniques at different stages of the life cycle of a pathogen.

Table 1. Advantages and disadvantages of methods used to diagnose plant pathogen attacks

Method	Sensitivity	Advantages	Disadvantages	Portable	Literature cited
Enzyme-linked immunosorbent assay (ELISA)	Medium	Low cost at commercial level	Low sensitivity for bacteria	No	23,25
		High-throughput potential	Not useful for early detection		
		Low limit detection for virus	Time consuming		
		Medium costs	Low potential for spatialization		
		Good reliability	Not always effective with all types of plant material		
Traditional end-point PCR	High	Capability to detect a single target in complex mixtures	Time consuming	No	40
		Multiple targets	Used by highly specialized staff		
		Detect of uncultivable pathogens	Requires lab infrastructure		
Real time PCR	High	Accurate detection and quantification	Time consuming used by highly specialized staff	No	46
		Rapid and specific early detection	Requires lab infrastructure		
Digital PCR	High	Absolute quantification, no standard for several applications	Limited dynamic range of detection	Yes	46
		Sensitivity and accuracy	Problems with very large amplicons		
		Applicable to complex mixtures	Complex work-flow		
		Linear response			
Rolling-circle amplification	High	Amplification of entire circular genomes	For identification it is necessary for linearization, plasmid cloning, and Sanger sequencing	Yes	99
		Amplification not restricted to target-specific primers			
		Low cost in terms of instrumentation			
Portable thermocyclers	High	Rapid detection	Possibly false-negative results	Yes	156
		Cheap	Need to adapt kits for different pathogen		
		Detection of multiple pathogens			
		Easy-to-use			
Lateral flow microarrays	High	Rapid and infield analysis	Possibly false-negative results when only host genes are analyzed	Yes	104
		Possibility to analyze multiple pathogens	Need to be validated		
		Early detection at asymptomatic stage			
LAMP and qPCR based methods	High	Rapid detection	Possible false-positive results	Yes	84–86
		Detection of multiple pathogens			
		Early detection			
Differential mobility spectrometer	Medium	Rapid detection	Possibly false-negative results when only host genes are analyzed	Yes	109
		Easy-to-use	Need to be validated		
		Detection at early symptomatic stage			
Paper spray mass spectrometry	Medium	Simple and low cost for the identification of matrices containing different ionized chemical compounds	Simple and low cost for the identification of matrices containing different ionized chemical compounds	Yes	110
Spectral imaging	Low	Detect early infections	Possibly false-negative and false-positive	Yes	121,122
		Combinable with drones and remote sensing	Need to be validated		
		Allows detection at early symptomatic stage			
		Infield analysis	Complex work-flow		

Table 1. Continued

Method	Sensitivity	Advantages	Disadvantages	Portable	Literature cited
Raman spectroscopy	High	Combinable with drones and remote sensing Detection at early symptomatic stage	Possibly false-negative and false-positive results Need to be validated	Yes	127,128
Smartphone-based fingerprinting methods	Low	Rapid detection Detection of multiple symptoms Early detection	Possible false-positive results	Yes	134
Recombinase polymerase amplification	High	It avoids cDNA production	Need to adapt kits for different pathogen	Yes	68,69
Oxford nanopore technologies	High	Allows detection of any pathogen present in the plant	Possibly false-negative results High costs per sample	Yes	157

calibration set and, then, deploying the model onboard an autonomous platform. A key challenge of this alternative method is the illumination effects caused by the interaction of light with the complex geometry of plants and their surroundings causing scattering effects that may mask the real spectral responses. Furthermore, advanced NGS-based techniques offer rapid detection and identification through high throughput analysis in a cost-effective way. The primary advantage of portable instruments is obtaining rapid and reliable results in the field. Similarly, methods that detect marker volatile compounds can detect plant disease even before symptoms occur in the plant. Thus, these portable instruments are able to detect not only pathogen signatures (DNA, RNA, proteins) but also induced responses in infected host plants (RNA, proteins, metabolites), representing a promising future of plant disease detection.[156,158,159] Several modern platforms and portable devices are becoming more readily available for the diagnosis of a substantial number of pathogens. The MinION Nanopore sequencing technology is a rapidly maturing technique that will represent a general trend in the future, allowing on-site detection by generating long reads in real-time. Indirect detection methods, field-deployable or not, like hyperspectral imaging, and RS, need a secondary confirmatory test by the direct

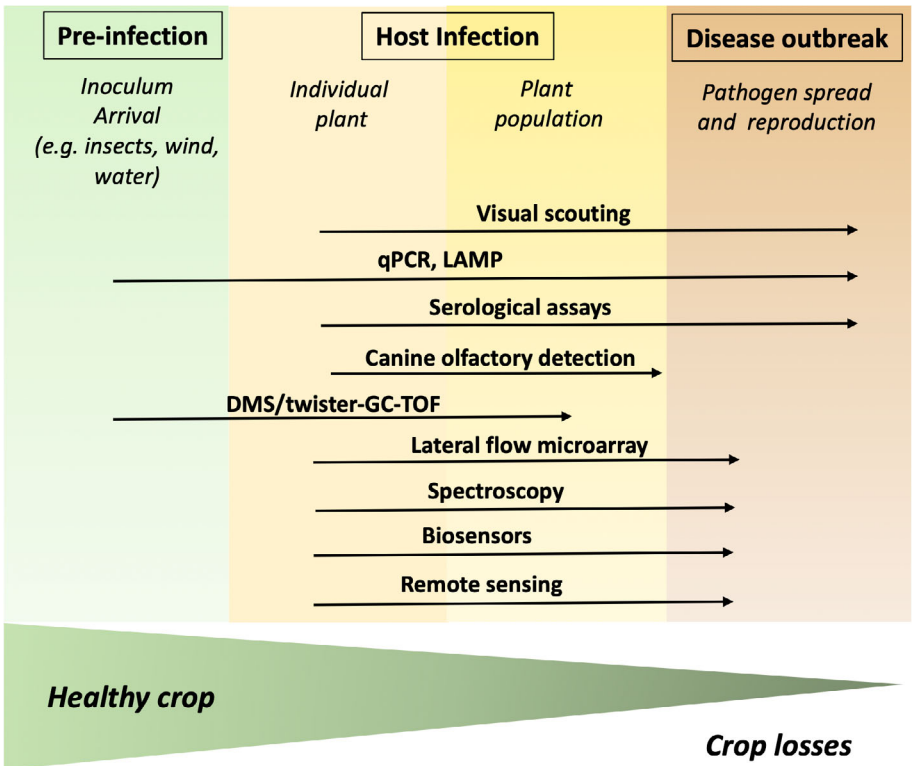

Figure 6. Diagnostic methods for detecting pathogens on crops. The various techniques show different sensitivities and allow detection of pathogens at different infectious stages in the host.

Figure 7. Detail of 'Il Campo dei Giganti', a land art work by Ulderico Tramacere (Boncore, Nardò, LE). The work begins in 2020 on a centenary olive grove of one and a half hectares, and 150 specimens of monumental olive trees are mapped in the Puglia Region and severely affected by *Xylella fastidiosa*. The intent of the work is to raise awareness of environmental issues through the artistic medium: infected olive trees are pruned and white painted. The gesture is intended as an act of care towards the senescent olive trees, whose secular memory is intended to be preserved and, at the same time, is inserted in the artistic languages of contemporaneity with the hope of activating an active rethinking of environmental policies in the area. Photo: Ulderico Tramacere.

method to validate their results. Alongside increasingly better techniques, the existence of good diagnostic skills remains an indispensable element for effective and prompt action. Obviously, earlier detection of infections allows earlier intervention. In this sense, portable thermocyclers, lateral flow microarrays, and volatile-based detection instruments can detect plant pathogens before secondary infections occur (Fig. 5). The spatial and temporal analysis of disease can be calibrated and coupled with reference data. This tissue-specific transcriptional fingerprint would greatly enhance early, asymptomatic detection of systemic pathogens characterized by low titers in infected tissues. So, the great challenge is to identify pathogen-specific host transcriptional signatures. Multiplex analysis of several host transcripts can improve the reliability, specificity, and sensitivity of host-based diagnostic methods. Therefore, with further optimization and improvements for use under field conditions, new portable techniques will offer unexplored avenues for the consistent diagnosis of highly threatening plant diseases such as OQDS (Olive Quick Decline Syndrome) (Fig. 7).

AUTHOR CONTRIBUTIONS

FM conceived the structure of the manuscript. FM and DT mainly wrote the manuscript. RS, MFB, SP, SD, AG, SO, NL, and SY participated in writing the manuscript. SY elaborated the table. All authors reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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