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The diagnosis of plant pathogenic bacteria: a state of art

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TABLE OF CONTENTS

1. ABSTRACT

Plant protection plays an important role in agriculture for the food quality and quantity. The diagnosis of plant diseases and the identification of the pathogens are essential prerequisites for their understanding and control. Among the plant pests, the bacterial pathogens have devastating effects on plant productivity and yield. Different techniques (microscopy, serology, biochemical, physiological, molecular tools and culture propagation) are currently used to detect and identify bacterial pathogens. Detection and identification are critical steps for the appropriate application of phytosanitary measures. The "harmonization of phytosanitary regulations and all other areas of official plant protection action" mean the good practices for plant protection and plant material certification. The prevention of diseases progression and spread by early detection are a valuable strategy for proper pest management and disease control. For this purpose, innovative methods aim achieving results within a shorter time and higher performance, to provide rapidly, accurately and reliably diagnosis. In this review, we focus on the techniques for plant bacterial diagnosis and on the regulations for harmonizing plant protection issue.

2. INTRODUCTION

Plant protection against plant disease played an obvious role in the food quality and production (1). Food losses due to pathogens crop infection (such as bacteria, viruses and fungi) were persistent issues in agriculture. The damages caused by pathogens, animals and weeds are responsible for losses between 20-40% of global agriculture productivity (2). Essential prerequisites for understanding and controlling plant diseases were the detection and identification of the causal agents. Systems for early detection of pests might prevent diseases spread and food losses.

At present, the whole budget invested to crop production limited the routinely use of pathogen's detection methods, except in special cases, e.g. certification and plant quarantine (3). Fang and Ramasamy (4) proposed the use of advanced disease detection methods to minimize and prevent the crop disease damages during growth, harvest and postharvest processing, as well as to maximize productivity and ensure agricultural sustainability.

Among the plant pests, we focused on phytopathogenic bacteria and related diagnostic techniques. These type of pathogens cause devastating effects on plant productivity and yield. Mansfield and colleagues (5) described the bacterial plant pathogens 'Top 10' list based on scientific/economic importance. The survey allowed the construction of a list that includes, in rank order: *Pseudomonas syringae* pathovars; *Ralstonia solanacearum*; *Agrobacterium tumefaciens*; *Xanthomonas oryzae* pv. *oryzae*; *Xanthomonas campestris* pathovars; *Xanthomonas*

axonopodis pathovars; *Erwinia amylovora*; *Xylella fastidiosa*; *Dickeya* (*dadantii* and *solani*); *Pectobacterium carotovorum* (and *Pectobacterium atrosepticum*). This list is partial since did not consider other important pathogens as *Clavibacter michiganensis* (*michiganensis* and *sepedonicus*) and *Pseudomonas savastanoi* (5).

Phytopathogenic bacteria might survive in diverse environments: in plants as pathogens and outside their hosts as saprophytes and epiphytes. Adverse environmental conditions might reduce bacterial survival and compromise disease initiation and dissemination. On the contrary, the infection cycle might occur when the condition become favorable. Rainfall, contaminated farming equipment and/or plant material and insects promoted the dissemination of phytobacteria. Disease symptoms caused by bacteria include leaf spots, blights, wilts, scabs, cankers, tumors and soft rots of roots, storage organs and fruit and overgrowth.

Some disease caused by bacteria recently raised public awareness such as in Italy where *Xylella fastidiosa,* a gamma-bacterium, caused an "inestimable negative impact on the economic, social, environmental, cultural and historical importance of the olive trees" (© FAO, 2017 I7075EN/1/04.1.7).

3. PLANT PROTECTION AND MANAGEMENT

The protection of natural and managed plant systems from alien and emerging indigenous pests is a strategic socio-economic issue (6). Globalization, climate change and landscape modification facilitated the dissemination and introduction of alien pests, the evolution of new races, biotypes and strains of indigenous pests; this dramatic change of scenario causes emerging diseases everywhere. Plant productivity, sustainability and biodiversity is compromised by pathogens (7). The cooperation in plant health have been established within the International Plant Protection Convention (IPPC, https://www.ippc.int/); in particular, the European and Mediterranean Plant Protection Organization (EPPO) is an intergovernmental organization responsible for cooperation and harmonization in plant protection within the European and Mediterranean region, under the International Plant Protection Convention (IPPC). The EPPO missions, as reported in the EPPO website aim at i) "developing an international strategy against the introduction and spread of pests that damage cultivated and wild plants"; ii) "harmonizing of phytosanitary regulations and all other areas of official plant protection action"; iii) "promoting the use of modern, safe, and effective pest control methods"; iv) "providing a documentation service on plant protection".

The harmonization of phytosanitary regulations and all other areas of official plant protection action meant the good practices for plant protection and plant material certification based on the application of diagnostic methods validated by test performance studies and proficiency tests. The application of appropriate validated guidelines produced universally acceptable results useful for phytosanitary action and regulatory decisions (8). International Organization for Standardization (ISO) standardized reference methods at the international level and by the European Committee for Standardization at the European level (9). The standardization process established reference documents in agreement with all parties and countries. As a result of the work undertaken by the panels of experts, nominated by their National Plant Protection Organizations (NPPO) under the supervision of the working parties, EPPO makes recommendations to the NPPO of its member countries. In order to ensure international acceptance, the draft standards went through a complex approval procedure during which all member countries had the opportunity to express their opinion. Final decisions are obtained by consensus and the EPPO council officially adopted EPPO Standards. EPPO Standards have been developed within the two main fields of EPPO activity: plant protection products and phytosanitary measures. The EPPO standard provide the diagnostic protocols, the pathogen identity, the disease symptoms, the sampling procedures, the bacterial extraction procedure, the screening and identification tests. For each test, the performance criteria are reported. A protocol is considered validated when the analytical sensitivity, analytical specificity, repeatability and reproducibility are reported (EPPO Standard PM 7/98 (2)).

A European and International Standard, ISO 16140, has been developed to provide a common reference protocol for validating alternative methods and determining the principles for their certification (9). The ISO 16140:2003 recently revised, defined the general principle and the technical protocol for the validation of alternative methods in the field of microbiological analysis which can be used in the framework of the official control.

For diagnostic laboratories in the "EPPO regions", the quality management systems and accreditation has become a concern. The Standard Pest Management (PM) 7/84 "Basic requirements for quality management in plant pest diagnosis laboratories", adopted in 2007, described the requirements to assist laboratories conducting plant pest diagnosis and in designing their quality management system. PM 7/98 (2) included additional requirements for laboratories applying for accreditation, based on the ISO/IEC Standard 17025 *"General requirements for the competence of testing and calibration laboratories"* (ISO/IEC, 2005). National accreditation bodies granted accreditation against the ISO/IEC Standard 17025. Accreditation is part of an overall system and provides an authoritative statement

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of the technical competence that ensure conformity with the applicable requirements.

4. BACTERIAL PATHOGEN DETECTION AND IDENTIFICATION

Plant symptoms observation, the first step in pest management, is carried out using optical methods (10). Detection and/or identification of pests actually represent critical steps for the appropriate application of phytosanitary measures as illustrated in a range of international documents (11). International Standards for Phytosanitary Measures (ISPM) No. 27 described the procedures and the methods for the official diagnosis of regulated pests and provided the minimum requirements for reliable diagnosis.

The protocols for official diagnosis foresee a first step of detection of the causal agent in the plant host and a second step for its identification. Therefore, a diagnostic method can be used as screening test for the detection of the bacterium in the plant matrix and/or as identification test for the characterization of the purified bacterial culture. The screening test evaluates the presence or absence of the pathogen directly in the plant matrices; it can be performed with different techniques. These assays were particularly useful when a rapid throughput diagnosis is required, as the case of big lots of seeds/plant propagation material, waiting for a quarantine diagnosis, object of commercial trade blocked at airport or port. In case of negative screening test result, the material might be promptly moved, conversely, a positive result required the isolation procedures and identification test on pure culture for result confirmation.

Several techniques based on different principles involving microscopy, serology, biochemical, physiological, nutritional, molecular tools and culture propagation are currently used as diagnostic tools.

Serology provided indirect evidence of bacterial diseases, whereas the use of broad-range polymerase chain reaction (PCR) based techniques, allowed specific detection and identification of bacteria. Indeed, isolation and culture propagation remained the “golden” method and were still crucial steps for an accurate diagnosis.

Isolation provides several irreplaceable advantages for studying emerging bacterial diseases, since allows complementing the pathogenicity assay but also developing antigenic studies, antibiotic or alternative compounds tests, susceptibility, experimental models, genetic investigations and taxonomic studies. In bacterial diagnosis, the pathogenicity tests and bioassays are necessary for a final confirmation, in particular in case of new syndromes or for the diagnosis of quarantine bacteria. Moreover, isolation permitted the constitution of culture collections for the maintenance of bacterial strains.

The isolation of bacterial plant pathogens occurred by using:

1. selective media in order to stop or delay the growth of non-targeted saprophyte or contaminant bacteria, (e.g. SMSA for Ralstonia solanacearum, KBC for Pseudomonas syringae pv. actinidiae)
2. media added with particular substrates that can be degraded only by the targeted bacteria (e.g. crystal violet pectate-CVP for Pectobaterium carotovorum, BS for Xanthomonas arboricola pv juglandis)

media that confers a particular color to the growing colonies (e.g. EMB agar medium for Brenneria nigrifluens).

The bacterial identification phase includes at least two tests based on different biological principles (e.g. combination of biochemical, serological and/or molecular test) or two molecular tests based on different DNA sequence targets in the genome of the pathogen. Indeed, the final identification of a suspected plant bacterial pathogen generally requires a pathogenicity test by properly inoculating their host plant. The hypersensitivity response test (HR) on tobacco is useful for some pathogens i.e phytopathogenic *Pseudomonas* and *Erwinia amylovora*, but for others bacterial species provided little or no response, i.e *Pantoea stewartii* or several *Xanthomonas* spp.

Nevertheless, conventional detection methods have several drawbacks (3) such as:

1. the bacterial culturing might often take days or weeks and this is critical when rapid and high throughput detection is required;
2. it may be difficult to discriminate a bacterial species on the basis of the sole morphological features overall if it is necessary to discriminate among closely related organisms (low specificity);

these methods did not permitted to detect the pathogen if present in a low load in the plant material (low sensitivity).

Different papers (12-14) dealt with the positive and negative aspects of the diagnostic techniques as well as the considerable advantages that the immunological and nucleic acid techniques offered over conventional diagnostic methods.

4.1. Serological methods

The serology-based methods for bacteria detection are analytical tools used for a wide range of targets and provide indirect evidence for causal relationships

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between the disease and the causal agent. Serology is helpful, timely and cost-effective for the large-scale analyses of symptomatic materials. The enzyme-linked immunosorbent assay (ELISA) is a test combining the specificity of antibodies and color change to identify a target. Antibodies may recognize specific antigens associated with a given plant pathogen (3, 10); polyclonal antibodies, successfully used for detecting the pathogen, are not always sufficiently specific whereas monoclonal antibodies are more specific, but even more expensive. There are a number of different ways for detecting antibody/antigen binding; often these involve coupling the antibody to an enzyme that can be used to generate a color change when a substrate is added. The ELISA test may be grouped in:

1. the direct ELISA detection assays in which assay the specific antibody is conjugated to the enzyme;

2. the indirect ELISA assay in which the specific antibody is detected by a second generic antibody, e.g. anti-rabbit or anti-mouse, which is conjugated to the enzyme (indirect detection) (15, 16);

3. the sandwich ELISA assay quantify antigens employing two layers of antibodies (i.e. capture and detection antibody). The schematic procedure of the sandwich ELISA can be resumed: a) a specific antibody coats the microtiter plate and trap the target antigen from the tested sample; b) the detecting antibody is added and binds the antigen; c) the enzyme-linked secondary antibody is added and binds to detecting antibody; d) the substrate is added and is converted by enzyme to detectable form;

4. The ELISA competition assays in which the higher sample antigen concentration corresponds a weaker the detection signal.

Many different commercial kits used for the preliminary screening of plant material for bacterial detection are based on indirect ELISA.

Another serological method widely used in bacteriology as screening and identification test is the indirect immunofluorescence (IF). IF is a fluorescence microscopy-based optical technique utilized to detect pathogen infections in plant tissues. For this technique, plant samples (thin tissue sections or extract) are fixed to microscope slides. Detection is achieved by conjugating a fluorescent dye to the specific antibody to visualize the distribution of the antigen (bacterial cell) throughout the sample (3).

IF is more accurate than ELISA because the interpretation of the result is based not only on the fluorescence emission (that can be aspecific), as for ELISA, but also allow the discrimination of bacterial cells by fluorescent microscopy directly into the plant tissue sample.

4.2. Molecular methods

The molecular tests can be based on hybridization or amplification techniques and can be highly specific. Most assays developed for bacteria pathogen detect DNA, which is easier to prepare, and more stable than RNA.

The hybridization techniques exploited probes as single stranded DNA or RNA molecules labeled with reporter molecules such as a radioactive isotope, an enzyme or a fluorescent dye (3). The probes recognize the complementary DNA/RNA sequences on the target samples and the signals might then be detected. Depending on the type of reporter molecules, the detection step might use radioactivity or light (chemluminescence). Probes can be generated from double stranded genomic, cloned or PCR-derived DNA.

Among the hybridization techniques, a modern evolution is represented by DNA microarray. It consisted of an array of thousands microscopic spots of specific DNA oligonucleotides (probes or reporters) on a solid substrate (usually a glass slide or silicon thin-film cell). The probes hybridize a DNA or cDNA samples (target) under high-stringency conditions. The probe-target hybridization is usually determined by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to assess relative abundance of nucleic acid sequences in the target sample. Considering the high number of spotted probes, a microarray can accomplish many genetic tests in parallel. Pelludat and collaborators (17) developed a DNA microarray for a rapid detection of quarantine phytopathogenic bacteria. Another hybridization technique used for detection of plant pathogenic bacteria is the Fluorescent *In situ* Hybridization (FISH). This technique used 16S or 23S rDNA oligonucleotide probes labelled with a fluorescent dye in combination with microscopy. FISH probes (20-30mers) recognize the plant extracts/cells fixed in microscopic slide and hybridize with target gene in plant samples. The probe-target hybridization can be visualized by incident light (fluorescent). FISH have been used with probes targeted for the 23S rDNA to detect *Ralstonia solanacearum* from potato peels (18).

In recent years, molecular diagnostic assays used PCR that is more sensible, simple and fast compared to hybridization techniques. However, use of probes integrated within PCR based techniques producing more sensitive, more specific or simpler diagnostic protocols compared to PCR alone.

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Among the amplification tests, different PCR-based methods are developed for detecting bacterial nucleic acid: end-point PCR (among which BIO-PCR, nested-PCR and duplex/multiplex-PCR), real-time PCR, LAMP (Loop mediated isothermal amplification) and digital-PCR. The DNA extraction method and the choice of the nucleic acid target are critical steps to be considered when a PCR based diagnostic technique is performed. The availability of commercial kit helps in the standardization of DNA extraction, ensuring the reliability and the quality of DNA from different plant species and matrices.

The end-point based PCR technique detect the presence of the amplified sequence by gel electrophoresis to discriminate positive or negative samples. Different end-point PCR methods are developed for the detection of plant pathogenic bacteria (19, 20).

It is worth mentioning that, for the PCR based method the combination with enrichment of bacterial cells in liquid or solid media may facilitate the detection from difficult matrices as seeds, soil samples or symptomless plant material and reduce the PCR inhibitors detection (19, 21). These PCR based methods usually named BIO-PCR have the advantage, compared to conventional PCR, to detect the presence of viable bacterial cells. Indeed one of the most relevant negative aspect of PCR is the inability to discriminate between viable and non-viable bacterial cells, because DNA is almost stable whether the cell is dead or alive. Ito and collegue (21) developed a PCR method to detect viable cell of *Ralstonia solanacearum* in soil using a semi-selective medium and PCR technique. Some workers have opted to start from RNA to proceed with a reverse transcriptase PCR (RT-PCR) assay or with an isothermic amplification technique, Nucleic Acid Sequence Based Amplification (NASBA) (22, 23). Up today, validated diagnostic protocol based on RNA to screening bacterial plant, pathogens are not yet available.

Another widely used PCR-based method is the nested-PCR that involves two sets of primers, used in two successive runs of PCR, the second set intended to amplify a secondary target within the first run product. This method is developed for the detection of plant pathogen bacteria as *Erwinia amylovora* (24) or *P. syringae* pv. *actinidiae* (25). It is useful for very rare templates, because is quite sensitive, however the risk of false positive results is high.

An advantage of PCR-based method is the possibility to detect simultaneously seeveral targets by, for instance, duplex or multiplex-PCR. The duplex/multiplex conventional PCR uses several primers in the same reaction and the products of different targets need to be of different sizes to ensure that they can be distinguished from one another using an agarose gel (26). Different DNA regions can be targeted to distinguish strictly related pathovars or subspecies. An example is the duplex-PCR developed to distinguish *P. syringae* pv. *actinidiae* from the phylogenetic-related pathovars *P. avellanae, P. syringae* pv. *theae* and pv. *tomato* (27) and from the kiwifruit-pathogen *P. syringae* pv. *actinidifoliorum*. Moreover, a multiplex-PCR has been developed to distinguish several subgroups within a bacterial population, as the case of the different *P. syringae* pv. *actinidiae* groups that are differentiated on the basis of their geographic origin (28).

The real-time PCR exploits the fluorescent emission of a specific dye to detect the targeted amplicons; it is possible to follow the amplification steps in real time, eliminating post-amplification time-consuming procedure. The fluorescence intensity is proportional to the amount of amplified products and for each sample the 'cycle threshold' (Ct) is calculated. The 'cycle threshold' (Ct) is the cycle number at which a statistically significant increase in fluorescence is detected. The Ct increases with decreasing amounts of target DNA. The real-time PCR used non-specific DNA binding dyes or fluorescent probes specific for the target DNA. The DNA binding dyes, such as SYBR green, represents a simpler and cheaper approach than using specific probes, e.g. TaqMan oligonucleotide probes (29); conversely, the SYBR green method may provide non-specific PCR products.

It is worth mentioning that, the specific probes have the advantage of reducing non-specific signals. Different papers demonstrated that nucleic acid-based methods are very sensitive and highly specific for the screening and the identification of different bacterial pathogens such as *Xylella fastidiosa, Pseudomonas syringae* pv. *actinidiae* or *Pantoea stewartii* (30-33).

Another advantage of real-time PCR is represented by the possibility to quantify the target microorganism (32, 33). The multiplex Real-Time PCR can also detect simultaneously more pathogen using probes with different fluorescent reporter dyes (34). Strayer and colleagues (34) improved a multiplex real-time PCR to detect and identify the bacterial spot pathogens *Xanthomonas vesicatoria*, *X. perforans*, *X. gardneri* and *X. euvesicatoria* employing the region of *hrpB* as target. The fragment amplified with the Taq Man real-time PCR revealed a highly conserved region within each species, with a single-nucleotide polymorphism (SNP) among the *X. vesicatoria* strains. *X. euvesicatoria* and *X. perforans*.

LAMP (loop-mediated isothermal amplification) is an isothermal amplification, highly specific, because it used about six specific primers, exploited the use of a fluorescent dye and it can be used in field when it is necessary to perform the

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analysis at large-scale of a contaminated area (35). The procedure indeed includes a rapid DNA extraction method using low quantity of plant material and is based on an easy-to-use system to be performed in field. However, to be considered effective by the scientific community and usable for official analysis, it needed of a validation by screening a large scale of samples which are representative of the different conditions (e.g. kind of pathogen, plant species, plant matrices). LAMP was recently proposed for the monitoring activity of *X. fastidiosa* from plant material and insects (35, 33). LAMP could be used to confirm isolate identity from culture collections and for epidemiological studies and disease surveys as reported by Harper and colleagues (31).

Recently, some authors proposed using in plant pathology diagnosis the Droplet Digital polymerase chain reaction (ddPCR) (36). This ddPCR is a novel technique that quantify the nucleic acids target without the need of an external calibrator. This method is a robust approach for diagnosis of plant pathogens also with a low target concentration. Among the advantages of the ddPCR the authors reports the potential for the quantitative detection of *Xanthomonas citri* subsp. *citri* with high precision, accuracy and a lower coefficient of variation compared to the qPCR (37). Among phytopathogenic bacteria, besides *Xanthomonas citri* subsp. *citri* the ddPCR was recently proposed for the detection of *Xylella fastidiosa* (38).

4.2.1. Which target for molecular detection?

The choice of target gene to discriminate plant pathogen represented a crucial point for the development of plant disease diagnosis systems and for the detection of the emergent plant pathogens. The 16S rDNA gene (ribosomal DNA) is traditionally used to ascribe a bacterial strain to a genus (3). The rDNA is present in many copies in each cell and allowed a very sensitive detection, when used as target. The rDNA genes are universally applicable; include regions that are highly conserved such as the 16S and 23S genes and others that are highly variable such as the internal transcribed spacer (ITS) regions. In many cases, the rDNA discriminates between taxa and at the required level: genus, species or below (39,40). The target based on rDNA conserved region showed problems due to lack of specificity, whereas the ITS regions were used by several authors to design primer to improve the primers specificity (19) discriminating between related organisms.

A variety of targets can be used to design specific PCR assays according to the specificity (41, 42). For instance plasmid DNA (43), genes associated with hypersensitive response and pathogenicity (27, 44-46) or with the phytotoxin production (47, 48). The ability to distinguish between different strains within a bacterial species is an important requisite for epidemiological surveillance and evolutionary studies. It is important to consider that very closely related strains can appear actually different on the basis of antigenic gene proteins, increasing the discriminatory power, (40), by contrast, the use of region with slow accumulation of variation (within house-keeping loci) would lead to a lack of discrimination between closely related strains. A correct evaluation of the genetic variability of a bacterial population is an important prerequisite for developing a diagnostic method. Frequently the genetic variability studies stated the basis for the identification of specific genome sequences that can be used as target for the diagnosis of a species (49, 50).

Multilocus sequence typing (MLST) is often used for a deep characterization of bacterial strains using several housekeeping genes. The 16S rDNA and the genes *rpoB*, *groEL* and *ftsZ* are employed for the microarray development enabling the differentiation of quarantine bacteria by 38 probes (18). The authors reported that for the direct identification in plant material, further improvement is needed to avoid cross-hybridisation among closely related pathovars (18). The housekeeping genes, highly conserved within the same genus made them a great target for barcoding (51, 52), for instance the *gyrB* employed for the *Xanthomonads* barcode and *rpoD* for the *Erwinia* barcode. MLST analysis are widely used for *Xylella fastidiosa* to define the subspecies of a new detected strain (53, 54).

The next-generation DNA sequencing projects provided the possibility to enlarge the availability of sequenced pathogen genomes and the molecular marker resources, making possible a significant improvement in the surveillance of emerging disease threats. Whole genome sequencing is helpful to find new targets for plant pathogens. Ash and colleague (55) developed a genomics-based LAMP (loop-mediated isothermal amplification PCR) assay for detection of *Pseudomonas fuscovaginae* from rice using its draft genome sequences. Its genome has been used to assess genomic diversity of *P. fuscovaginae* isolated from rice in South America, Asia, Australia, and Africa and to guide the development of a selective and sensitive LAMP detection assay.

4.3. Image spectroscopy techniques

The application of spectroscopic and imaging techniques in agriculture give the possibility of an automated non-destructive method of plant disease detection (56). Among these, fluorescence, multispectral or hyperspectral imaging, infrared spectroscopy, fluorescence spectroscopy, visible/multiband spectroscopy and nuclear magnetic resonance (NMR) spectroscopy are methods used

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for plant disease detection (12). In plant bacterial diseases, fluorescence spectroscopy has been adopted (56) to detect stress caused by citrus canker (caused by *Xanthomonas citri – X. axonopodis* pv. *citri)* and mechanical injury. The author discriminated between the mechanical and diseased stress. Lins and collegue (57) conducted field experiments to discriminate the abiotic citrus canker-stressed leaves from chlorosis-infected (caused by *Xylella fastidiosa*) and healthy leaves. In this technique, the chlorophyll fluorescence is measured on the leaves as a function of the incident light and the change in fluorescence parameters provided an indication of pathogen infections. Visible and infrared spectroscopy have been used as a rapid, non-destructive, and cost-effective method for the detection of plant diseases (12, 58, 59). Hyperspectral imaging is gaining *momentum* for its application in precision agriculture (60) and stress detection as also reported for different plant pathogens (61) by measuring the changes in reflectance resulting from the biophysical and biochemical characteristic changes upon infection.

4.4. Electronic nose and volatile organic compounds

A non-optical indirect method for plant disease detection is based on the profiling of the volatile chemical signature of infected plants. Infected plants could release specific volatile organic compounds (VOCs) that are highly indicative of the type of stress experienced by plants. Recently, some study (62) evaluated the volatile metabolic gas profile analysis to identify plant diseases. The common methods used for assessing the VOC profile are gas chromatography (GC)-based and electronic nose system-based techniques. Specific volatile biomarker may be used to detect plant diseases as reported for the bacterial soft rot, caused by *Erwinia carotovora*. Prithiviraj and collegue (63) assessed the volatiles released from onion bulbs infected with *Erwinia carotovora* to discriminate the infected once.

4.5. Point of care

Ideally, the diagnosis should be made at the point of care, so that treatment can start immediately and it is not depending on the availability of a laboratory or of highly trained staff. The point-of-care (POC) tests for plant diseases could be used to improve disease management in resource-limited settings. POC approach is gaining momentum for the ability to perform diagnostic procedures in a precise and rapid manner at the site where it is needed. These systems are easy to use and results are clear, providing quantitative data. The application of POC (64) on-site and mapping the results to define positions via the global positioning system (GPS), would enable the farmer to perform a precise and targeted application of pesticides and thereby reduce and optimize the use of agrochemicals (64).

4.6. Biosensors

Environmental monitoring and medical diagnosis recently improved by the development of novel sensors. The analytes may be detected using sensors exploiting electrical, chemical, electrochemical, optical, magnetic or vibrational signals. The sensitivity and specificity could be improved respectively by the use of nanomaterial matrices as transducers (such as metal and metal oxide nanoparticles, quantum dots, carbon nanomaterials) and by the use of bio-recognition elements (such as DNA, antibody, enzymes etc.). Various types of nanostructures have been evaluated as platforms for the immobilization of a bio-recognition element to construct a biosensor. The immobilization can be achieved using various approaches including biomolecule adsorption, covalent attachment, encapsulation or a sophisticated combination of these methods (4). Fluorescent silica nanoparticles combined with antibody as a biomarker have been utilized for detecting *Xanthomonas axonopodis* pv. *vesicatoria* that causes bacterial spot in Solanaceae plant (65). Nano-chips made of microarrays, which contain fluorescent oligo probes are also reported for detecting single nucleotide change in bacteria and viruses with high sensitivity and specificity based on DNA hybridization (66).

5. SUMMARY AND PERSPECTIVE

Over recent years, several studies in plant pathogen detection allow developing innovative methods to achieve results within a shorter time and sometimes with higher performance than conventional microbiological assays. However, these new methods need to be validated to guarantee their performance at least as well as the corresponding reference methods. The prevention of diseases development and spread by early detection is a valuable strategy for proper pest management and disease control. Plant disease detection, should be provided rapidly, accurately and reliably at early stages, by exploiting novel sensor systems in open field. The spread of disease can be avoided by early diagnosis of infected plants. Asymptomatic plants can be a reservoir of the pathogen and the development of diagnostic methods with improved sensitivity, specificity is helpful for the identification of plant pathogens, even in the absence of disease symptoms or evident signs of the causal agent.

The current breakthrough for the disease diagnosis is found in the spectroscopic, imaging techniques and the volatile organic metabolites as biomarkers, however the technology is constantly evolving and we can expect new inputs in the field of

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diagnostics. The new techniques need to be as much as possible non-invasive and applicable at the point of interest. The interest of the farmers is in solving the problems caused by plant disease, identifying the pests' infection in rapid, real-time, non-destructive methods allowing the application of timely intervention and preventive treatments for the containment of infections and the reduction of crop losses. These strategies can also permit the reduction of massive application of chemicals, localize the sprayings and perform the applications timely to reduce the costs and the deleterious side effects of the control management.

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Abbreviations: European and Mediterranean Plant Protection Organization (EPPO); International Plant Protection Convention (IPPC); International Organization for Standardization (ISO); National Plant Protection Organizations (NPPO); Nuclear Magnetic Resonance (NMR); LAMP Loop-mediated isothermal Amplification PCR; Volatile Organic Compounds (VOCs); Nucleic Acid Sequence Based Amplification (NASBA); International Standards for Phytosanitary Measures (ISPM); Hypersensitivity Response (HR); ImmunoFluorescence (IF); Enzyme-Linked Immunosorbent Assay (ELISA); Fluorescent *in situ* hybridization (FISH); Droplet Digital polymerase chain reaction (ddPCR); Internal Transcribed Spacer (ITS); MultiLocus

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Sequence Typing (MLST); POC (point-of-care); Global Positioning System (GPS)

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