Chapter 13
A Concise Compilation of the Diverse Detection Methods to Study Plant-Microbe Interfaces at the Cellular and Molecular Level: The Past, Present and Future

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Abstract Plant-microbe interaction can be classified under certain distinctive categories like synergistic, associative or pathogenic. The degree of friendly or hostile consortium depends on the kinds and species of microorganisms involved. These interactions are observed at various physiological planes of the host plant which in turn build the basis of molecular and genetic modifications. These changes then direct the path for biochemical reactions which occur between the plants and microbes. As a result of which, nutrient sequestration, mineral solubilization, nitrogen fixation, etc. are embarked upon by the plants, and in exchange the microbes get building blocks for energy conservation in their system. Due to this coevolutionary existence in the same niche, both have acquired mechanisms to defend each other's non-complementary company as well. Before the scientific advent of modern molecular instruments and technologies, traditional methods such as culturing on solid media, light and electron microscopic observations and biochemical tests provided initial insight into a broader realization of how these two beings communicate. As years passed, the dire need of new, effortless techniques with contemporary serological and molecular-based methodologies like isozyme assays, polymerase chain reaction (PCR), enzyme-linked assays (ELISA, RIA), microarrays (lab-on-chip), nucleic acid-based techniques (next-generation sequencing, whole genome sequencing, etc.) surfaced. Also, the invention of particular high-resolution microscopic techniques like video microscopy, confocal laser scanning microscopy (CLSM) and fluorescence microscopy brought a whole set of new information at cellular level. Apart from these, high spectral imaging also proved to be efficient enough to detect the disease symptoms at an early stage based on volatile organic compound profiling. A compilation is presented.

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13.1 Introduction

The term 'interaction' simply draws a picture in one's mind of active involvement of two entities under given circumstances. When we try to explore the humongous impact of the living micros on a crucial group of eukaryotic organisms, i.e. the plants, we must not avoid the most prodigious issue of all, the type of plant-microbe interactions taking place in nature every single microsecond. A precisely elucidated definition of plant-microbe interaction would not be able to accommodate the vastness of several kinds of relationships that a plant builds with the associated microscopic creatures. These organisms can communicate with a variety of plants in either of the following ways or in conjunction with phylloplane and phyllosphere (leaf surfaces and surrounding zone), rhizoplane (root surfaces), rhizosphere (an expansive zone around the roots in soil) and finally vascular system of plants.

In mutualism the interaction benefits both the host and the infecting microbe by allowance of increased nutrient availability, susceptibility against drought, heightened immunity against pathogens, usable forms of minerals from soil, production of plant hormones, nitrogen fixation, etc. In return, the exudates secreted by plants such as amino acids, sugars, vitamins and certain growth factors enhance the colonization of the microbes more efficiently. This can also be denoted as a synergistic relationship (Mitchell and Gu 2010; Madigan et al. 2012; Talaro and Chess 2012).

Commensalism is a type of symbiotic relationship where the *commensal* generally benefits, whereas the other partner is neither harmed nor benefitted by the association. Usually, a different form of the ingested nutrient by the plant is by-passed to the microorganism living in close contact with its host (Willey et al. 2009). Amensalism states the reciprocal action where the adverse effect of one organism's activity affects the other in a negative manner (Willey et al. 2009; Madigan et al. 2012). The sole purpose of these interactions, in most cases, is to confiscate nutrients which are otherwise difficult to utilize, for both the plants and the microbes. Also, it adds up some beneficial features to the plants which make them resistant to grazing, flood conditions so that they can hold on to the soil tightly (e.g. dense mycorrhizal network), infection by other group of pathogenic microorganisms (e.g. bacteria, fungi, nematode, algae, etc.) by increasing immunity, being devoured by herbivores, etc. Parasitism is a type of long-term, co-existing relationship that evolves over a period of time to favour the parasite to feed onto its host figuratively. This intimacy gives beneficial gains to the predator at the expense of the host member. Usually, if the host defence mechanism is too strong, then the interaction equilibrium will favour the host over the parasite, but in the opposite scenario, the host organism becomes ill and may eventually die depending on certain factors, like the infecting species. One remarkable aspect about parasitism is that the parasite is 'expectant' of the host to remain alive as long as possible to draw maximum nutrition to prolong the reproduction process as a mode of continuum for infecting new hosts (Willey et al. 2009).

13.2 Definition of Plant-Microbe Interaction

Acquisition of nutritional substances from environment is indulged by both parties involved in a synergistic plant-microbe relationship. These nutrients are essential for all metabolic activities that take place in vivo (Talaro and Chess 2012). Plant's exudative properties and rhizoplane-allied microbial communities depend on certain factors such as nutritional standing of the plant, species and, most importantly, the developmental stage of the plant. Root microbiome, in return, helps in carbon and nitrogen sequestration, solubilization of macro- and micronutrients and synthesis of growth factors and protective substances (Mitchell and Gu 2010; Talaro and Chess 2012). This co-operative behaviour is encouraging for both plants and the microbes. Although, there are a handful of examples present which negate this collaboration.

13.2.1 Differentiation Between Beneficial and Harmful Effects

Few of the outstanding examples of plant-microbe interactions in bacteria and fungi are symbiotic nitrogen-fixing bacteria and the mycorrhizal fungi, respectively (Bever et al. 2001; Buscot 2015). Examples of parasitic associations are numerous and have been elaborately outlined in various studies. Many leguminous plants and some others have acquired the propensity to fix atmospheric dinitrogen by forming associations with bacteria. This feature is widely distributed among prokaryotes, but is unknown and undefined in eukaryotes. The bacteria *Rhizobium* and *Bradyrhizobium* (rhizobia) and the actinomycetes *Frankia* form nodules on plant roots and are major contributors to symbiotic nitrogen fixation. During the course of evolution, the leguminous plants developed a couple of systems to attain mutual symbioses with rhizobia and mycorrhizae. The genetic requirements for rhizobial and mycorrhizal interactions in plants overlap in a common symbiosis pathway (CSP) leading to successful, mutually beneficial associations (Imaizumi-Anraku et al. 2005; Kouchi et al. 2010).

13.3 Plant-Bacteria Interactions

When a host plant is threatened by any microbe, due to the process of co-evolution, a cascade of defence strategies are turned on in both. *Phytoanticipins* and *phytoalexins*, two low molecular antibiotic compounds, are products of such defence mechanism. The former is usually present in plants inherently, but the latter is synthesized as soon as the microbes confront plants, as a mode of recognition (VanEtten et al. 1994). Studies suggest that the production of extracellular matrix (ECM) is a more precise and irreversible signal definitive of cellular specification

once the initial yet firm root surface adhesion takes place (Allard-Massicotte et al. 2016). This ECM principally consists of exo-polysaccharides, nucleic acids and diverse groups of proteins which are the basis of biofilm formation (Branda et al. 2005). With the help of microfluidics concomitant with laser confocal microscopy, the distribution of bacterial cells such as *Bacillus subtilis* (soil-dwelling microorganism) and *Escherichia coli* (common contaminant of manure fertilizer) in distinctive parts of the root has been well studied. It has been found out that root exudates play a major role as chemical attractants in the colonization of growing root tips by bacteria, which, after a certain point, is well distributed throughout the root surface as the cell density decreases and eventually forms a bacterial plug (Englert et al. 2010; Grossmann et al. 2011; Mendes et al. 2011; Parashar and Pandey 2011; Nezhad 2014; Jiang et al. 2014; Panke-Buisse et al. 2015; Shapiro et al. 2016).

Bacteria are ubiquitous. They are capable of occupying ecological niches as well as colonizing hotspots like plant rhizosphere and rhizoplane. By the process of rhizodeposition, plants release up to 40% of their photosynthetically fixed carbon through the roots into the vicinity (Barber and Martin 1976; Lynch and Whipps 1991; Marschner 1995; Hütsch et al. 2002). The colonization of the root itself (rhizoplane) and the surrounding soil zone (rhizosphere) directs towards a crucial link between the plant roots and soil zone (Lenc et al. 2011; Bulgarelli et al. 2013; Reinhold-Hurek et al. 2015). Bacteria utilize this constant flow of organic plant-based substrates and in return promote plant growth by mobilizing and providing inorganic nutrients and plant growth-promoting substances (Spaink et al. 1998; Brimecombe et al. 2007; Nannipieri et al. 2007; Compant et al. 2010). Investigation of such a continual stream of affairs in a complex and happening habitat is a major challenge.

Biochemical studies of tissue extracts are not very ideal to study plant-microbe interactions, because they are initiated at the level of the cell and need high-resolution studies of cellular responses. The potential to visualize or detect such interactions at a single-cell level becomes particularly important. More than about a decade and a half ago, imaging techniques have addressed the question to a certain extent. Video microscopy (Inoue and Spring 1997), confocal laser scanning microscopy (CLSM), laser trapping, image processing using a wide variety of commercially available software programs (Russ 1999) and fluorescence microscopy have been especially used and applied to a few plant-microbe systems (Heath 2000).

Some examples of bacterial phytopathogens that have been documented over the years include comprehension of the biology underlying disease initiation and progression in *Erwinia chrysanthemi* (Collmer and Keen 1986; Hugouvieux-Cotte-Pattat et al. 1996; Bouchart et al. 2007), *Pseudomonas syringae* (Loubens et al. 1993; Bohin and Lacroix 2006), *Xanthomonas campestris* (Minsavage et al. 2004) and *Xylella fastidiosa* known to cause citrus variegated chlorosis (Chang et al. 1993), Pierce's disease in grapevine (Davis et al. 1978) and leaf scorch in oleander, mulberry, coffee, almond and plum (Purcell and Hopkins 1996).

13.3.1 Phylloplane Interactions

Distribution of bacteria on all over the plant foliage is heterogeneous, and in turn their occurrence is affected by factors like orientation of the leaves within the herbage, climatic conditions, chemical composition of the cuticles and competition with other group of microbes, plant-eaters, etc. (Bodenhausen et al. 2013). Sometimes a co-existing foundation is observed between bacteria and fungi, where they form aggregates as they myriad spread over the leaves' surface at the depressions formed at the joints of epidermal cells and stomata, alongside the veins and at the base of trichomes (Remus-Emsermann et al. 2014). These structural features of the leaves also enhance the nutrient availability by facilitating percolation of photoassimilates onto the leaf surface (Leveau and Lindow 2001; Vorholt 2012).

13.4 Plant-Virus Interaction

Unlike many mutually beneficial acts of bacterial and fungal cells with plants, viruses always play a catastrophic role when they are in close contact with their hosts. This intimate involvement ultimately leads to infection, resulting in plant diseases by causing certain physiological changes. Technically, plants are not affected by viruses via the so-called receptor-binding mechanism due to the lack of it and rather are threatened when there is a mechanical damage by either vectors or environmental causes. The molecular basis of this interaction is dependent on the formation of multi-structural complexes between the host and virus proteins. Almost around 80% of the viruses (Mandahar 2006) have single-stranded RNA (mRNA) molecules (either singly or in multiple copies) as their genetic material (rice stripe virus, maize stripe virus, tomato spotted wilt virus, etc.), but few examples have also been documented on DNA (ssDNA and dsDNA) viruses (cauliflower mosaic virus, soybean crinkle leaf virus, para-retroviruses, single-stranded Gemini viruses).

Spread of infection depends on viral factors and host component interactions. This is specified by targeting viral RNA to plasmodesmata (PD) and increasing the PD pore size to allow the viral ribonucleoprotein (RNP) complex (i.e. viral genetic material/infectious viral RNA particle along with the movement proteins). For promoting the diffusion of these virus particles, the size exclusion limit of PD is exploited by the movement proteins (MP) which otherwise limits transport of macromolecules between cells. This natural phenomenon is also called 'PD gating' (Wolf et al. 1989).

It is to be noted that the whole process sometimes takes place by diffusion of the aforementioned substances through endoplasmic reticulum (ER)-Golgi complex secretory pathway or simply in association with the ER, independent of the above route. Varied set of reactions are generated in a cascade during plant-virus interactions at every stage of the infection favouring either the host or the infectious agent. Depending on the type of interaction mentioned earlier, viral replication and their

movement are halted as they are tightly coupled (Heinlein 2015). A more stable interaction is formed if the virus particles are not recognized by the host plant, but the obverse is true if the viral particles are detected. This follows an incompatible match for both and is unfavourable for the virus (Hammond-Kosack and Jones 2000). In order to counteract this ordeal, some major defence mechanisms have been imposed to plants by nature. One of these is RNA silencing, in which Dicer or an RNA-induced silencing complex (RISC) recognizes and degrades the virus genome (Soosaar et al. 2005; Dunoyer and Voinnet 2005; Li and Ding 2006; Valli et al. 2009; Shiekh 2014). But, these nonliving particles have learned to deal with that too by producing suppressor genes against the defence (Incarbone and Dunoyer 2013) in the host and accelerate replication by coinfecting viruses to possess multiple infections (Pruss et al. 1997; Fukuzawa et al. 2010; Syller 2012).

13.5 Plant-Fungal Interactions

Plant health can in part be attributed to their ability to team up with filamentous microorganisms. The primary plant part for nutrient and water uptake is the root system, which is inhabited and surrounded by a complex microbial community referred to as the root microbiome (Hacquard et al. 2015). It may be of special importance to mycologists and plant pathologists to comprehend the underlying mechanisms. Microbiologists have been drawn to this field of research mainly because of the need for identification of microbial agents responsible for causing infectious diseases in economically important crop plants (Montesines 2000).

Plant pathogenic fungi fall into one of the three categories on the basis of their growth within the tissues of their hosts, namely, necrotrophs (perthotrophs), biotrophs and hemibiotrophs. The enzymes and toxins are used by necrotrophs, which kill the host cells, in advance of their hyphal proliferation. Eventually they grow between and into dead and dying cells. On the other hand, biotrophs obtain nutrients from living host cells and serve as typical examples of fungi exhibiting a parasitic mode of nutrition. Hemibiotrophs like the anthracnose fungus *Colletotrichum lindemuthianum* initially require living host cells but soon cause their destruction like necrotrophs (Alexopoulos et al. 2010). The culprit which caused the Irish famine, namely, *Phytophthora infestans*, continues to cause dramatic yield losses in crops such as potato and tomato (Fry 2008). There is an increasing impact of plant diseases in crop plants; plant-pathogen research has resulted in extensive documentation on plant defence mechanisms.

There is considerable evidence of fossilized fungal structures inside plant cells (Remy et al. 1994), and further nearly about 80% of all existing higher plants are colonized by arbuscular mycorrhizal (AM) fungi (Wang and Qiu 2006; Prasad et al. 2017). Plants also engage in beneficial root associations, namely, endophytic mycorrhizal interactions (Parniske 2008). The fungal partner is known to provide mineral nutrients such as phosphorus, etc. Conversely, plants supply carbohydrates generated via photosynthesis. It is also to be understood that plant carbohydrates may also

serve as attractant molecules to root-infecting filamentous pathogens, namely, fungi and oomycetes. Of particular importance are the arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi because they actively participate in plant development (Smith and Read 2008).

13.6 Methods Used to Detect Plant-Microbe Interactions: Conventional and Molecular Methods

Conventional techniques in the detection of phytopathogenic microorganisms involve the use of culturing methodologies on specific media with subsequent morphological and biochemical characterization (Lopez et al. 2003). These traditional methods are extremely time-consuming and laborious and require expert and skilled personnel for the purpose of identification. In the 1970s, phytopathogenic viruses have been detected based on isolation, electron microscopy, electrophoresis, biological indexing and serological techniques. Meanwhile the advent of ELISA and PCR have revolutionized phytopathogen detection, especially in the last quarter of the twentieth century, and have become commonplace, being routinely used by plant pathologists (Lopez et al. 2003).

Erwinia chrysanthemi is a Gram-negative enterobacterium that is a causative agent of soft rot diseases in ornamentals and vegetables. Plant hosts become vulnerable to this bacterium because of its ability to secrete a number of enzymes that are responsible for degrading plant cell wall components. Techniques such as 2-DE and MALDI/TOF MS have been carried out to characterize the secretome and compared with mutants of *Erwinia chrysanthemi*. The secretome of *Xanthomonas* pv. *campestris* was analysed by another research group elsewhere (Watt et al. 2005) using the aforementioned techniques.

In fact, the first phytopathogenic organism to be sequenced completely was *Xylella fastidiosa* following which the annotation of its 2849 genes was found in the chromosome and two extrachromosomal plasmids (Simpson et al. 2000). Consequent to the determination of the complete genome sequence, efforts were made to analyse the whole bacterial cell proteome as well as the secreted protein profile (*secretome*), which led to the identification of 142 different proteins including some that were homologous to proteins involved in different cellular adhesion systems (Smolka et al. 2003).

The genetic basis of this robust specificity of plant-bacteria interaction can be described by gene-for-gene elicitor-receptor model (Flor 1955; Baker et al. 1997). It goes in a way where the avirulence (*avr*) gene acts like a resistance (R) gene counterpart in hosts. A complementary amalgamation of the *avr* and R genes ends up in unsuitable plant-pathogen interaction, and plant defences are triggered, but in a compatible reaction, infection takes place (Bent 1996; Ellis et al. 2000; Hammond-Kosack and Jones 2000). A hypersensitivity response (HR) is provoked in host plants due to the presence of these HR genes and pathogenicity genes (a set of genes

consisting of these two). With the help of these genes, viruses can also elicit HR in non-host plants (Lindgren 1997; Nakahara and Masuta 2014; Rodriguez et al. 2015) (same is applicable for viral infection as well). In contrast to the positive side of the relationship, a set of genes are also involved in pathogen-host relation.

While studying the principles of colonization, it is disadvantageous to separate plant pathology and symbiosis systems on different plant species. *Arabidopsis thaliana*, the choicest plant for numerous plant-pathogen interactions, has not been found to be ideal to study the feeding structure formation by endomycorrhizal fungi and has thus found limited use. On the other hand, *Phytophthora* and beneficial AM fungi in legumes follow analogous steps to establish an interaction.

Gehrig et al. (1996) are of the opinion that the establishment of early land plants was facilitated by the interaction with symbiotic fungal association, ever since the evolution of land plants more than 700 million years ago as suggested by molecular clock estimates (Heckman et al. 2001). The exposition of the plants to microbes is a continual and ongoing process. To exhibit pathogenesis, most microbes must gain entry and access to the plant interior. The entry process may be direct by the formation of specialized structures (e.g. in bacteria, haustoria and appressoria in fungi) or indirectly through wounds or natural openings such as stomata, thus overcoming the first line of host defence. Digit-like haustoria formed by *P. palmivora* in *Nicotiana benthamiana* roots have been documented using green fluorescent protein (GFP) (Rey and Schornack 2013). Subsequently, the microbes are required to conquer the cellulose-based support, namely, the rigid cell wall and the host plasma membrane, where they encounter extracellular surface receptors that recognize pathogen-associated molecular patterns (PAMPs). During the course of evolution, microbes have found ways and means to suppress the PAMP-triggered immunity (PTI) that supposedly alter resistance signalling and responses in plants (Chisholm et al. 2006).

13.6.1 Conventional Methods

Traditional methods involve the isolation of the fungal pathogens onto suitable standard mycological agar media (general purpose, routine, semisynthetic, synthetic, semi-selective, selective, specialized media), studying the cultural characteristics such as colony colour, obverse and reverse colony morphology and micromorphological characteristics like sporangiospores, conidiophores, conidiospores, chlamydospores, etc., identifying sexual structures such as ascospores, asci, cleistothecia, perithecia and miscellaneous structures like Hulle cells, etc. However, culture-dependent techniques allow the phenotypic analyses of only culturable strains and limit its use in the case of fungi that cannot be isolated on artificial media. Temporary mounts using cellophane and agar plug techniques have also been successfully used to study fungal propagules. Study of *Fusarium* species has been extensively elaborated by many researchers using standard protocols (Booth 1971; Nelson et al. 1983; Leslie and Summerell 2006). Other examples of fungi include

Phytophthora bischeri species (Erwin and Ribeiro 1996; Abad et al. 2008), *Botrytis* species (Mirzaei et al. 2008) being some of them.

13.6.2 Biochemical Methods

13.6.2.1 Detection of GUS Activity

Gene reporters enable valuable insight into gene expression. The GUS gene reporter system is one of the popular and common plant reporter systems to establish the cause of certain diseases. Fungal transformant strains of *Cladosporium fulvum* infecting tomatoes and *Leptosphaeria maculans* infecting brassica crops expressing β-glucuronidase activity have been produced and used to histochemically detect the presence of the hyphae in infected host plant tissues. Oliver et al. (1993) also reported that this activity could be used as a measure of fungal biomass in the cotyledons of infected tomato seedlings.

13.6.2.2 Isozyme Analysis

Isozyme analysis is a powerful biochemical analysis tool whose usefulness is obvious for the detection, differentiation and identification of morphologically similar or closely related species, varieties and *formae speciales*. This in turn helps in the 'fingerprinting' of strains by protein profiling. This technique which was used more than three decades ago is still relevant and can be applied to study fungal interactions with plants. Isozyme analysis has been performed with *Peronosclerospora* spp. from maize for distinguishing *P. sorghi*, *P. sacchari* and *P. sacchari-phillipinensis* complex (Bonde et al. 1984) and in other studies (Bonde et al. 1985, 1989, 1993; Pan et al. 1991; Kaufmann and Weidemann 1996). In a particular study, uniform isozyme patterns were noted from different *Fusarium* species, independent of the geographical origin and hosts from which they were isolated. The different strains studied include *F. cerealis*, *F. culmorum*, *F. graminearum* and *F. pseudograminearum* (Láday and Szécsi 2001, 2002).

13.6.3 Immunoassays and Nucleic Acid-Based Assays

Immunodiagnostic methods fall into two broad groups of (a) direct and (b) labelled methods. While immunoprecipitation, immunoagglutination and immunodiffusion are examples of direct methods, enzyme immunoassay (EIA), radioimmunoassay (RIA) and immunofluorescence are categorized as labelled methods. The legacy dates back when Tempel (1959) developed a gel diffusion test for the detection and differentiation of *formae speciales* of *Fusarium oxysporum*. Later on, there have

been arrays of studies which different research groups have undertaken in different parts of the world. Some of the assays include detection of *Botrytis cinerea* using RIA (Savage and Sall 1981), *Phytophthora cinnamomi* by ELISA (Hardham et al. 1986) and turf disease causers, namely, *Pythium* species, *Rhizoctonia solani* and *Sclerotina homeocarpa* (Rittenburg et al. 1988), using commercial kits of ELISA and its variants. Similar studies based on antigen-antibody-based detection has been carried out (Kitagawa et al. 1989; Sundaram et al. 1991; Plantiño-Álvarez et al. 1999) in the detection of *F. oxysporum* f. sp. *cucumerinum*, *Verticillium dahliae* and *F. oxysporum* f. sp. *radicis-lycopersici*, respectively. ELISA tests have been used for the detection of *Phytophthora* at the generic level and *P. ramorum* at the species level using the diagnostic method in combination with TaqMan PCR (Kox et al. 2007). Karthikeyan et al. (2006) have used ELISA and PCR for the detection of *Ganoderma lucidum* known to cause *Ganoderma* disease in coconuts.

13.7 Assays for Virus Detection

Identification of viral phytopathogens by traditional methods like culturing is not easily achievable. For that reason, serological methods and advanced molecular methods have been employed to guide their detection regime methodically. ELISA, being a highly sensitive virus detection tool, uses targeted epitopes which bind specifically with a desired antibody conjugated to an enzyme. Upon irreversible binding, a colour reaction (Clark and Adams 1977) is generated as a suitable substrate is added to the reaction mixture. This binding specificity can in turn be enhanced by using monoclonal (using hybridoma technology against single epitope on a particular cell line) (Holzloehner et al. 2013) or recombinant antibodies (Gorris et al. 1994; López et al. 2001). Initially, PCR was invented for the robust detection of bacterial and viral pathogens (Cai et al. 2014).

Polymerase chain reaction and its modifications have successfully been used to enormously detect plant pathogens over the years. RT-PCR (mostly used for its high sensitivity to detect RNA viruses) (Lopez et al. 2003), multiplex PCR (simultaneous detection of different DNA and RNA in a single reaction) (Osiowy 1998; Pallisgaard et al. 1998; James 1999; Williams et al. 1999; Nassuth et al. 2000), real-time PCR (for viral DNA identification in real time for improved diagnosis of diseases and on-site as well) (Schaad and Frederick 2002; Lievens et al. 2006). Nucleic acid sequence-based amplification (NASBA) is another example of excellent technique for amplification and identification of RNA sequence containing viruses (Klerks et al. 2001; Olmos et al. 2005), the predominant category responsible for viral plant diseases. Loop-mediated isothermal amplification has been extensively in use to detect plant virus such as plum pox virus (PPV) (Varga and James 2006). This method is very simple to use without incorporating the need of an expensive thermocycler, where the amplicons are detected by observing solution turbidity through photometric analysis (Mori et al. 2001). SYBR green is usually used as a colour detector. Microarray is another molecular method where the RNA of interest

(mRNA) is isolated from cells and is reverse transcribed to its complementary DNA. After labelling, they are hybridized with prefixed probes on the chip and detected for light signals.

13.8 Challenges and Lacunae in the Detection of Microbes

The advancement in the field of plant pathology over years has made it easier for the scientists to detect and identify several different kinds of diseases and the etiological agents related to them. Also, this progress has led farmers and growers achieve a contamination-free cultivation with minimum economic loss. Apart from being extremely advantageous, these unique detection methods also have certain downsides. Among the direct methods for detection, PCR (normal PCR, QPCR, qRT-PCR, etc.) is one such molecular method with higher ability, specificity and sensitivity for a particular pathogen. The technique is however challenged at the point, where it demands species-specific probe design to amplify target DNA which is cost-prohibitive and time-consuming and can be applied for high target value analytes. Alongside this, parameters like buffer concentration of nucleotide solution, polymerase activity, etc. may compromise with the quality of result expected.

Another issue to be addressed is that the infection can be detected only on the onset of disease, i.e. when the host starts expressing disease symptoms. As soon as the infective viral particle (RNA/DNA) is found, it undergoes a tiresome sample processing for detection, which includes isolation and purification of the genetic material, complementary DNA synthesis and amplification. The whole method makes it less of a choice for large-scale preventive measure. ELISA is another serological technique which is highly sensitive for detecting viral plant diseases but fails to do so in case of bacterial infections as it is poorly sensitive. Next-generation sequencing of the disease-causing pathogens is also a robust method, but the high analysis cost per sample, duration of sample processing, complexity of the data analysis and compromisation for low-titre virus samples make it unfit economically. Certain protocols are available as commercial kits (fluorescent in situ hybridization, immunofluorescence) which require skilled personnel to operate and prepare samples and to decode the data.

Volatile organic compounds (VOCs) are released by plants as a mode of metabolic activities which have a distinct pattern and vary from each species of plant to the other and also under stressful conditions. This change can be measured with using gas chromatography (GC), but the limitations to this method are as follows:

1. Time taken to process the diseased plant parts which release the VOCs.
2. Differentiate between the variation in VOCs released naturally which might cover up the ones secreted as an outcome of environmental stress or diseased condition. This requires the usage of characteristic volatile markers specific for a plant and a diseased condition that will be different from when its produced under environmental or nutritional stress.

3. Performing instrumentation and analysis of the complicated data which demand skill and expertise.

Another crucial point to be mentioned in this context is that all these direct measures of detection restrict their application in cultivation field which is the biggest drawback. This problem could partly be solved with the help of thermography or high spectral imaging, but again their object instability to environmental changes and sensitivity towards climate change is extreme which makes them unsuitable in field detection. A plausible way to prevail over this situation would be to gather more knowledge about a particular wavelength range which can be sensitized for a particular plant disease and at the same time would also be least affected by the changes in surrounding.

The void that has been left unfulfilled by all these preventive measures could now be resolved by the application of nanotechnology as is evident in a number of research studies. Nanoparticles like nanorods, nanocrystals, nanotubes and microcapsules can effectively carry chemical and biological pesticides, host immune factors, defensive molecules against pathogens, etc. and can release them as per host's requirement and will contain the infection. The other potential next-generation large-scale field detection method would be biotrophic sensors and VOC sensors which are yet to be brought into limelight. These overwhelming technologies could detect plant pathogens at the earliest stages of infection with robustness before symptoms appear. Interestingly it could serve as an edge over other molecular or serological methods, way before by detecting early induced volatiles when pathogens make their way through the host system.

13.9 Future Prospects and Conclusions

The need to detect, identify and eliminate all primary sources of inocula in disease production in plants through microbial interactions is of great consequence. This is of vital importance to prevent infection and spread of microbial plant pathogens which can cause major economic losses in crop plants. While there are beneficial aspects also ruling these interactions, the need of the hour is the use of methods to distinguish pathogens and closely related species and strains. Conventional and rapid assays have always proved handy for this purpose. However, the former techniques are quite laborious, time-consuming and incommodious. Rapid assays such as immunoassays and nucleic acid-based methods are not only fast but also specific and reliable. Further, these methods are distinctly advantageous over immunoassays because microbial antigens or propagules are complex in nature, especially varying depending on the stages in their life cycle. On the contrary, the nature of the genomic elements remains constant thus enhancing specificity and sensitivity with PCR-based detection being one of the answers to problems faced while studying such interactions. In nature, diverse microorganisms reside in, on and around plants as endophytes and epiphytes (Hallmann et al. 1997; Mano et al. 2007; Whipps et al.

2008). Many questions underlying these plant-microbe relationships remain unanswered (Saito et al. 2007; Hardoim et al. 2008). Both beneficial and harmful interactions exist, which are a resultant of relationships between plants and bacteria, fungi, viruses, viroids, etc. Symbiosis is the living together of two or more organisms involved in this association (Ogle and Brown 1997). All the organisms involved in this association derive benefits. Parasitic interactions on the other hand lead to derivation of benefit from one of the partners, while the other associate could face highly detrimental effects, and the association ceases to exist with the passage of time. The challenges for the present and the next decades include understanding the complex behaviour of microbes in their natural habitats. Plant-microbe interactions have been extensively studied and researched upon in habitats such as the rhizosphere and phyllosphere. The underlying principles are microbes, which like human beings, want to survive, even if conditions are inhospitable or hostile, want to feed on nutrients, grow, multiply and proliferate on the onset of favourable environments.

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