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Review

Advancements in the diagnosis of bacterial plant pathogens: An overview

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The timely detection and appropriate identification of causal agents associated with disease of crop plants or seeds are considered to be the most important issue in formulating the management strategies for plant diseases. This is particularly important for plant diseases of a bacterial nature, where disease-free planting materials is the only effective way to restrict the disease. The detection of bacterial pathogens still largely depends on cultural, morphological and biochemical properties. The protocol requires skilled taxonomical expertise and is also time and labor intensive. Moreover, it cannot discriminate between closely related strains of same bacterial pathogens. With the advent of polymerase chain reaction (PCR), nucleic acid based techniques have made the diagnostic procedures for plant pathogens, including bacteria, easier than the conventional approaches. The wide acceptability of nucleic acid based techniques is due to them being more sensitive, more accurate, more specific, and much faster than conventional techniques. The serology-based diagnoses are very often preferred over nucleo-based techniques as they are more user-friendly and less cumbersome, besides being sensitive, accurate, specific, and much faster than conventional techniques. This review critically analyzes the recent developments and scope of various nucleic acid- and serology-based techniques for the diagnosis of bacterial plant pathogens.

Key words: Bacterial pathogens, diagnosis, detection.

INTRODUCTION

Bacterial pathogens cause substantial loss to the productivity of major crop plants. Unlike fungal pathogens, bacterial pathogens cannot be contained effectively through chemical methods. Early detection in seeds, planting materials, or ensuring disease-free planting materials through rapid diagnostics are likely the effective means of reducing bacterial disease incidence. The traditional detection protocol, based on cultural, morphological and biochemical properties, requires skilled taxonomical expertise to confirm the identity of the causal bacterium. Though recent automation in the field of conventional biochemical approaches like Biolog-phenotyping, fatty acid methyl esterase (FAME) analyser etc. have reduced data analysis and interpretation part to a great extent for diagnostician but these approaches are still time and labor intensive requiring incubation and processing time of 2-7 days before being subjected for automation. Also, they cannot discriminate closely related races within pathovar-populations of affecting bacterial pathogens. This review focuses on scope and status of different molecular diagnostics including nucleic acid and serology-based techniques for bacterial plant pathogens.

NUCLEIC ACID-BASED TECHNIQUES

Nucleic acid (NA) based techniques are widely recognized and powerful plant pathogen detection techniques. The target region mostly exploited for bacterial diagnostic is ribosomal DNA, which are present

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Table 1. Important housekeeping marker genes for the detection of bacterial pathogens.

Gene	Encoded proteins	References
16S rRNA	16S Ribosomal protein subunit	(Lee et al., 1997a; Alvarez, 2004)
23S rRNA	23S Ribosomal protein subunit	(Maes et al., 1996a)
16S-23S rRNA	Internal transcribed spacer region between 16S and 23S ribosomal subunit	(Maes et al.1996b; Song et al., 2004)
rpoB	β subunit of RNA polymerase	(Hocquellet et al., 1999)
groEL	Heat-shock protein	(Yushan et al., 2010)
gyrB	β subunit of DNA gyrase	(Mondal et al., 2012)
recA	Recombinase A protein	(Eisen, 1995; Waleron et al., 2002; Young and Park, 2007)
atpD	ATP synthesis β chain	(Young and Park, 2007)
dnaK	Heat shock protein 70, molecular chaperone DnaK	(Young et al., 2008)
rpoD	Sigma -70 factor of RNA polymerase	(Young et al., 2008)
fyuA	transmembrane protein, TonB-dependent receptor	(Young et al., 2008)
efP	Eleongation factor P protein	(Bui et al., 2010)
glnA	Glutamine synthetase I	(Takle et al., 2007)

in all bacteria at high copy number per genome with highly conserved regions, allowing for very sensitive detection (Mondal et al., 2004). Since the specificity of DNA-based techniques only relies on primer and probe sequences, such assays are also easy to develop and can be transposed into virtually every pathosystem. Molecular markers like restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), as well as *hrp* genes, *pth* gene based markers have been extensively employed to detect and identify pathogenic bacteria affecting different crop plants (Williams et al., 1990; Leite et al., 1994; Opio et al., 1996; Kerkoud et al., 2002, Berg et al., 2005). Besides the aforementioned biomarkers, the important housekeeping marker genes used for multi locus sequence typing (MLST) of bacterial pathogens are listed in Table 1.

Restriction fragment length polymorphism (RFLP)

RFLP analysis has been extensively used in detection and identification of plant pathogens (Mondal et al., 2004). A small DNA segment from a known bacterium, pathogenic to the host plant in question, is used as a probe. The DNA from both the known (as positive control) and suspected bacterial pathogen (isolated from infected plant samples) are digested with the same restriction enzyme(s). If the bacteria in question contain DNA with slightly different base sequences, some of the assayed restriction sites will be missing or in different locations. Therefore, the restriction enzyme-digested DNA will produce unique fragment numbers and sizes. The samples of both control as well as unknown digested bacterial DNA are placed side by side in an agarose gel, and are then separated by size using electrophoresis. The double-stranded DNA fragments are then chemically denatured into single-strands. Then the DNA fragments in gel are transferred to a nylon membrane which fixes their positions and maintains them as single-stranded DNA. The nylon membrane is washed with a solution containing many copies of a radioactive DNA probe. The probe is a very short, single-stranded DNA that will hybridize with its complementary sequence wherever the sequence is found among the DNA fragments on the nylon membrane. This further increases the specificity of this technique. Finally, an x-ray film is exposed to the nylon membrane. When this film is developed, a dark band will appear at each location where the probe hybridized to

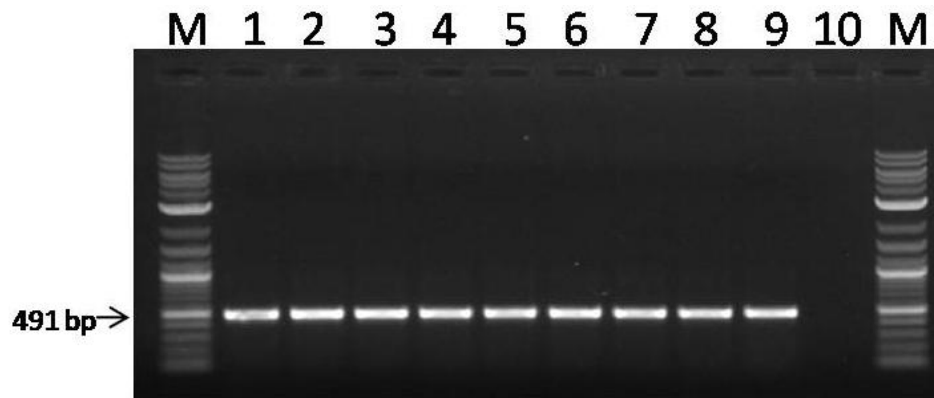

Figure 1. PCR detection of *Xanthomonas axonopodis* pv. *punicae* in pomegranate plant. M 100 kb ladder, Lanes 1 to 8 indicate pomegranate leaf samples, 9 = positive control (purified bacterial DNA) and 10= negative control (*Pantoea agglomerans,* out group bacteria).

complementary DNA. These banding patterns will determine whether the unknown specimen is same to the bacterium that was used as probe.

PCR-aided nucleic acid based diagnosis

The polymerase chain reaction (PCR) is one of the greatest inventions in science by Kary Mullis in 1984 (Mullis, 1987). With the advent of PCR, DNA based techniques have rapidly become the gold standard for detection, and identification of plant pathogens, including bacteria (Jensen et al., 1993; Bereswill et al., 1994; Alexander et al., 2004). This is due to the fact that these techniques overcome many of the shortcomings due to their sensitivity, greater accuracy, specificity, and more rapid results than conventional techniques (Schaad *et al., 2001*). The PCR technique primarily involves three steps in sequential events, namely denaturation, primer annealing and DNA synthesis or chain extension with the help of a thermal cycler. The specificity of the technique depends upon designing of primers that are unique to target pathogens. With the introduction of PCR, the nucleic acid mediated detection of plant pathogens has become easier and more sensitive (Janse, 1988; Henson and French, 1993; Cubero and Graham, 2002). Recently, Mondal et al. (2012) demonstrated a rapid and reliable PCR based detection of *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight of pomegranate. The primer set (KKM5 and KKM6) was synthesized based on sequence alignment of 530 nucleotides of *C*-terminal region in the *gyrB* genes from 15 different bacterial strains. The primer set was validated for amplification of 491 bp of *gyrB* gene. The developed technique could detect the pathogen from infected pomegranate plant samples including leaf (Figure 1), fruit and stem within 3 h, at a detection limit of 0.1 ng of template DNA μl^{-1}.

Random amplified polymorphic DNA (RAPD)

The RAPD markers are demonstrated to be useful for determining polymorphisms among phytopathogenic bacteria (Rafalski et al., 1994; Chen et al., 2003; Grover et al., 2006; Mondal et al., 2008). The utility of random primers for differentiating bacterial blight pathogens in beans, *Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var fuscans has been well documented (Birch et al., 1997). RAPD based polymorphism comprising Indian isolates of three bacterial pathogens causing common blight, fuscous blight and halo blight in grain legumes was studied (Mondal, 2009). Among the random primers used, SBSB02 yielded polymorphic amplicons (the sequence of the primer SBSB02 is 5'-TGATCCCTGG-3'). The primer specifically amplified two polymorphic fragments of ~300 bp and ~1600 bp in fuscous blight bacterium, but not in common and halo blight bacterium, suggesting that the amplified regions are conserved within the fuscous blight isolates. Thus one of these amplicons could be exploited as sequence characterized amplified regions (SCAR) marker after sequencing and designing primer specific to the region.

Amplified fragment length polymorphism (AFLP)

AFLP involves amplification of specific region of genomic DNA through PCR (using a single primer) followed by the cleaving of the amplified fragments using restriction endonuclease (Figure 2). The technique is thus a combination of RFLP and PCR techniques and is extremely useful in detection of polymorphism between closely related bacterial pathogens. The genomic fingerprints are produced without any prior knowledge of

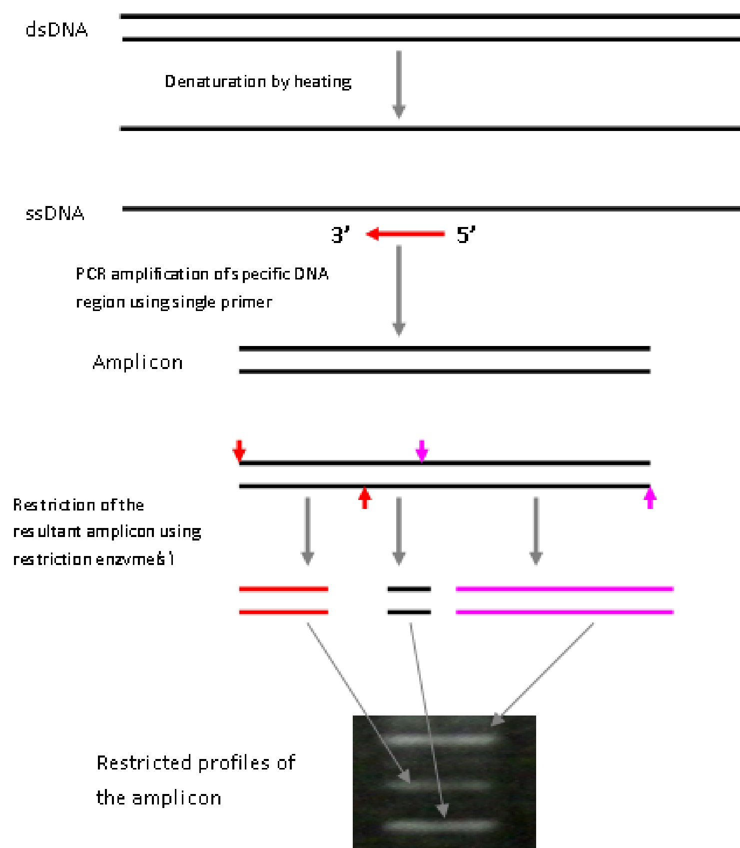

Figure 2. Schematic steps involved in AFLP.

sequences using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer set. The AFLP technique is reliable, since stringent reaction conditions are used for primer annealing. AFLP is now preferred over RFLP as it is PCR linked and is very often employed in the detection and differentiation of several bacterial plant pathogens including *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans, Pseudomonas syringae* pv. tomato, using primers specific to ribosomal genes or rRNA operons (Manceau and Horvais,1997; Mahuku et al., 2006). A comparative analysis of RAPD and AFLP techniques was undertaken to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae* (Clerc et al., 1998). Both techniques showed high discriminating power because strains of *P. syringae* pv. tomato and *P. syringae* pv. Maculicola, which were indistinguishable by other techniques including pathogenicity tests on tomato, were separated into two groups by both RAPD and AFLP analyses. However, the AFLP method was more efficient for assessing intrapathovar diversity than RAPD analysis and allowed clear delineation between intraspecific and interspecific genetic distances, suggesting that it could be an alternative to DNA pairing studies (Clerc et al., 1998).

Real time PCR

Real time PCR is the most advanced version of PCR wherein the amplification of target sequence can be quantified after each PCR cycle and amplification is pictographically displayed through an attached monitor (Espy et al., 2006). Real time PCR has gained popularity as it not only eliminates the post-PCR processing steps including gel electrophoresis, but also eliminates the exposure to carcinogenic chemicals, ethidium bromide, radioactive isotopes, UV-radiation. It also poses a reduced risk of sample contamination with amplicon and a greater amenity to multiplexing, simultaneous testing for multiple pathogens or samples. The technique can also be implemented in the field by using portable real time PCR machines. There are currently four different fluorescence-detection techniques that are used to detect amplicons, include SYBR green dye based detection, TaqMan probes, fluorescent resonance energy transfer (FRET) probes, and molecular beacons (Schaad and

Frederick, 2002; Mackey et al., 2002).

SYBR green dye based detection

The use of fluorescent dyes such as SYBR Green I (SG) in real time PCR have become increasingly important for diagnostic applications of plant pathogens (Kiltie and Ryan, 1997; Vitzthum and Bernhagen, 2002). The SG binds to the minor groove of the DNA double helix. In solution, the unbound dye exhibits very little fluorescence, however, fluorescence is greatly enhanced upon DNA-binding. At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted out during computer analysis. During primer annealing steps, a small amount of dye molecules bind to the double strand. The dye binding results in a dramatic increase of the SG molecules to emit light upon excitation. Subsequently, during elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls. Fluorescence measured at the end of the elongation step of every PCR cycle indicates the increasing amount of amplified DNA. A real-time SYBR Green I assay was developed for the detection of *X. arboricola* pv. *pruni*, the causal agent of bacterial spot disease of stone fruit (Palacio-Bielsa et al., 2011).

TaqMan probes

The TaqMan assay is based on the 5′-3′ exonuclease activity of *Taq* polymerase. In this assay, an oligonucleotide probe is labeled with two dyes; a reporter fluorescent dye attached to its 5′ end and a quencher dye attached to its 3′ end. Usually, 6-carboxyfluorescein (6FAM) is used as reporter dye at 5' and 6-carboxytetramethyl-rhodamine (TAMRA) is used at 3' end as quencher dye (Holland et al., 1991). When the probe hybridizes to its target template DNA, the reporter dye is cleaved by the 5′ nuclease activity of *Taq* polymerase. As a result of cleavage, the reporter dye emits fluorescent signal, since it is no longer suppressed by the quencher dye. TaqMan probes overcome the error of reducing signals due to mispriming or primer-dimer formation – superior over SYBR green chemistry with DNA binding dyes (more prone to error since any non-specific PCR products, and primer-dimer artifacts, can generate a signal). TaqMan amplicon is generally 60-70 bp, thus reaction is more efficient (contrary to standard PCR, where an amplicon of at least 200 bp is required to detect efficiently by electrophoretic separation). TaqMan PCR was demonstrated to be useful in detecting *R. solanacearum* strains (Weller, 2000).

Fluorescent resonance energy transfer (FRET) probes

There are two FRET probes used in this method (Didenko, 2001). Probe 1 contains a fluorescent label at its 3' end, and probe 2 is labeled at its 5' end with a different dye such as light cycler red 640. The two probes are designed so that when they hybridize to the amplified PCR product they are aligned head to tail to bring the two fluorescent dyes in close proximity to each other. The dye attached to the first probe is excited by the light source of the light cycler unit and it emits a green fluorescent light at a slightly longer wavelength. When 2nd probe is in close proximity, the energy emitted by the 1st probe excites the light cycler red 640 dye attached to the 2nd probe and the red fluoresces at a longer wavelength, which is now detected at 640 nm. Fluorescence is measured during annealing step of each cycle.

Molecular beacons

Molecular beacons are short fluorescent oligonucleotide probes that are designed to form stem-loop folding (Didenko, 2001). The probes contain a fluorescent chromophore at the 5' end and a quencher molecule at the 3′ end. The probes at resting stage (after end each amplification cycle) form a stem-loop structure due to annealing of the complementary arm sequences that are added on both sides of the probe. The probes at stem-loop form do not emit fluorescence as the energy from fluorophore molecule get transferred to the quencher molecule. However, the probes emit fluorescence when the fluorophore and quencher become separated from each other that is, at the time of annealing to the target template DNA during PCR amplification (Cockerill and Smith, 2002).

Nested-PCR

In nested PCR two rounds of PCR are performed, the second using a primer set internal to those used in the first round. Sometimes immunocapture is used to concentrate pathogenic cells prior to PCR, thus increasing detection sensitivity. It is a technique with increased detection sensitivity through multiple displacement amplification (MDA). MDA exponential amplification of DNA is carried out using random hexamer primers resulting in more or less complete genome amplification for essentially all DNA present in

sample (Dean et al., 2002). By performing MDA prior to PCR on a sample containing trace amounts of pathogen genomic DNA one can exponentially increase the quantity and thereby help in pathogen detection. Furthermore, MDA can be used to generate large quantities of genomic DNA from a very limited sample (few bacterial cells in the infected samples), thus providing a supply of genomic DNA that can be used for multiple tests and stored for future use. The nested-PCR has been demonstrated to be useful in ultrasensitive detection of various plant pathogenic bacteria including *Clavibacter michiganensis* subsp. s*epedonicus* (Lee et al., 1997b), *Erwinia amylovora* (Llop et al., 2000) and *Pseudomonas savastanoi* pv. *savastanoi* (Bertolini, 2003b).

Bio-PCR

The sensitivity of a PCR based detection assay can be improved using a short culture enrichment step preceding PCR-amplification and is referred as Bio-PCR (Schaad et al., 1995). Increased sensitivity of the detection assay using culture enrichment step prior to PCR was observed during detection of many bacterial plant pathogens including *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts, *Acidovorax avenae* ssp. *avenae* in rice seeds (Schaad et al., 1995; López et al., 2003; Song et al., 2004).

Multiplex PCR

Multiplex PCR is useful for the simultaneous detection of multiple pathogens in a single reaction. In multiplex reaction more than one set of primers are used. Multiplex reaction can be run either in a conventional or real time PCR machine. Using multiplex reaction we can reduce the total detection time required for individual pathogens (viral, fungal, bacterial) associated with a given crop samples. A multiplex nested reverse-transcription PCR detection protocol was developed for four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* affecting olive trees (Bertolini, 2003a).

Loop-mediated isothermal amplification (LAMP)

The LAMP technique has been shown to be good approach for amplifying nucleic acid with high specificity, efficiency, and rapidity without the need for thermal cycler (Kubota et al., 2007). It is a method for the detection of specific nucleic acid sequences and has the potential to overcome many of the limitations of PCR-based methods. The ability of LAMP to amplify a target nucleic acid sequence under isothermal conditions eliminates the need for thermal cycling equipment, allowing testing to be carried out with minimal equipment (a water bath or heated block). Furthermore, this simplicity of LAMP-based technique facilitates its use in the field or in less well-resourced settings. LAMP method has been successfully used for detection of many plant pathogenic bacteria including epiphytic *E. amylovora* in pear and apple (Temple et al., 2008).

Repetitive sequence based PCR (Rep-PCR)

Diagnosis of bacterial plant pathogen using primers corresponding to specific repetitive sequences like enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (Rep) and repetitive BOX elements (BOX), which are dispersed throughout the bacterial genome, has well been documented (Martin et al., 1992; Louws et al., 1994, 1998; Barak and Gilbertson, 2003). The distribution pattern of these repetitive sequences varies from one bacterium to other, and this comprises the basis of differentiation in a bacterial population. PCR based on these repetitive sequences (often termed as rep-PCR) were found to be effective in identification of bacterial plant pathogens even at race level (Mahuku et al., 2006). The primers corresponding to Rep region are rep-1 R-1[5'-IIIICGICGICATCIGGC-3'] and rep-2 [5'-ICGICTTATCIGGCCTAC-3']; and to ERIC region (Enterobacterial repetitive intergenic consensus sequences) are ERIC1R [5'-ATGT AAGCTCCTGGGGATTCAC-3'] and ERIC2 [5'-AAGTAAGTGACTGGGTGAGCG-3']; to BOXA region (a subunit of the BOX element) is BOX-A1R primer [5'-CTACGGCAAGGCGACGCTGACG-3']. The ERIC-PCR has been demonstrated to be an effective method in determining the genetic diversity among population of many bacterial plant pathogenic genera, including *Xanthomonas* and *Pseudomonas* (Weingrat et al., 1997; Mondal and Mani, 2009). Recently, the use of BOX-PCR in determining the genetic variability among the bacterial flora associated with pomegranate leaf (Figure 3) was documented (Mondal, 2011).

Microarrays

Identification of bacterial plant pathogens by conventional PCR is prone to potentially reporting false-positives. Further, scoring of a gel band continues to be problematical, especially with smeared backgrounds or low and high intensity bands. Criteria for inclusion or exclusion of bands above or below a given size are arbitrary. Eliminating these problems, microarray-based assays has come up as an effective method for the identification as well as differentiation of pathogenic

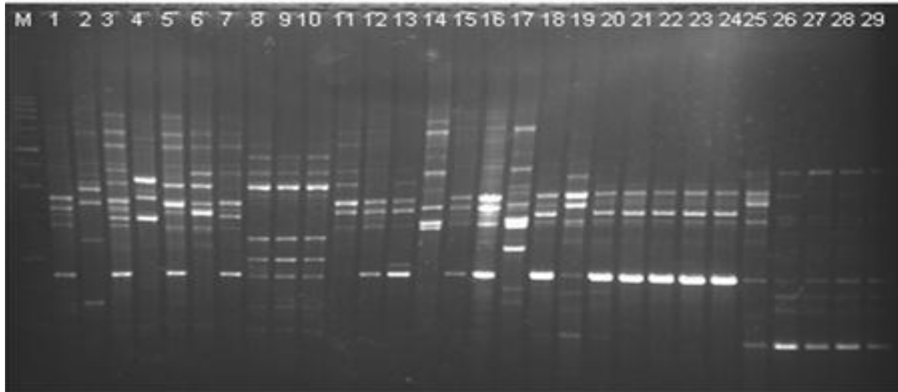

Figure 3. Genomic fingerprints of major bacterial flora associated with pomegranate leaf generated by BOX-PCR. M=1 kb plus ladder (Fermentous Co.) 1-29 bacterial isolates.

bacteria from mixed-culture environmental samples without the problem of false-positivity (Willse et al., 2002; Burton et al., 2006). The method is alternatively known as oligonucleotide arrays (Call et al., 2001). This technique has a greater multiplexing capability compared to conventional PCR, real time PCR or other NA-based techniques. After amplification by PCR, the resultant amplicon is hybridized with species/pathovar-specific DNA probes. The microarray-based techniques are applicable to any microorganism, without requiring *a priori* knowledge of specific nucleic acid signatures (Beattie, 1997; Loy et al., 2002). Thus dozen or hundreds of plant pathogens can be detected at a time, and this method is most useful in cases when the species being detected is unknown.

Genome-wide comparison between pathogenic and nonpathogenic strains within a species is a useful strategy for identifying candidate genes important for virulence. DNA microarray-based genome composition analysis is a good alternative to full genome sequencing and has been used in comparative studies to analyze various bacterial pathogens including *Mycobacterium tuberculosis* (Behr et al., 1999), *Helicobacter pylori* (Salama et al., 2000), *Pseudomonas aeruginosa* (Wolfgang et al., 2003), *Yersinia pestis*, and *Y. pseudotuberculosis* (Hinchliffe et al., 2003). A nonpathogenic strain J1a12 of *Xylella fastidiosa* associated with citrus could easily be differentiated from the pathogenic strain 9a5c causing variegated chlorosis, through DNA microarray-based comparison (Koide et al., 2004). It was revealed that 14 coding sequences of strain 9a5c are absent or highly divergent in strain J1a12. An arginase and a fimbrial adhesin precursor of type III pilus were confirmed to be absent in the nonpathogenic strain by PCR and DNA sequencing. Thus, the absence of both genes can be associated with the failure of the J1a12 strain to establish and spread in citrus and tobacco plants.

The gene distribution among strains of *Ralstonia solanacearum*, a highly polymorphic plant pathogenic bacterium, has been a priority area to study the status of known or candidate pathogenicity genes. Based on the use of comparative genomic hybridization on a pangenomic microarray for the GMI1000 reference pathogenic strain, researchers could compare the repertoires of genes among a collection of 18 different strains representative of the biodiversity of the *R. solanacearum* species (Guidot et al., 2007). Presently, a list of 2,690 core genes has been identified in all tested strains. As a corollary, a list of 2,338 variable genes within the *R. solanacearum* species has been defined. The hierarchical clustering based on the distribution of variable genes is fully consistent with the phylotype classification, which was previously defined from the nucleotide sequence analysis of four different genes. The presence of numerous pathogenicity-related genes in the core genome indicates that *R. solanacearum* is an ancestral pathogen.

Advantages of NA-based detection techniques

There is no debate about the value of NA-based techniques to an applied plant pathology programme. The main advantages are high sensitivity (Janse, 1988), rapidity, specificity and the quantifiability. Of further note are the following advantages:

(i) Detection and identification of bacterial pathogens in seed and plant samples for post- and pre-entry quarantine check.
(ii) Quantification of bacterial biomass in host tissue or in environmental samples.
(iii) Identification of bacteria that grow slowly or difficult to grow (fastidious bacteria).
(iv) Genomic characterization of genus, species, pathovars,

of bacterial pathogens.
(v) To provide a more dependable diagnosis in the specific cases where the routine diagnostics through cultural, morphological and biochemical are not conclusive enough.

Disadvantages of NA-based detection techniques

(i) NA-based tests very often yield false positives and false negatives, therefore, plant pathologist should not rely exclusively on NA-based test as the sole evidences for new reports, nor for other important samples.
(ii) Depending on the complexity of the diseased sample, a positive PCR test may not be sufficient evidence for a high-confidence diagnosis.
(iii) Further verification of the identity of PCR amplicons generated using species-specific primers is sometimes required to confirm results. These include analysis of RFLP, sequencing or hybridization to a specie-specific DNA probe. This makes the diagnostic protocol more complex, cost and time intensive.

SEROLOGY BASED DIAGNOSTIC TECHNIQUES

The basic principle of sero-diagnostic lies on the fact that the antigen (target pathogens) is detected using antibodies (specific to the target pathogens concerned); and this reaction is visualized through an enzyme-substrate hybridization. The detection of bacterial plant pathogens with antisera is still the method of choice for many plant diagnostician because of the relative low costs and presence of technical infrastructure based on automated enzyme liked immunosorbent assay (ELISA).

Preparation of antigen and production of antisera

The antigen preparation is emulsified with an equal volume of Freund's complete adjuvant. The emulsified antigen (0.5 ml/animal) is administrated to rabbits through sub-cutaneal mode and foot pad at multiple sites. The antigens (100-150 µg), emulsified in an equal volume of Freund's incomplete adjuvant, are administered to rabbits to produce hyper immune sera. Rabbits are bled through the ear vein 7 days after the last booster dose and sera are separated, and then stored at -20°C.

Mono- and poly-clonal antibodies are required to develop for specific immunological diagnostic assays. Polyclonal antibodies (pAb) are typically raised in rabbits, goats or sheep, and their popularity is evident by the fact that they are frequently used in immunosensor-based assays for bacterial detection. It should be noted that the different epitopes of pAbs may often be recognized on a single cell. Under specific cases where this is undesirable, such as in the case where high specificity is a requirement, monoclonal or recombinant antibodies may be more applicable. Monoclonal antibodies are generated using the hybridoma technology. The bone marrow, primary lymph nodes and the spleen are selected as a source of antibody-producing B cells which are harvested and fused to immortal myeloma cells. The resulting hybrid cells (referred to as hybridomas) subsequently secrete full-length antibodies that are directed towards a single epitope. Suitable candidates, identified by ELISA-based analysis, are then 'cloned out' to ensure that a single cell, producing antibody specific for an individual epitope, is present and the antibody generated can be used for assay development.

Cell parts of different pathogens possess antigenic properties that are employed for antibody production. Like soluble cytoplasmic antigen, insoluble cell wall antigen and lyophilized mycelial antigen in fungi. In bacterial plant pathogens, lipopolysaccharides, exopolysaccharide, cell wall protein, flagellar protein, and outer membrane proteins are used as antigens. The monoclonal antibody when reacted with bacterial antigen (like lipopolysaccharides) yields a low mobility ladder pattern on immuno-blotting experiment.

Enzyme linked immunosorbent assay (ELISA)

Through this technique, the bacterial pathogens can be detected directly in the infected plant materials without culturing the bacteria. Owing to its simplicity, sensitivity and adaptability, this technique has gained popularity in different diagnostic laboratories. Both poly and monoclonal antibodies are used for the detection of phytopathogenic bacteria using ELISA assay. The sensitivity of ELISA assay ranges between 10^5–10^6 CFU ml^{-1}. There are several reports where monoclonal antibody have been employed to detect bacterial plant pathogens including *Xanthomonas campestris* pv. *begoniae, X. campestris* pv. *pelargonii* (Benedict et al., 1990), *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (Benedict et al., 1989), *Corynebacterium sepedonicum* (DeBoer and Wieczorek, 1984), and *Erwinia amylovora* (Gorris et al., 1996). Shanmugam et al. (2002) used polyclonal antibody developed against whole bacterial cells (Courtesy: International Potato Research Centre, Lima, Peru) of *R. solanacearum* infecting potato to detect the survival of *R. solanacearum* in ginger rhizomes stored at different temperatures.

The sensitivity of ELISA can be increased to many folds by adding ethylene diamine tetraacetic acid (EDTA) and lysozyme in the extraction buffer (which helps to release lipopolysaccharide into solution). Pre-heating of bacterial samples to destroy proteins also improves the sensitivity by increasing the signal: noise ratio (Jones et al., 1997).

Lateral flow devices

The lateral flow devices are principally based on ELISA techniques where different types of filters are used as solid support for capturing the antigen-antibody binding reaction (Alvarez, 2004). Lateral flow devices are now available for rapid detection of bacterial pathogens including *Ralstonia solanacearum* (Danks and Barker, 2000), *Clavibacter michiganensis* subsp. *michiganensis* and *X. hortorum* pv. *pelargonii* (Alvarez, 2004).

CONCLUSION

The detection of pathogenic bacteria through conventional strategies involves the aseptic transfer of inoculum from an infected source (soil, plant parts etc.) to a suitable growth medium and subsequent transfer to selective or differential media. The colonies that appear on such media can be distinguished based on their distinctive colony morphologies by ocular inspection and their identification confirmed by rigorous biochemical (sugar utilization, etc.), physiological assays. Though the conventional colony based approach provides an inexpensive and straightforward protocol for quantitative and qualitative bacterial pathogen detection, a major disadvantage of this approach is the lengthy times required to obtain visible and identifiable bacterial colonies. The sero-diagnostics for bacterial pathogens are often preferred being more user-friendly, higher sensitivity, and much faster than conventional techniques. However, the success of any antibody-mediated test depends on the specificity of the antibody. Adequate care should be taken to minimize the cross-reactivity with related antigens that should be checked while evaluating important antigens. The nucleic acid based detection of bacterial pathogens has emerged as a supplement to overcome these bottlenecks. The recent advancement in the area of PCR based approaches further extended its versatility. Assays like real-time PCR, multiplex PCR, nested PCR, Bio-PCR, repetitive PCR, LAMP are among the detection options that provides rapid data analysis with specificity. However, one has to choose the best or combination of options depending upon the needs. For example, when multiple pathogens are to be detected in a minimum time multiplex-PCR would be the best options. While, the pathogen detection limit in a sample is at zero tolerance level, nested PCR, bio-PCR should be carried out to detect even the lower number of bacterial cells in the tested samples and this would also help to differentiate between viable and non-viable cells. And for routine diagnosis of the bacterial pathogens, integrated approaches including 16S rDNA sequencing, MLST, Biolog-phenotyping, fatty acid methyl esterase (FAME) profiling and pathogenicity assay following Kotch's postulate are to be preferred.

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