Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Recent Res. Devel. Microbiology, 9(2005): ISBN: 81-7736-243-7

# Recent developments in diagnostics of plant pathogens: A review

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## Abstract

*Accurate detection and identification of plant pathogens are fundamental to plant pathogen diagnostics and thus plant disease management. The lack of rapid, accurate and reliable means by which plant pathogens can be detected and identified has been one of the main limitations in plant disease management and has prompted the search for alternative diagnostic techniques. The advent of enzyme-linked immunosorbent assay (ELISA) and polymerase*

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*chain reaction (PCR) has caused a shift towards the use of molecular approaches in modern plant pathogen diagnostics. Nowadays, many techniques have been developed for the detection and identification of plant pathogens, each requiring its own protocol, equipment, and expertise. In addition, some of these techniques permit reliable quantification of the target pathogen as well, and supply the information required to estimate potential risks regarding disease development, spread of the inoculum, and economic losses. The major challenge at the moment is the development of multiplex assays that allow accurate detection and quantification of multiple pathogens in a single assay. In this chapter, we discuss recent advances in molecular plant pathogen diagnostics that are likely to impact future plant disease controlling and preventing strategies.*

# Introduction

The ability to accurately detect and identify a potentially plant pathogenic organism is fundamental to plant pathogen diagnostics and plant disease management. Conventional methods to detect plant pathogens have often relied on interpretation of symptoms, morphological identification, usually following isolation and culturing of the organism and, sometimes, further characterization based on pathogenicity tests (1). Although these methods are fundamental to diagnostics, the accuracy and reliability of these methods largely depend on skilled taxonomical expertise. In addition, diagnosis requiring a culturing step is time consuming and labor intensive. Finally, these techniques rely on the ability of the organism to be cultured *in vitro*. This latter aspect is a serious limitation of the applicability of these techniques since possibly less than 1% of the microorganisms in an environmental sample may be cultured *in vitro* (2).

In contrast, more recently developed methods that are based on molecular approaches are increasingly being used to detect and identify plant pathogens. These include immunological (or serological) and nucleic acid-based techniques. Compared to conventional assays, these techniques are more suitable for routine analyses since they are generally faster, more specific, more sensitive and more accurate, and can be performed and interpreted by personnel with no taxonomical expertise. In addition, since no culturing step is required, these techniques are equally suitable for the detection of culturable as well as non-culturable microorganisms.

Many different molecular assays have been described for the detection and identification of pathogens, each requiring its own protocol, equipment, and expertise. In this review, we outline some recent advances in molecular plant pathogen diagnostics with an emphasis on fungal molecular diagnostics. Methods that have been used in the past, the most predominant methods used nowadays, and some future perspectives of these methods are discussed.

# Serological techniques

A first development towards techniques for molecular pathogen detection was the advent of serological or antibody-based detection methods almost 30 years ago. These techniques were originally developed to detect viruses, as those can not be cultured *in vitro*. Serological techniques are based on the binding between diagnostic antibodies and specific antigenic determinants of the target pathogen. Several serological plant pathogen detection methods have been described (3,4) of which the enzyme-linked immunosorbent assay (ELISA; 5) is by far the most common technique. Although different types of ELISA have been developed, all involve an enzyme-mediated color change reaction to detect and often also quantify antibody binding as a measure for pathogen presence. Since its introduction in the late 1970s ELISA assays have been routinely used for virus and bacteria detection because of their high-throughput capacity, the rapid, relatively cheap and simple nature, and the possibility to quantify the target pathogen (6,7).

A major limitation for the development of serological methods is the labor-intensive procedure to obtain reliable assays, often due to the difficulty to generate selective antibodies. Although polyclonal antibodies, which recognize multiple epitopes of the pathogen, have been used successfully for detecting many viruses, they do not always have the desired degree of specificity and, importantly, the specificity may vary with each newly produced batch. The accuracy of detection is often improved by using either monoclonal or recombinant antibodies. Both of these allow the selection of specific target epitopes to avoid "false positives". However, developing antibodies with the required degree of specificity is difficult for complex organisms such as bacteria and fungi. In those cases, it is often hard to find reliable species-specific epitopes that are ubiquitously shared within a species but not with other species. Therefore, most antibody-based assays currently available are for the detection of relatively unsophisticated organisms such as plant viruses (8,9) while those available for the detection of fungi and bacteria are less common (10,11).

# Nucleic acid-based techniques

Before the possibility to amplify nucleic acid sequences existed, the sensitivity of detection based on those sequences totally relied on the method to translate their presence into a detectable signal (12-14). Since the introduction of amplification methods for nucleic acids, in particular the polymerase chain reaction (PCR; 15), nucleic acid-based methods are increasingly developed for the detection and identification of plant pathogens. This trend is enhanced by the growing availability of pathogen sequence data

in public databases like GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and COGEME (http://www.cogeme.man.ac.uk/) (16,17).

A crucial step in the development of nucleic acid-based diagnostic assays is the selection of sequences that can be employed for pathogen identification. In general, there are two main approaches that can be used to select target sequences. The first, and most widespread, strategy involves the use of ubiquitously conserved genes. The second strategy involves the screening of random parts of the genome in order to find sequences harboring the desired selectivity.

Currently, the nuclear ribosomal DNA (rDNA) operon is the most commonly used target for bacteria as well as for fungi for a number of reasons. First, it has been found that this gene provides a powerful means for analyzing phylogenetic relationships over a wide range of taxonomic levels, including for example genus, species (18), and even below (19). Apart from this potential, the large amount of ribosomal sequences in public databases allows to determine genomic regions that can be used to design selective primers or probes. This is facilitated even more by the structural nature of this type of gene since it contains alternating regions with high and low degrees of conservation. This allows to design primers on sequences that are conserved between species which span variable domains that can be used for species identification (18,20). In addition, the multiple copies of the gene present in each cell permit a very sensitive detection.

Although rDNA is the main target of many nucleic acid-based analyses, other targets for detecting fungi include β-tubuline (21,22), actin (23), elongation factor 1 alpha (24,25), and mating type genes (26,27).

However, if these genes do not display the desired degree of selectivity, other regions of the genome need to be assessed. The screening of arbitrary regions in the genome to find sequences with the required selectivity can be achieved by several techniques, including RAPD (random amplified polymorphic DNA; 28) and AFLP (amplified fragment length polymorphism; 29) technology. Diagnostic markers identified with these approaches can be sequenced and are used to design specific SCAR (sequence characterized amplified region) primers (30). Nevertheless, as these sequences can be derived from anywhere in the genome, there often is few sequence data available for comparison to multiple other organisms. Therefore, extensive screening is required to validate the specificity of the marker.

Two specific problems can hamper detection of plant pathogens based on nucleic acid-based techniques because they complicate the identification of reliable markers. First, misclassification of strains is a regularly occurring phenomenon in fungal taxonomy. Historically, taxonomists have grouped closely related fungi in a single genus or species largely based on similarities of structural and morphological characteristics. However, especially large

fungal genera and genera containing asexual fungal species are known to often contain unrelated species. As a result, taxonomic relationships are not always reflected by the evolutional relationships that are often revealed using nucleic acid-based techniques. Therefore, finding selective sequences shared by all members of a species is complicated for certain species. A second problem for molecular detection of certain plant pathogens is the existence of fungal species that harbor pathogenic as well as non-pathogenic or even beneficial strains. This is a known phenomenon for complex species such as *Fusarium oxysporum* and *Rhizoctonia solani* (31). In those cases, target sequences should preferably be directly associated with virulence traits which severely limits the number of sequences (32,33).

Nucleic acid-based techniques can be divided into DNA- and RNA-based technologies which will be discussed separately.

## 1. DNA-based techniques

DNA is a highly attractive target for the detection of plant pathogens in biological samples because it is easier to handle and more resistant to degradation than RNA. With improved extraction methods (34) and commercially available extraction kits (35,36) highly purified DNA can rather easily be obtained from complex environmental samples.

**Polymerase Chain Reaction (PCR)**

Using PCR, millions of copies of specific DNA sequences may be rapidly synthesized in a thermocyclic process that consists of repetitive cycles of DNA denaturation, primer annealing, and extension using a thermostable DNA polymerase (15). If a DNA sequence unique to a particular organism is determined, specific PCR primers or probes can be designed that enable determination of the presence or absence of that sequence, and thus of the specific organism. The presence of amplified DNA is traditionally detected by gel electrophoresis, but alternative detection formats including colorimetric and fluorimetric assays do exist (21,37). PCR-based detection methods are very sensitive and can detect minute quantities of pathogen DNA, even the amount derived from a single fungal spore (38). To improve specificity, but sometimes also sensitivity, PCR products may also be detected using a probe (39). Other approaches to increase sensitivity and specificity include the use of immunocapture PCR (IC-PCR) or nested PCR. IC-PCR utilizes antibodies to isolate the pathogen from a sample prior to PCR amplification and has mainly been used to detect plant pathogenic viruses (40). Nested PCR involves two consecutive PCR reactions, the second one using primers that share a sequence within the target DNA fragment that is amplified in the first reaction (41). As a result, a specific reaction products that are generated in the first PCR reaction should not be amplified in the second reaction.

Many reports describe specific applications of PCR technology in plant pathology (42-47). In addition, increasingly companies providing diagnostic services are using PCR to routinely detect and identify plant pathogens.

Quantification of the amount of pathogen DNA, supplying the information required for disease management decisions and for monitoring the effects of these decisions, has also been pursued using PCR-based methods. Although it is relatively easy to quantify the amount of amplicon generated, it is more difficult to relate this quantity to the initial amount of target DNA present in a sample. This is caused by the typical non-linear kinetics of template amplification. Nevertheless, in theory, the exponential nature of PCR allows the initial amount of DNA to be calculated from the amount of product at any time point in the reaction. In practice, however, as the reaction proceeds reagents become limiting and a plateau level is reached where the amount of product is no longer proportional to the original amount of template. Target DNA can be quantified using competitive PCR, which is based on the co-amplification of target DNA and a competitor DNA, both with the same primer pair (48). The amount of target DNA is subsequently determined on agarose gel by comparing the relative amounts of target and competitor PCR product. This method has been used to successfully quantify, for instance, Verticillium wilt pathogens (49).

**Real-time PCR**

Especially with respect to quantification purposes real-time PCR is a powerful development (50). This technology differs from conventional PCR by monitoring products on-line while they accumulate at each reaction cycle in a closed tube format, without the need of post-reaction processing such as gel electrophoresis. As a consequence, real-time PCR is generally faster than conventional PCR, enabling high throughput analyses. In addition, the risk of post-PCR carry-over contamination of amplicons is eliminated. Real-time PCR allows accurate template quantification during the exponential phase of the reaction, before reaction components become limiting. The initial amount of target DNA can be related to a threshold cycle, defined as the cycle number at which fluorescence increases above the background level. Target DNA is quantified using a calibration curve that relates threshold cycles to a specific amount of template DNA. Typically, DNA amplification is monitored each cycle based on the emission of fluorescence (51).

Amplicons can be detected using several chemistries, which can be divided into either amplicon non-specific (52) and amplicon specific (53-59) methods, using DNA-binding dyes and sequence-specific probes, respectively (Fig. 1). The use of DNA-intercalating dyes such as SYBR Green® is a more straightforward and less expensive approach compared to using probes, but it is also less specific since the dye binds to all double stranded DNA (dsDNA)

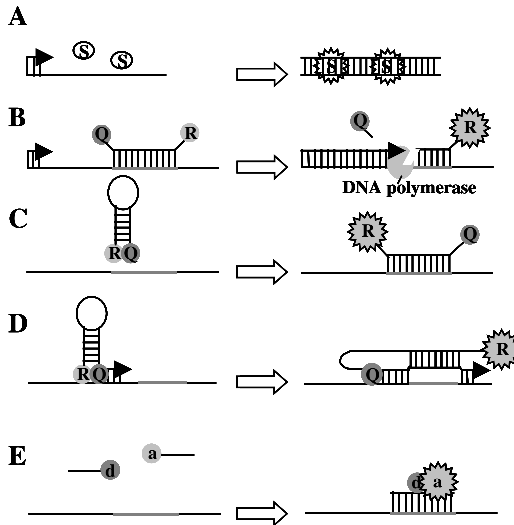


**Figure 1. Main chemistries for amplicon detection in real-time PCR applications.** (A) As a DNA-intercalating dye such as SYBR Green® (S) binds to dsDNA, fluorescence is recorded. (B) Taqman® probes, (C) Molecular Beacons® as well as (D) Scorpion® primers use a strategy to extinguish fluorescence at certain conditions using a reporter fluorophore (R) and a fluorogenic quencher (Q). Upon physical separation of both molecules fluorescence is emitted. (E) The use of FRET probes involves the hybridization of two labeled oligonucleotides in close proximity. When both probes bind to the target fragment, energy is transferred from the donor (d) to the acceptor (a) molecule resulting in fluorescence.

present in the sample (Fig. 1). In addition, the interpretation of results can be disturbed by formation of primer-dimers or aspecific reaction products. It is therefore crucial to use highly specific primers and to determine optimal reaction conditions (51,60). In addition, melt curve analysis at the end of the PCR reaction allows evaluating the accuracy of the amplification reaction. In contrast to amplicon non-specific chemistries, probe-based assays often offer the advantages of increased specificity, certainly in combination with specific primers, and reducing signals due to mispriming or primer-dimer formation (54). Most applications to date have used TaqMan® probes (54,57). These probes are single stranded, short oligonucleotides which are labeled with a fluorophore and a fluorogenic quencher (Fig. 1). Because of the close proximity of both groups, the fluorescent signal is quenched. During the annealing phase of each PCR cycle the probe hybridizes to a specific region within the target amplified fragment. The probe is degraded by 5’ exonuclease

activity when the DNA polymerase extends the primer. Consequently the fluorophore and the quencher are released independently, resulting in a fluorescent signal (Fig. 1). Variants of this quenching chemistry include hairpin shaped Molecular Beacons® (Fig. 1; 55,59) and Scorpion® primers (Fig. 1; 58). Whereas the loop portion of these molecules contains the probe sequence, the stem, which is formed by complementary sequences added to both ends of the probe, holds a fluorophore and a quencher in close proximity. In addition, Scorpion primers couple the stem-loop based probe to a PCR primer. Specific binding of the probe to its target opens the structure, producing a fluorescent signal (Fig. 1). A completely different detection chemistry comprises the use of fluorescent resonance energy transfer (FRET) probes (Fig. 1; 56). With this technology, two oligonucleotide probes are designed such that they hybridize in very close proximity to the amplified fragment. Whereas one of the probes contains a donor fluorophore at its 3' end, the other probe is labeled at its 5' end with an acceptor fluorophore. When both probes properly hybridize to the target fragment, the energy excited by the donor is transferred to the acceptor resulting in a fluorescent signal (Fig. 1).

Closely related microbial species often only differ in a single or a few bases of ubiquitously conserved genes of for instance the rDNA. The high degree of specificity of real-time PCR technology allows, independent of the detection chemistry, the detection of single-nucleotide polymorphisms (SNPs), meaning that specificity is determined by a single base pair(57-60). Therefore, this technology offers many opportunities in plant pathogen diagnostics. In recent years, real-time PCR assays have been developed for accurate detection and/or quantification of specific plant pathogens (61-64) as well as for monitoring pathogen infections (65). Although not yet used routinely in phytodiagnostics, real-time PCR has much potential for future applications.

**Ligase Chain Reaction (LCR)**

The ligase chain reaction (LCR) uses two complementary pairs of oligonucleotides that hybridize in close proximity on the target fragment (Fig. 2). Only when the oligonucleotides correctly hybridize to the target sequence, the remaining nick between the oligonucleotides is ligated by a DNA ligase and a fragment equating to the total sequence of both oligonucleotides is generated. Similar as in a PCR reaction, the products of one reaction serve as templates for subsequent cycles, resulting in an exponential amplification of the desired fragment (Fig. 2). To further enhance sensitivity and sometimes also specificity, LCR can also be used following a PCR preamplification (66,67). Detection of LCR products can be performed by polyacrylamide gel electrophoresis. With this technology, SNPs can easily be differentiated (68). Although LRC is regularly applied in human disease detection (66,68,69), it has rarely been reported for detection of plant pathogens (67,70).

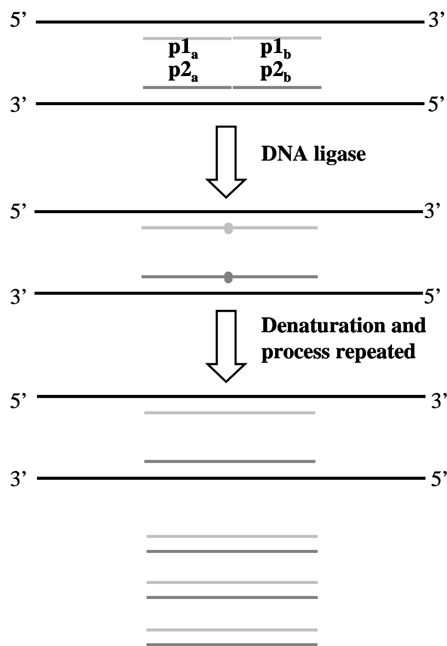


**Figure 2. General principle of the Ligase Chain Reaction (LCR).** Two complementary pairs of adjacent oligonucleotides ($p1_a$ and $p1_b$; $p2_a$ and $p2_b$) bind to the target sequence. Only if the oligonucleotides bind in close proximity DNA ligase seals the nicks and the cycle can be repeated.

## Rolling Circle Amplification (RCA)

Originally, padlock probes (71) were developed as a new approach for molecular analysis of complex DNA samples, including analysis of alleles and point mutations in the human genome (72). A padlock probe consists of a single stranded linear oligonucleotide of about 70-100 nt in length with a target-complementary region at both ends and a linker segment in between. The 5’ and 3’ end regions are designed to hybridize next to each other on a target strand. When properly hybridized to the target sequence, the molecule can be circularized upon ligation. Because of the need for precise base pairing at the junction where ligation should take place and the simultaneous hybridization of two different fragments, padlock probes ensure high specificity (72).

For sensitive pathogen detection, however, signal amplification is a prerequisite. One approach for the amplification of padlock probes is a PCR reaction using primers that hybridize to sequences within the spacer region of

the probe (73). Another method to amplify padlock probes is rolling circle amplification (RCA), analogous to replication mechanisms of several viruses with circular genomes (73-76). Two types of RCA have been described: linear and hyperbranched RCA. In the first procedure, a primer hybridized at some point on the circular DNA is extended continuously using a DNA polymerase that lacks exonuclease activity. As a result, a long linear fragment composed of many tandem repeats of the complement to the circularized molecule is generated. In addition, hyperbranched (or cascade) RCA (Fig. 3) uses a second primer that binds to each generated RCA repeat. During elongation, the exoncuclease deficient DNA polymerase displaces the polymerized strand in front of it. Next, the displaced strands which are tandem repeats with identical sequences to the original padlock probe, serve again as template for the first primer, resulting in a cascade of DNA amplification (Fig. 3).

As for conventional PCR, detection of amplified products can be achieved using gel electrophoresis (75,77) or labeled probes (76) enabling real-time monitoring of the amplification process. However, although RCA is considered to be one of the most sensitive amplification methods, the procedure is fairly complicated (78) and relatively expensive. Therefore, it is

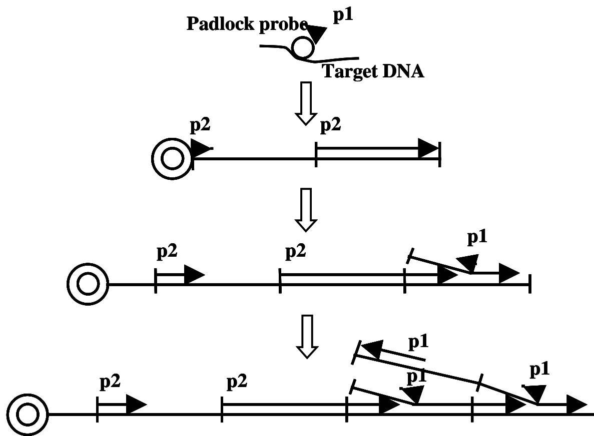


**Figure 3. General principle of hyperbranched Rolling Circle Amplification (RCA).** The 5' and 3' ends of a linear padlock probe are designed to hybridize next to each other on a target strand. When properly hybridized, the molecule is circularized by ligation. Synthesis of the complementary strand of the circularized padlock probe is initiated by primer p1. As a strand of linear tandem repeats is generated, a second primer (p2) hybridizes to each newly generated repeat. During elongation, the exonuclease-deficient DNA polymerase displaces the polymerized strand in front of it which, in turn, serves as template for the first primer.

important to realize what level of sensitivity is required for a method to be used for plant pathogen diagnostics. The most sensitive technique will probably not be required when assessing whether measures have to be taken in a certain crop to prevent yield losses. Often such a decision requires a threshold level to be crossed which can be detected by many less sensitive techniques. In contrast, sensitivity is very important when it comes to zero tolerance of quarantine diseases.

All DNA-based methods have in common that they might detect DNA from dead or non-active organisms as well. Therefore, detection of non-viable propagules cannot be ruled out. However, DNA from dead cells in soils should be degraded fairly rapid due to the high microbial activity, suggesting that interference by DNA from dead cells might be negligible (79,80). Nevertheless, the rate of DNA degradation depends on soil type (81) and moisture content (82). As DNA degradation occurs slower in desiccated soils (82), accurate diagnosis of samples from dry fields may be biased by detection of dead organisms. However, since long-lasting soil dessication generally does not occur in horticultural or agricultural practice, this should not be a major concern.

To exclude detection of dead organisms, a culturing step on or in a suitable medium, or even in planta, prior to PCR amplification could be included. This technique is referred to as BIO-PCR. Because only active propagules will be able to grow, this technique enables selection of viable organisms. In addition, PCR sensitivity is increased by the culturing step (83,84). However, disadvantages are its labor intensive and time consuming nature, and the inability to detect non-culturable organisms. As an alternative, attempts are made to use DNA-binding dyes such as ethidium monoazide (EMA) to distinguish viable from non-viable organisms (85,86). Since the membranes of dead cells quickly disintegrate, EMA is able to selectively enter dead cells where it covalently binds to dsDNA upon light-exposure. EMA-bound DNA is blocked for PCR amplification, thus enabling the selective amplification of targets from living organisms. Another alternative to prevent detection of dead organisms is the use of RNA as target molecule. Since RNA is less stable than DNA, RNA will be degraded much faster in dead organisms. In addition, the stability of DNA can be the cause of persisting contaminations in diagnosis laboratories where large numbers of samples which often contain the same pathogen are processed. In addition, messenger RNA (mRNA) is only produced in metabolically active cells, making mRNA attractive to selectively detect living microorganisms (87-92). However, extraction of RNA from environmental samples should be a careful procedure.

## 2. RNA-based techniques

Whereas DNA-based detection techniques are increasingly being used to detect and identify pathogenic fungi, bacteria as well as nematodes, RNA-based

techniques are mainly used to detect plant viruses since most of them have RNA genomes. However, since mRNA may more accurately reflect metabolically active pathogen material, these techniques can also be used to selectively detect viable pathogen propagules (87-92).

### Reverse Transcriptase PCR (RT-PCR)

Since PCR can only amplify double stranded templates such as DNA, RNA should be converted to DNA (called complementary DNA or cDNA) prior to use in a PCR-based assay. Typically, such reverse transcriptase PCR (RT-PCR) consists of an annealing step for one primer and an extension step to synthesize the complementary or second strand, followed by a (real-time) PCR reaction (93,94). In plant pathology, RT-PCR is a common strategy to detect plant viruses (95).

### Nucleic Acid Sequence Based Amplification (NASBA), Transcription Mediated Amplification (TMA), or Self-Sustained Sequence Replication (3SR)

Nucleic acid sequence based amplification (NASBA), also known as transcription mediated amplification (TMA) or self-sustained sequence replication (3SR), has been used for the direct amplification of RNA (96). In contrast to conventional PCR, amplification is carried out in an isothermal process (avoiding the need for a thermocycler) using three different enzymes, including a reverse transcriptase, RNase H, and T7 RNA polymerase (Fig. 4). Initially, a primer containing an RNA polymerase promoter sequence at its 5’ end and a target-specific sequence at its 3’ end is extended by reverse transcription to produce a cDNA strand. The resulting hybrid is a substrate for RNase H, which degrades the original RNA strand. Subsequently, a second DNA strand is produced from a primer designed to bind to the 3’ end of the cDNA, resulting in a dsDNA molecule that contains the sequence information of the original RNA and the promoter sequence of the T7 RNA polymerase. In a next step, T7 RNA polymerase initiates DNA transcription leading to the production of a large number of antisense RNA molecules. Each antisense RNA molecule is used to generate new dsDNA molecules based on the same principle, and initiates a new round of replication (Fig. 4).

The amplification products can be visualized using a specific labeled probe which hybridizes to the RNA amplicons (97,98). In addition, amplicons can be monitored in real-time using a specific detection probe such as a Molecular Beacon®. This procedure is referred to as AmpliDet RNA and combines the advantages of both NASBA and real-time PCR (90,99).

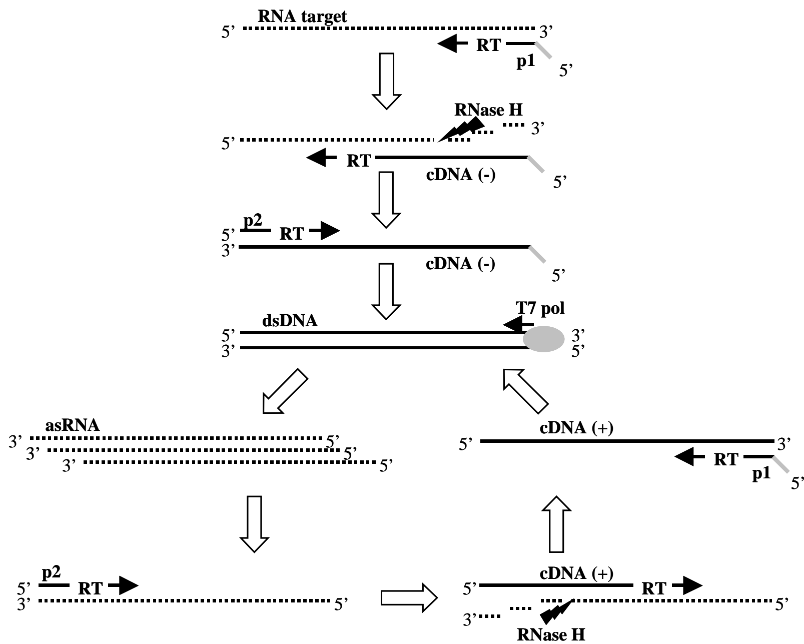


**Figure 4. General principle of Nucleic Acid Sequence Based Amplification (NASBA).** Upon binding of primer p1 that is tailed with a T7 RNA polymerase promoter, reverse transcriptase (RT) generates a cDNA strand. The resulting hybrid is a substrate for RNase H, which degrades the original RNA strand. Subsequently, reverse transcriptase generates a complementary strand to the fist cDNA strand using a second primer (p2), resulting in double stranded DNA (dsDNA) with a T7 RNA polymerase promoter. This is a template for T7 RNA polymerase (T7 pol) that transcribes a large number of antisense RNA molecules (asRNA) which, in turn, are converted into dsDNA for a next amplification cycle.

# Multiplex detection

One of the limitations of most detection procedures, whether serological, DNA- or RNA-based, is that only a single pathogen is detected per assay. However, as most crops are subject to be infected by multiple pathogens, there is a need to detect several pathogens at the same time. Therefore, multiplex detection, enabling to detect and identify a large number of pathogens in a single assay, is a major challenge in plant disease diagnostics and disease management.

Multiplex PCR assays, using several primers in the same reaction, have been developed for the simultaneous detection of several microorganisms

(100). However, the development of efficient and accurate multiplex formats is often difficult and typically requires extensive optimization of reaction conditions in order to properly discriminate at least a few amplicons per reaction. Besides designing selective primer pairs that can be used under the same conditions, the generated PCR products need to have different sizes to ensure clear discrimination of amplicons on agarose gels (101). This latter limitation does not apply for a real-time PCR applications based on amplicon-specific detection probes since different fluorophores can be used for the labeling of different probes. Nevertheless, for this technology the total amount of PCR reactions in a single tube is limited by the number of different fluorescent dyes available and the use of a monochromatic light source in real-time PCR instruments (51). As a result, detection of more than a handful of different pathogens is currently impossible.

Currently, array technology is the only technology that can be used to, in principle, detect an unlimited number of different organisms in one single sample. When combined with nucleic acid amplification it will result in high degrees of sensitivity, specificity, and throughput capacity (20,102,103). With this technology, detector oligonucleotides, each specific for a DNA or RNA sequence of a respective target organism, are immobilized on a solid support to create the array. In addition, multiple specific target molecules of a single pathogen can be spotted on a single array, thus increasing overall specificity. In general, target DNA (including genomic DNA, cDNA or even padlock probes harboring a specific random sequence in the spacer region (104)), is amplified using universal PCR primers, labeled, and subsequently hybridized to the array under stringent conditions. This technology, enabling the simultaneous detection of several different SNPs in one test (105, 106), was originally developed as a technique to screen for human genetic disorders (105, 107), but has also been successfully applied to detect and identify human and animal pathogens of diverse nature (108-110). In plant pathology, this technology has been successfully applied to identify oomycete, fungal, nematode, and bacterial pathogens (20, 111-114).

Nowadays, the most advanced array platforms are high-density microarrays using a glass slide (115-119) or beads (120,121) that carry many detector probes on a small scale. For disease diagnostic use in plant pathology, however, macroarrays using conventional nitrocellulose or nylon membranes are more suitable for a number of reasons. First, since spots on a macroarray often contain higher amounts of immobilized probes, macroarrays are generally more sensitive than microarrays (122,123). Secondly, whereas highly specialized instruments are needed for microarray production and reading, a macroarray-based detection procedure for plant pathogens does not require such specialized equipment and has therefore been proven to be more cost effective. This is an extremely important aspect in commercial plant pathogen

diagnostics relative to human clinical diagnostics since the readiness to spend money on expensive plant disease diagnosis is limited. After all, profit margins in agriculture are often low. Increasingly, companies that provide disease diagnostic services are using macroarray-based assays for multiplex detection of microbial pathogens (Fig. 5). An example of such a commercial array is called the "DNA Multiscan®"(www.DNAmultiscan.com), which can be used to simultaneously detect over 45 different plant pathogenic soilborne fungi and 10 bacteria in its current format. Each diagnosis can be achieved within 36 hours after sampling and results for pathogen presence or absence are read as a checklist. This new strategy thus provides a substantial improvement in diagnosis time from several days to several weeks, typically experienced with currently recognized conventional culture plating and microscopic methods, to less than two days. This should result in timely accurate advice to cure infected plants, prevent spread of the disease, and minimize economic losses.

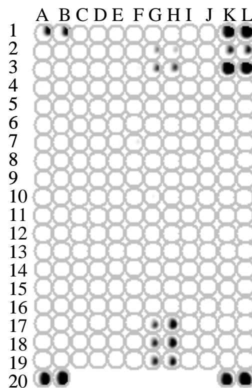


**Figure 5. Diagnosis on a diseased tomato seedling using a commercially available DNA array.** Each detector oligonucleotide is spotted in duplicate. Specificity of the analysis is enhanced by using multiple oligonucleotides for each target species. In addition to the immobilized pathogen-specific oligonucleotides, the array contains control oligonucleotides for the hybridization (1A & B, 1K & L, 20A & B, 20K & L) and a reference for detection and calibration (2K & L, 3K & L). PCR-labeled amplicons hybridize to genus-specific oligonucleotides for *Pythium* (2G & H, 3G & H) and species-specific oligonucleotides for *P. ultimum* (17G & H, 18G & H, 19G & H).

# Conclusion and future directions

The development of tools for plant pathogen diagnostics has increased enormously with the introduction of molecular tools, and more in particular with the advent of PCR. For disease management of many crops parallel

detection of a large number of pathogens and quantification of their presence are of high importance. Detecting multiple pathogens in a single assay is desirable with respect to efficiency, cost, time, and labor. Pathogen quantification can be used to estimate potential risks regarding disease development, spread of the inoculum, and economic losses. Currently, DNA array technology is the only technique to detect large numbers of pathogens in a single assay. Especially with regard to quantifying pathogen presence real-time PCR is a powerful development. Combining both methods, i.e. qualitative detection using a DNA array followed by accurate quantification using real-time PCR for the detected pathogens, can form a cornerstone for accurate disease management decisions. Nevertheless, the development of a single assay leading at the same time to identification and quantification of multiple pathogens should be pursued. Therefore, attempts are made to implement a quantitative aspect to DNA array technology for disease diagnosis, leading to a qualitative and quantitative multiplex assay (36). Ultimately, such multiplex approach must lead to a complete pathogen assessment method to detect and quantify all relevant pathogens of a specific crop. With timely and regular qualitative as well as quantitative diagnoses, preventive treatments can be properly prescribed and performed and, in case actual infections are monitored, the afflicted plants can be cured or removed to avoid spread of the disease. Until now, preventive treatments were applied frequently, based on the theoretically calculated risk for disease incidence. Preventive treatments based on actual monitoring of pathogen populations will probably reduce the number of treatments and thus result in reduced environmental impacts.

Without any doubt, the future will bring novel methods for detecting plant pathogens. Often such novel technologies are developed in the field of clinical medicine and whenever appropriate they find their application a little later in plant pathogen diagnostics as well. Most progress can be expected from the development of simple and rapid devices for on-site pathogen detection. Recently, new formats using antibody-based detection for very rapid presumptive on-site diagnosis have become available. These do not require specialized equipment or knowledge. Most of them use a membrane-based lateral flow assay, in which capillary forces generate a migration of the sample extract over specific antibodies (Fig. 6; 124, www.pocketdiagnostic.com). In case the antibodies recognize specific antigenic determinants a visual signal is generated immediately. One of the drawbacks, however, is the relatively low sensitivity, impeding widespread use. Nevertheless, because these assays are relatively inexpensive and require little labor and knowledge, there is a gaining interest to use these tests for in-field plant pathogen diagnostics (125).

In clinical diagnostics biosensors are currently developed (126). These are small devices that use a biological recognition element (e.g. specific antibodies or DNA sequences) coupled to a physical transducer that translates recognition

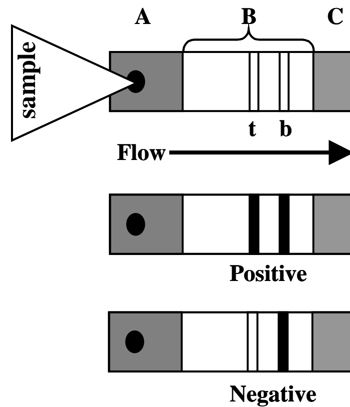


**Figure 6. Membrane-based Lateral Flow Assay.** Capillary forces establish the migration of a sample extract from the sample pad (A) to the absorbent pad (C). The sample pad contains antibody-coated latex beads that capture target antigens if present in the sample. The mixture migrates along the detector strips (B) containing target-specific antibodies (t) and bead-specific antibodies (b). Beads containing antigenic determinants are trapped on the test line, whereas superfluous beads are trapped on the control strip as a control for the assay. Binding of beads to the detector strips immediately results in a visible line.

into measurable electronic signals such as light or current. Since target molecules may include antigens (127) or DNA sequences (126) these devices could be highly attractive for rapid in-field plant pathogen diagnosis too.

Another interesting development in medical diagnostics is the integration of multiple processes (from sample preparation to detection) in a single sophisticated instrument called lab-on-a-chip (126,128).

However, only some of these novel methods will be used on a large scale by companies that provide diagnostic services. Which technologies will subsist and which not is guided not only by the sensitivity, specificity, and analytical quality of the assays, but also by the cost effectiveness of the test.

Apart from the development of molecular detection and identification strategies, a number of important issues need to be addressed in the future to enable intelligent use of novel technologies. Appropriate sampling strategies need to be developed to account for possible spatial variability of inoculum, especially since the amounts of material necessary for analysis reduces with the development of more sensitive technologies. In addition, monitored pathogen densities have to be translated in accurate advices to growers. Therefore, ecological studies are necessary, studying the behavior of a pathogen in its biological, physical, and chemical environment.

Since the introduction of PCR, molecular plant pathogen diagnostics is progressing quickly. Technologies are developed, improved and introduced. However, only when new detection technologies become integrated with conventional tools and human expertise they will lead to a better understanding and, ultimately, prevention of plant diseases.

## Acknowledgments

The authors would like to thank the ‘Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek-Vlaanderen’ (IWT-010121; IWT-040169; HOBU-030109) and De Ceuster Corp. (Sint-Katelijne-Waver, Belgium) for their financial support to our work. B.P.H.J.T. is supported through receipt of a VENI grant by the Research Council for Earth and Life sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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