



## Pathogens identification and pest control in plant disease diagnosis

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### Abstract

Feeding the rapidly growing population of the world is a major challenge that human beings face today and ensuring food security of all individuals is a major goal. This is never truer than in the case of India with a population of 1.32 billion expected to touch 1.65 billion by 2050. The present rates of crop production while significantly increased due to the steps taken during the green revolution of the 1960's and 1970's will not be able to meet the demands of the predicted 1.65 billion people. Plant diseases caused by numerous pathogens such as bacteria, viruses, fungi, phytoplasmas are a major problem causing major economic losses worldwide and hence cost effective, rapid, accurate and reliable methods to detect these pathogens are vital to limit the potential damage these pathogens cause by preventing their spread and to enable the cultivators to implement the correct management strategies. The present review recognizes this need and reviews and describes the technologies and techniques that have been used to detect pathogens beginning with the traditional methods before moving on to the methods currently in use- the direct and indirect methods of detection of plant pathogens comparing the benefits and limitations of these methods. It also looks the methods which are still being developed or have just been introduced and the possibilities these new methods hold. Finally, we consider the future directions plants pathogen diagnosis may take and assess the potential of portable on-site plant pathogen detection devices.

**Keywords:** pathogens identification, pest control, disease diagnosis

### Introduction

Food security, described as "a condition in which all people at all times have access to adequate, secure, nutritious food to sustain a healthy and active life" at the 1996 World Food Summit, is a critical problem in the contemporary world <sup>[1]</sup>. This is because the world population is predicted to rise from around 7 billion in the present day to 9.5 billion by 2050 - a 35% increase <sup>[2]</sup>. Moreover, a major chunk of this population is expected to be urban and so the requirement of processed foods will rise, and it is estimated that the world will need an 85% increase in the production of primary foodstuffs by 2050 to meet these demands and produce these processed foods <sup>[3]</sup>. If the current global rates of production per hectare of the major food crops are sustained, they would be unable to meet these requirements and a serious drop in supply to demand seems likely. Currently, over one billion people worldwide lack food security suffering from varied situations of malnutrition either because they do not have access to food supply or because they do not have get the required nutrients in the sufficient quantities in their diet to meet their daily nutrition needs. The situation is all the more concerning in a country like India with an estimated population of around 1.32 billion people which continues to rise. Despite nearly 54.6% of the population being engaged in agriculture and allied activities (census 2011), contributing around 18% of the total GDP, the rapidly diminishing amount of cultivable land in the country means that producing the estimated 450 million tons of food grains to feed a predicted population of 1.65 billion in 2050 is an extremely difficult task <sup>[4]</sup>. Furthermore, a stagnation in the yields per hectare of the

major crops seems to have set in with the effects of the green revolution of the sixties and seventies appearing to have waned. This is highlighted through the example of rice, the staple diet in several Asian countries with China, India and Indonesia the major producers showing a 36% rise in yield per hectare between 1970 and 1980 but the rise in yield per hectare has dropped to 7% between 2000 and 2010 <sup>[5]</sup>. This drop can be attributed to reduction in agricultural land area that results a decrease in productivity. Damage caused by pests and pathogens also plays a significant role in crop losses throughout the world. Although no precise estimates of total crop loss in India due to insects, diseases, or weeds could be found, the Union Minister of Agriculture stated on March 2, 2007, in response to a question in the Rajya Sabha, that crop losses due to pests, diseases, and weeds are estimated to be between 10 and 30 percent of crop production. Pests, diseases, and weeds destroy approximately 30-35 percent of the yearly crop output in India, according to P.K. Chakrabarty, assistant director general (plant protection and biosafety) of the Indian Council of Agricultural Research (ICAR) <sup>[6]</sup>. Crop production losses owing to pathogen infections vary from 20% to 40% globally. Crop infections are thought to be responsible for a loss of approximately 12% of the entire crop of maize, barley, rice, and soybean. Pathogen-related losses in groundnuts and potatoes are projected to be about 24%, while losses in wheat and cotton are predicted to be around 50% and 80%, respectively. Disease-related post-cultivation losses are estimated to reach 30-40 percent <sup>[7]</sup>. The world today thus faces three massive challenges: -

- Meeting the rapidly increasing demands of the growing population for food
- While simultaneously taking care to ensure that the methods adopted to meet these demands are environmentally and socially sustainable
- Ensuring that even the poorest of people have access to food.

As a result, increasing agricultural output to satisfy the needs of a fast-expanding population for safe and healthy food is critical. In addition to the obvious approaches to meeting these demands, such as developing crop strains with higher productivity or resistance to abiotic stresses, one of the major ways to meet the growing demands of a constantly growing population is to detect plant pathogens and pests and then protect crops from these pests and pathogens. Plant protection methods have become more important in overall crop production programs for sustainable agriculture, with the goal of reducing crop losses caused by insect pests, diseases, weeds, nematodes, rodents, and other pests. Furthermore, these techniques must be very precise, dependable, and effective, with high sensitivity and specificity, since many diseases may stay latently asymptomatic in plants, causing harm that is not recognized until years later. Thus, detection of crop pathogens is necessary for the following reasons:

- To determine presence and quantity of pathogens to take appropriate plant protection measures and prevent further spread of the pathogens to adjacent plants, fields and other crops.
- To assess the effectiveness of the various physical, chemical and biological methods of containing pathogens.
- To determine the extent of disease incidence and consequent yield loss caused.
- To detect and identify new pathogens rapidly and prevent their further spread.

Diseases impact all plant species, and these plant diseases may be caused by biotic (pathogenic) or abiotic (physiogenic) factors. The physiogenic causes may be nutrient deficiencies, toxicity due to excess of minerals, lack or excess of soil moisture, temperature extremes, air pollution, variations in soil pH, etc [8]. Pathogenic diseases can be transmitted from infected plants to healthy plants whereas the physiogenic diseases are not transmissible, and the affected plant may recover from the disease if the adverse condition is removed. The pathogenic diseases are far more numerous and varied than the physiogenic diseases, and hence the diagnosis of such diseases becomes more difficult. Pathogens that cause illnesses include fungus, oomycetes, bacteria, viruses, viroid's, mollicutes, phytoplasmas, protozoa, nematodes, and parasitic plants, which vary in size from ultramicroscopic entities to well-defined multicellular creatures. They are responsible for major economic losses in the agricultural industry worldwide. The major crop plants are affected by numerous such pathogens. These pathogens cause economic losses in two ways firstly by reducing the agricultural yield and the second by forcing cultivators to implement costly pest management strategies. The history of occurrence and evolution of plant pathogens is closely linked to the history of agriculture itself. Archaeologists estimate that human beings sustained their existence for over 2.5 million years as foragers and gatherers and did not attempt to interfere or

manipulate the lives of the plant and animal species on which they were dependent for their existence. Roughly an estimated 10,000 years ago however the entire situation changed and what archaeologists call the "agricultural revolution" occurred with humans beginning to manipulate the lives of the plant and animal species they were dependent upon and even to this day all the crops cultivated to meet the ever-growing demands of human beings are derived from the handful of plants our ancestors domesticated in the period between 9500 BC and 3500 BC [9]. Interactions between plants and pathogens have gone on ever since agriculture began and has resulted in a constant never-ending cycle of adaptation, competition and detection. Plant pest control practices too began at the same time as these plant-pathogen interactions. There are 2 alternative hypotheses about the origin of the pathogens - the first suggests that the agricultural practices of humans altered the geographic, environmental, and genetic environments of species, and created new niches for diversification of human, plant, and animal pathogens while the second theorizes that coevolutionary processes in natural populations of the crop's ancestors drove divergence of pathogen species [10]. The early farmers mainly resorted to superstition when it came to dealing with the occurrence of pests and pathogens on their crops. The discipline of plant pathology which is the study of plant diseases caused by both biotic stresses and abiotic stresses only began in the year 1767 when Giovanni Targioni Tozzetti first reported *Puccinia graminis* (which causes "rust of wheat") as the cause of the famine in central Italy [11]. The methods to detect and treat plant diseases have come a long way since then and it is the methods for the detection and identification of the plant pathogens responsible for the biotic plant diseases which are the main topic of interest of this review. The present paper reviews the methods and techniques in place to detect and characterize diseases caused by pathogens. It begins with traditional pathogen identification methods such as microscopy and biochemical tests before moving on to more modern methods such as polymerase chain reaction (PCR), immunofluorescence (IF), fluorescence in-situ hybridization (FISH), and enzyme-linked immunosorbent assay. It then moves on to indirect pathogen identification methods such as spectroscopic methods, imaging techniques, and vol. This review also investigates the new innovative methods still under development or just being introduced including lateral flow microarrays, next generation sequencing and biosensors before considering what the future holds for plant pathogen diagnosis, investigating the potential of on-site detection using portable detection devices. Figure 1 gives an overview of the various techniques for plant pathogen detection which will be covered in this review.

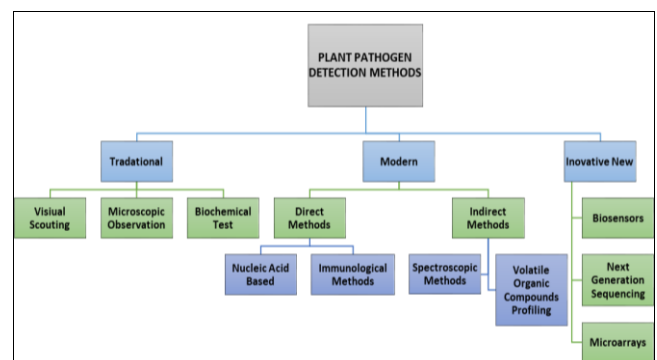


Fig 1: Overview of plant pathogen detection methods

## Methods for Crop Disease Detection

### Traditional Detection Methods

The first method of plant pathogen diagnosis was by simple visual scouting or scanning of cultivated plants to find any signs or symptoms of plant diseases. The major drawbacks with this strategy are that it is extremely consuming and would need a large workforce to manually scan each and every plant, furthermore differentiating between different pathogens based simply on visual symptoms is extremely difficult. In addition, this method is not reliable or accurate when plants are asymptomatic and thus detection of the disease may be too late. Microscopic observation of the pathogens infecting a plant is one of the most straightforward traditional methods for detection and involved isolation of the disease-causing organism from the infected plant and then observing the infected parts under the microscope to identify the pathogen, however this approach of isolation of pathogens from infected plant and subsequent classification based on taxonomic criteria after microscopical observation has its drawbacks – it is time consuming, labour intensive and expensive. Biochemical assays or chemodiagnostic tests were also developed for detection of plant pathogens and involved use of various chemicals that reacted with the pathogens isolated from infected plant and based on this reaction would result in a unique and clearly discernible and visible result such as a change in colour or effervescence and this serves as the basis to distinguish between the pathogens infecting plants. These methods all lack the specificity and sensitivity needed for routine analysis of multiple samples. The biochemical tests are used for plant pathogen detection to detect fungi, bacteria, viruses, viroids and phytoplasmas. The biochemical tests used for detection differ depending on the pathogen and are briefly discussed here.

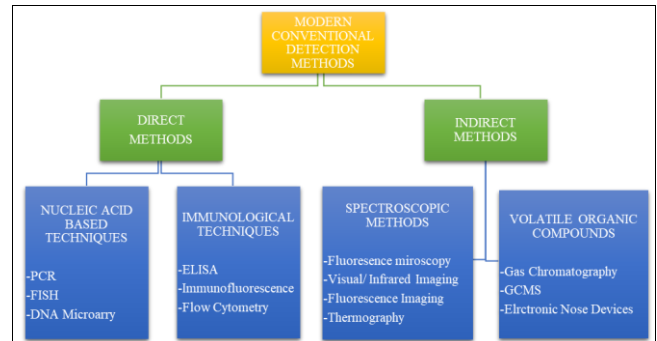
**Biochemical Tests to Detect Fungi:** Both visual scouting and microscopic observation are quite inaccurate in identification of fungal pathogens and so biochemical tests serve as an alternative. The fluorescein diacetate assay (FDA assay) in which fluorescein diacetate a cell permeable esterase substrate is used to assess enzymatic activity was reported to help detect presence of viable fungal spores in samples of plants <sup>[12]</sup>. Analysis of the glycoproteins in fungal cell wall was also shown to be successful in detection of fungal pathogens <sup>[13]</sup>.

**Biochemical Tests to Detect Bacteria:** There are several biochemical tests for bacterial pathogen detection these include tests for indole acetic acid production, nitrate reduction, lipolytic activity, hydrogen sulphide production and tyrosinase activity to name a few.

**Biochemical Tests to Detect Viruses, viroids and Phytoplasmas:** The tests to detect viruses, viroids and phytoplasmas are grouped according to the chemicals involved into the test. Accordingly, the tests used are protein tests, carbohydrate tests, polyphenol tests to name a few.

The present world situation with regards to agriculture makes it abundantly clear that rapid, cheaper methods for the detection, identification and characterization of plant pathogens is imperative to enable formulation and application of effective strategies to manage the plant diseases and prevent further spread of the disease. Thus, to fulfil this need several different methods have been

developed. These modern methods are classified as the direct methods where the pathogens are directly detected to identify disease/pathogen and the indirect methods which are non-invasive methods to identify and characterize pathogens through assessment of numerous parameters like changes in morphology or volatile organic compounds the infected plant releases.



**Fig 2:** Summary of the direct and indirect modern conventional methods for plant pathogen detection covered in this review

### Modern conventional detection methods

#### Direct Detection Methods

Two major properties of the pathogens have been taken advantage of to design the direct detection methods the first being the presence of specific epitopes on the surface of the pathogen and the second being the genetic contents of the pathogen. Figure two summarizes the various direct and indirect modern conventional methods of detection. The direct methods of detection are classified as

#### 1. Immunological Methods for Plant Disease Diagnosis

The immunological techniques for plant pathogen diagnosis involve the use of antibodies. These antibodies are usually generated by the immune systems of mammalian organisms to identify any invading pathogens. This concept formed the basis of development of immunological plant pathogen detection techniques which were initially developed for viral pathogen detection because there are several difficulties when it comes to culturing or cultivating viruses. Today thousands of bacteria, fungi and viruses can be detected using monoclonal and polyclonal antisera and the methods to produce these antibodies for plant pathogen diagnostics and their applications have been addressed in great detail over the years by a number of authors including Halk & De Boer 1985 <sup>[14]</sup>; Dewey et al., 1991<sup>[15]</sup>; Werres & Steffens 1994 <sup>[16]</sup>; Dewey & Thornton, 1995 <sup>[17]</sup>; Barker, 1996 <sup>[18]</sup>; Dewey et al., 1997 <sup>[19]</sup>; Torrance, 1998 <sup>[20]</sup>; Schots, 1995 <sup>[17]</sup>, Ward et al., 2004 <sup>[21]</sup>; and will not be discussed in detail due to availability of extensive and comprehensive existing literature. All the immunodiagnostic methods for plant pathogen detection are based on the ability of the generated antibody to bind to a specific antigen and then numerous assays or techniques are used to first detect and then quantify the antigen-antibody complexes formed. The assays used for the purpose of detection and quantification can range from simple visual detection of macroscopic or microscopic precipitates or involve the use of fluorescent antibodies. Some of the immunological methods that are commonly used which will be discussed in more detail are: -

##### ▪ Elisa

The enzyme-linked immunosorbent assay (ELISA), first developed in the 1970's is the most extensively used of all the immunodiagnostic methods and the reason for this is its

high throughput potential <sup>[22]</sup>. Antibodies raised against the pathogen of interest and coupled to an enzyme target the antigen produced by the pathogen of interest. The color shift caused when the conjugated enzyme attached to the antibody acts on a particular substrate is used to detect and quantify the antigen antibody complexes. The amount of antigen-antibody complex present is directly proportional to the intensity of the colour produced and can be measured using a microwell plate reader or an ELISA plate reader. The relatively low cost and easy visual detection which is possible due to colour change are the two major advantages of this technique of detection of plant pathogens.

Schaad et al.2002, stated that the sensitivity of ELISA varies depending on numerous factors including the organism producing the antigen, freshness of sample, titer etc. <sup>[23]</sup>. Using specific monoclonal antibodies can significantly reduce the limits of detection to around  $10^5$ – $10^6$  CFU/mL. However, the sensitivity of detection when it comes to bacteria is low and this is a major drawback as it means that ELISA is not a viable option for early detection of bacterial pathogens and can only serve to confirm diagnosis once the symptoms are already visible <sup>[24]</sup>.

#### ▪ Immunofluorescence (IF)

This is a fluorescence microscopy based immunodiagnostic method which involves preparation of very thin sections of plant tissue samples collected from infected plants and fixed on microscopic slides. The prepared tissue sections are stained using a fluorescently labelled antibody that binds to specific target molecules. Thus, enabling detection of a specific characteristic target antigen associated with plant disease while also simultaneously providing a view of the distribution of the antigen in the tissue sample. The ability to visualize the distribution of the antigen is one of the major advantages of immunofluorescence along with the low cost and high sensitivity. The potential of cross-binding or non-specific binding of antibodies to pathogenic bacteria is one of the technique's downsides or limits. Photobleaching is a major risk in case of immunofluorescence and can lead to false negative results. The risk posed by photobleaching can be reduced by using more fluorophores which are not as sensitive to photobleaching. Immunofluorescence was used for the detection of the gram-negative plant pathogenic bacteria *Ralstonia solanacearum* which infects a number of different plants causing bacterial wilt with a high level of sensitivity of around  $10^3$ – $10^4$  CFU/mL <sup>[25]</sup>.

#### ▪ Flow Cytometry

A laser impedance based optical method which allows for rapid acquisition of multi parametric data related to cells using electronic detection apparatus as cells flow through a liquid stream. It is primarily used for cell counting and sorting, in cell cycle studies, to differentiate between viable and non-viable cells, to estimate susceptibility of cells to antibiotics and other compounds to name a few applications. It has also been utilized in the detection of plant diseases; however, this use is reliant on the availability of fluorescently labeled probes such as antibodies specific to target molecules (antigens) generated by pathogens infecting plants. The major advantage of flow cytometry when it comes to plant disease detection is that it allows for the simultaneous measurement of multiple parameters such as cell viability, cell count stage of cell cycle, structural

integrity etc Another key benefit it has over other methods for detecting microbial infections is that it takes much less time to conduct the experiment, with accurate findings to the level of 104 CFU/mL being reported by Chitarra et al. in 2003 <sup>[26]</sup>. However, scientists face a major challenge when it comes to using flow cytometry in plant pathogen detection and this is with regards to standardization of the tests which is difficult due to the fact that many biological characteristics of microbial organisms particularly bacteria vary depending on the growing conditions. Another major concern is with regards to the cost of the technology and the various other requirements.

## 2. Nucleic Acid Based Methods for Plant Disease Diagnosis

The nucleic acid-based methods for pathogen detection are also referred to as the molecular techniques for plant pathogen detection. The major techniques listed under this category include DNA based techniques including FISH (Fluorescence In Situ Hybridization), DNA Microarray and PCR (Polymerase Chain Reaction) along with numerous variants of PCR including Nested PCR (nPCR), multiplex PCR (mPCR), real time PCR or quantitative PCR (qPCR), Cooperative PCR (Co-PCR) and RNA based techniques like Reverse Transcriptase PCR(RT-PCR).

#### ▪ PCR

The polymerase chain reaction, discovered by Kary Mullis was a technique used for amplification of DNA fragments using primers <sup>[27]</sup>. It relies upon the fidelity of DNA hybridization and replication. Initially the technique was employed for identification of bacterial and viral diseases using specific primers and was later also applied for the identification of plant pathogens. PCR has several advantages as a method for plant pathogen detection including the ability to detect a single target in complex mixtures, rapid and specific detection of multiple targets, and the ability to detect certain unculturable pathogens. It is also a well-established cost-effective technique. The accuracy and reliability of PCR in plant disease detection depends on the quality of the DNA or RNA isolated from samples and specificity of the primers used, concentration of the deoxynucleotide triphosphates, PCR buffer, activity of polymerase enzyme and presence of any inhibitors or contaminants in the reaction mixture for the assay. Usually the PCR reaction takes between two to three hours to be performed but PCR variants like Real Time PCR allow for much faster results with high specificity and sensitivity particularly for plant virus detection <sup>[28]</sup>. Nested PCR (nPCR) performed with two steps and involving internal primers also shares similar specificity and sensitivity for pathogen detection but the use of internal primers can result in interference during amplification by the primers <sup>[29]</sup>. Co-PCR or Co-operative PCR overcomes the drawbacks of nested PCR through a single reaction and has a high sensitivity and specificity for plant virus and bacteria detection <sup>[30]</sup>. Multiplex PCR which enables rapid simultaneous amplification of multiple RNA or DNA targets in a single reaction has also proved to be useful especially in cases when multiple pathogens infect a single plant <sup>[31]</sup>.

#### ▪ Fish

Fluorescence In Situ Hybridization (FISH) is a technique originally designed for use in cytogenetic studies <sup>[32]</sup>. It

involves the use of fluorescent probes that bind to specific sites on the chromosomes and enables detection of specific DNA segments or regions of chromosomes. However, it has also been used in plant pathogen detection in combination with fluorescence microscopy and DNA probes that hybridize to target genes of the pathogens in the sample being tested. One of the most common target genes was the ribosomal RNA (rRNA) gene which is specific to each pathogen. FISH has been successfully demonstrated to be able to detect bacterial, fungal and viral pathogens. One of the major advantages of FISH is the high sensitivity and specificity of the technique with the limit of detection lying in the range of  $10^3$  CFU/mL. One of the major limitations of this technique is false positives due to autofluorescent materials which lower the specificity [33]. The second limitation, which is the same as in the case of immunofluorescence is photobleaching which can result in false negatives.

#### ▪ DNA Microarray

DNA microarray is a powerful high throughput technology that involves using specially prepared chips consisting of arrays of immobilized oligonucleotide probes with sequences complementary to those of specific target sequences in the sample DNA fragment. This technology has also proved to be useful in plant pathogen detection and has successfully been used to detect and identify plant pathogens in potatoes, tomatoes, and apples [34-36].

#### Indirect Detection Methods

One of the major disadvantages of direct detection methods, particularly molecular methods such as PCR, is that they take time, require elaborate procedures, and have customized requirements such as sequence specific primers, which means that these techniques are better for confirming the presence of a specific pathogen but are impractical as a preliminary scanning technique to assess multiple samples. The indirect detection methods are non-invasive, non-destructive methods which attempt identify and characterize pathogens through assessment of numerous parameters and these have shown great potential to overcome this drawback.

### 1. Spectroscopic and Imaging Techniques for Disease Detection

Spectroscopic and imaging techniques including fluorescence imaging, hyperspectral imaging, infrared spectroscopy, fluorescence spectroscopy and visible/infrared spectroscopy have been tested as potential techniques for detection of plant diseases and have shown great promise for future incorporation into a portable device or biosensor for on-site detection non-invasive of both symptomatic and asymptomatic plant diseases and this could prove to be extremely useful for early detection and prevention of spread of infections consequently limiting the losses incurred. Some of the major spectroscopy and imaging techniques are discussed in the following paragraphs.

#### ▪ Fluorescence Spectroscopy

This is a spectroscopic method which deals with measurement of fluorescent light emitted by an object after excitation through incidence of light. Leaves produce two types of fluorescence firstly blue-green fluorescence (400–

600nm) and the second chlorophyll fluorescence (650–800nm). Fluorescence spectroscopy has been utilized to monitor both abiotic stresses like nutrient deficiencies and diseases in plants. Belasque et al. (2008) employed fluorescence spectroscopy to detect stress caused by citrus canker, a bacterial disease caused by *Xanthomonas axonopodis* [37].

#### ▪ Visible/Infrared Spectroscopy

Fluorescence microscopy, like visible and infrared spectroscopy, is a quick, non-destructive, and low-cost way to identify plant diseases. Under greenhouse circumstances, Spinelli et al. (2006) employed a near infrared approach to identify fire blight disease in asymptomatic pear plants [38]. Purcell et al. (2009) investigated the application of NIR spectroscopy for detection of Australian sugarcane disease and Fiji leaf gall [39].

#### ▪ Fluorescence Imaging

This approach is based on the idea that when a pathogen infects a plant, changes occur inside the plant's photosynthetic machinery, causing changes in the plant's fluorescence properties. This method detects pathogenic diseases in plants by measuring the fluorescence of chlorophyll in the leaves and using that measurement as a parameter. Bravo et al. (2004) used fluorescence imaging for detecting yellow rust in winter wheat while Chaerle et al. (2007) measured changes in the blue-green fluorescence of chlorophyll to assess the effectiveness of this technique in detecting the tobacco mosaic virus and its development in tobacco plants [40].

#### ▪ Hyperspectral Imaging

Hyperspectral Imaging is gaining increasing popularity as a tool in large scale agriculture for crop disease identification. It enables us to obtain information about changes in biophysical and biochemical properties of plants a disease may cause by measuring changes in reflectance between 350 nm and 2500 nm. The reflectance value reported in each spectral region in the range between 350 nm and 2500 nm gives us information regarding different properties of the plant. For instance, the reflectance at visible wavelength provides the information on the leaf pigmentations while, reflectance at infrared wavelength provides the physiological condition of the plant. *Venturia inaequalis* an ascomycetes fungus that causes apple scab infection of apple trees have been identified and reported using hyperspectral imaging techniques (Delalieux et al., 2007). Chen et al. (2008) investigated hyperspectral reflectance to identify Verticillium wilt in cotton plants [41, 42].

#### ▪ Thermography

This is non-invasive, non-destructive technique to monitor physiological status of plants and can also be used to detect diseases in plants.

Thermography involves generating an image of the differences in surface temperature of plant leaves and canopies by measuring thermal differences in the emitted infrared radiation using thermographic cameras. Lindenthal et al., 2005 showed that thermography made possible presymptomatic detection of cucumber downy mildew [43]. The practical application of thermography for disease monitoring is limited due to its high sensitivity to the change of environmental conditions during measurements. It

also cannot distinguish between diseases that produce similar thermographic patterns.

## 2. Volatile organic compound profiling for disease detection

Plants release a large number of volatile organic compounds (VOCs) into their immediate environment, which are low molecular weight biomolecules with a high vapor pressure and low boiling point that perform important roles in growth, communication, defense, and survival. Plant and tree-produced volatile organic compounds (VOC) account for almost two-thirds of all VOC emissions in the atmosphere [44]. Some commonly released VOCs by plants include terpenoids, volatile fatty acids, benzenoids, amino acid volatiles and phenylpropanoids. Physico-chemical elements such as humidity, temperature, and light, as well as biological factors such as the plant's growth and developmental stage, insects, and illnesses, all impact the metabolic profile of VOCs generated by a plant. Thus, abiotic and biotic stressors may cause a change in a plant's volatile organic compound profile, which has been used to diagnose plant illness. To identify any unknown compounds in the volatile sample and to detect the presence of the specific VOC that is indicative of a particular disease, the unique VOC profile for a plant could be analyzed using either gas chromatography (GC) alone or gas chromatography in combination with mass spectrometry (GC-MS). Another technique used for VOC profiling is using electronic nose devices with integrated gas sensors each of which is sensitive to a particular gaseous compound. In comparison to spectroscopic and imaging methods the major advantage of VOC profiling is the high specificity which means it provides more information about a plant disease and has higher accuracy. VOC profiling was used by Moalemiyan et al. (2006, 2007) to identify two fungal infections in mangoes: *Lasioidiplodia theobromae*, which causes stem end rot, and *Colletotrichum gloeosporioides*, which causes anthracnose [45]. *Phytophthora cactorum* is a kind of *Phytophthora*. p-ethylguaicol and p-ethylphenol are released from the diseased area of the strawberry plant/fruit by a fungus that causes crown rot illnesses in strawberries and modifies the VOC profile of the affected plant. One of the major drawbacks of many of the indirect detection methods is that while they can detect the presence of a disease even in asymptomatic state they often fail to identify the specific pathogen.

### Innovative new Methods of Detection

#### ▪ Lateral Microarrays

Lateral flow microarray (LFM) is a rapid hybridization-based technique for nucleic acid detection [46]. The technique depends on availability of well-established and reliable pathogen biomarkers discovered through transcriptomics studies which are used to prepare arrays which are miniaturized lateral flow chromatography chambers on nitrocellulose membranes. Dandekar et al., 2010 devised an integrated omics approach using lateral flow microarrays for detection and diagnosis of huanglongbing a highly destructive disease of citrus fruits caused by a motile bacteria *Candidatus liberibacter spp.* in the asymptomatic stage of the disease with high accuracy [47].

#### ▪ Sequencing and Plant Pathogen Detection

Sequencing refers to analysis of the entire genome of an organism to determine the order in which the nucleotides are

arranged. DNA sequencing has had massive beneficial implications in the biomedical and biological fields. Sequencing began with the dideoxy chain termination method or sanger sequencing which was automated and served as the basis of the revolutionary human genome project and several other projects that followed. In the recent years the advent of next generation sequencing (NGS) platforms has drastically dropped the time taken and the cost of sequencing and it now viewed as a viable potential technique for plant pathogen detection. The major advantage of using sequencing for plant pathogen detection lies in the fact that it has the potential to identify both the previously known pathogens and new unidentified pathogens and provides information about the genes responsible for the pathogenicity of the microbe through rapid sequencing of nucleic acid sequences isolated from samples collected from infected plants. (Egan, A. N et al., 2012,). Adams, I.P et al., 2009 developed a next generation sequencing based protocol for the identification of plant viruses and tested it for the identification of two viruses – first the *Pepino mosaic virus* which infects tomatoes and in the second case they took samples from an ornamental plant *Liatris spicata* with unidentified infection and detected a new cucumovirus which they named the 'Gayfeather mild mottle virus' [48, 49]. Although genome sequencing is a highly promising approach for plant pathogen detection data analysis as well as the fact that the method cannot be adopted for an on-site approach are two severe limitations [50].

#### ▪ Biosensors

Sensors have been developed and are in commercial use for a number of different purposes, including monitoring of environment and medical diagnostic purposes [51]. The conventional modern detection methods presently used have a number of shortcomings - they are time consuming, labour intensive and require complex instruments and as such are not really suitable for real time on-site detection and identification of pathogens infecting plants. This is the major reason there is great interest in developing biosensors as a means for on-site real time non-invasive detection and identification of plant pathogens with high sensitivity and specificity. Biosensors for plant pathogens use a variety of receptors, such as antibodies, DNA probes, bacteriophages, and nanomaterials, to improve the specificity and detection limit of biological identification [52]. Antibody-based biosensors are the most common of all the biosensors used for plant pathogen detection in plants, seeds and post-harvest crops and allow for rapid qualitative and quantitative analysis of pathogens with label free options also available [53]. However, a major drawback of the antibody-based biosensors is the risk of false positive results due to cross binding or non-specific binding. DNA based biosensors do not face this limitation and are based on the techniques for nucleic acid amplification which means they have greater sensitivity and could detect infections even when they are in the latent or asymptomatic phase. However, both Fang and Ramasamy, 2015; Hushiarian et al., 2015 report that these biosensors face limitations related to the synthesis and selection of specific DNA probes [51]. Bacteriophages are viruses infecting bacteria and then replicating within the bacteria before causing lyses of the bacteria to propagate. Phage therapy has been developed for treatment of human and plant diseases. Phage based

biosensors for detecting and subsequently targeting bacterial infections in plants are being developed <sup>[51]</sup>. These phage based biosensors have been used for controlling *Dickeya solani*, the bacteria infecting potatoes <sup>[54]</sup> and tomatoes <sup>[55]</sup> and also for the detection of *Pseudomonas cannabina pv alisalensis* which infects cruciferous vegetables <sup>[56]</sup>. The major advantage of these phage-based biosensors is the high selectivity, low cost and their ability to differentiate between living and dead bacterial cells thus there are no chances of false positive results. However, these biosensors can only be applied for detection of bacteria and not for fungi, virus or phytoplasma detection and this is a major limitation.

#### **The future of plant pathogen detection: On site detection**

Each of the methods for plant pathogen detection and identification discussed in this review has its own advantages and limitations be it the traditional methods, modern conventional methods or the new innovative methods being developed, or which are just beginning to be implemented. The need of the hour in the field of plant pathology is for reliable, accurate, cost effective and quick techniques which can provide rapid results detecting and identifying the disease and the pathogen which is the underlying cause on site in real time. Early detection and identification of pathogens particularly when in the latent or asymptomatic stage could be key to minimizing losses enabling the earlier implementation of disease management strategies that reduce the impact of the disease. On-site real time detection using portable detection tools which can be operated by the cultivators themselves is seen as a key step to achieve this vital goal as compared to the conventional process of collecting samples from infected plants and sending them to diagnostic laboratories. If we consider the methods currently in use for plant pathogen detection when it comes to the direct methods of detection none of them seem to be suited for on-site real time detection. Techniques like PCR and FISH while highly effective in disease detection and pathogen identification have a number of requirements including sophisticated machinery and specially trained personnel. The same holds true for techniques like DNA microarrays, ELISA, immunofluorescence and Flow Cytometry. An exception is seen in case of Real Time PCR with on-site detection using portable real time PCR devices having been reported <sup>[57]</sup>. In case of the indirect method hyperspectral imaging which can be carried out using cameras operated by unmanned vehicles could be used to scan through fields and detect diseases with high specificity and sensitivity. Techniques like fluorescence imaging and thermography lack specificity to distinguish between diseases and identify specific pathogens and are also influenced by fluctuations in various environmental factors. Volatile organic compound profiling requires the use of sophisticated instruments like GC-MS (gas chromatography- Mass Spectrometer) and is highly impractical for on-site detection due to the time-consuming data analysis necessary to detect diseases. Biosensors incorporating this indirect detection techniques could help overcome their shortcomings and in the long run provide highly robust and effective on-site detection tools. Specialized image processing algorithms have been developed recently and used with the help of smartphones as a potential portable detection device. These image processing algorithms in combination with statistical

analysis have been quite effective in detection of endemic diseases in European wheat strains. While there are many positive reports with regards to the development of portable devices for on-site rapid detection and identification of plant diseases and pathogens further research is necessary into an extremely critical issue with far reaching consequences particularly taking into account the global aim to ensure food security to all which is only possible by maximizing crop yield.

#### **Conclusion**

Fast, reliable, accurate techniques for plant pathogen detection and identification are the need of the hour and their importance is all the more critical looking at both the present and predicted world situation with respects to food security and the demands of a constantly rising population. In this article we reviewed the methods of plant detection beginning right from the traditional methods such as visual scouting, microscopic observation and biochemical tests to the conventional modern-day methods presently being widely used. These included the direct methods which are the immunodiagnostic methods - ELISA, flow cytometry and immunofluorescence and nucleic acid based methods – FISH (fluorescence in situ hybridization), DNA microarray, PCR (polymerase chain reaction) and the PCR variants including nested PCR, Real Time PCR, Co-operative PCR and Multiplex PCR and the indirect methods- Spectroscopic methods and volatile organic compound profiling. While the direct methods have high accuracy, reliability and sensitivity they all require high levels of expertise and are time consuming. Moreover, they are not suitable for rapid on-site diagnosis or for preliminary scanning and analysis of multiple samples. The issues faced by these direct methods are overcome by the non-invasive, non-destructive indirect methods but the major limitation that many of these methods face is that while they successfully detect the presence of the disease in the plant even in the asymptomatic stage they fail to identify the specific pathogen which is the root-cause of the disease because of a lack of specificity when it comes to the different pathogens. Thus, a combination of first using the indirect methods for preliminary scanning to detect diseases and subsequently using the direct methods to identify and confirm the specific disease-causing pathogen seems to be the best approach at present. Newer innovative methods – lateral flow microarrays, next generation sequencing and biosensors were also considered, and their potential, advantages and disadvantages were discussed. Lastly, we looked at the future possibilities in the field of plant pathogen detection and reviewed the potential use of on-site portable devices for disease detection and pathogen identification. The potential of the current conventional methods for on-site detection was also assessed and most of these techniques cannot provide real time on-site detection. A few such as fluorescence spectroscopy and thermography can be used for on-site real time detection, but numerous environmental factors and fluctuations in them have been found to influence results. While the biosensors have undoubted potential to fulfil the need of rapid, accurate real time diagnosis on site and thus could help minimize pathogen induced crop damage to a great extent further research into this area is necessary.

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The authors declare that they have no competing interests.

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Nil

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