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REVIEW ARTICLE

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Innovative tools for detection of plant pathogenic viruses and bacteria

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Abstract Detection of harmful viruses and bacteria in plant material, vectors or natural reservoirs is essential to ensure safe and sustainable agriculture. The techniques available have evolved significantly in the last few years to achieve rapid and reliable detection of pathogens, extraction of the target from the sample being important for optimising detection. For viruses, sample preparation has been simplified by imprinting or squashing plant material or insect vectors onto membranes. To improve the sensitivity of techniques for bacterial detection, a prior enrichment step in liquid or solid medium is advised. Serological and molecular techniques are currently the most appropriate when high numbers of samples need to be analysed. Specific monoclonal and/or recombinant antibodies are available for many plant pathogens and have contributed to the specificity of serological detection. Molecular detection can be optimised through the automatic purification of nucleic acids from pathogens by columns or robotics. New variants of PCR, such as simple or multiplex nested PCR in a single closed tube, co-operative-PCR and real-time monitoring of amplicons or quantitative PCR, allow high sensitivity in the detection of one or several pathogens in a single assay. The latest development in the analysis of nucleic acids is microarray technology, but it requires generic DNA/RNA extraction and pre-amplification methods to increase detection sensitivity. The advances in research that will result from the sequencing of many plant pathogen genomes, especially now in the era of proteomics, represent a new source of information for the future development of sensitive and specific detection techniques for these microorganisms.

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Introduction

Plant pathogenic viruses and bacteria are responsible for increasing economic losses worldwide. They can cause a large range of symptoms in most cultivated plants, which can be affected in their different parts with various agronomic impact. Viruses and bacteria cause plant diseases that are difficult to control because of the lack of efficient products for chemical treatment under field conditions. Consequently, preventive measures to avoid planting of contaminated material are of the highest importance in the context of an integrated approach to control. Among such measures, testing of planting material for pathogen-free status is an important, although not exclusive, method for controlling bacterial and viral diseases of plants [26, 36]. As many pathogenic viruses and bacteria remain latent in the planting material, and in very low numbers, methods of high sensitivity, specificity and reliability are required. Public institutions and the agro-food industry used to control the sanitary quality of seeds, fruits and plant material by microbiological testing for bacteria and biological indexing for viruses. These methods were often expensive and time-consuming and some of them were not sensitive and specific enough. In addition, biological indexing cannot be applied on the large scale required.

Detection deals with establishing the presence of a particular target organism within a sample, with special emphasis on symptomless individuals. Diagnosis relates to the identification of the nature and cause of the disease problem, thus concerning plants showing symptoms [35]. Accurate routine disease detection requires high levels of specificity, sensitivity and speed. In this context, specificity is defined as the capability to detect the

organism of interest in the absence of false positives and negatives. Sensitivity relates to the lowest number of pathogens reliably detected per assay or sample. For bacteria, the sensitivity levels of different techniques for detection in plant material are as follows [in colony forming units (cfu)/ml]: isolation, about $10–10^2$; immunofluorescence (IF), 10^3; conventional PCR, $10^3–10^4$ and ELISA, $10^5–10^6$.

Detection protocols typically target viruses or bacteria for which the primary disease management strategy is avoidance. To reduce the risk of disease and to prevent the spread of inoculum, the plant material may be subject to regulated zero tolerance, as in quarantine pathogens, or to desired zero tolerance as in many seedborne pathogens or quality pathogens. The need for techniques of high sensitivity, and of course specificity, is especially required in the case of quarantine viruses and bacteria. Such techniques must be applied in sanitary and eradication programmes where, in addition, detection is required as early as possible. Detecting very low pathogen populations by laboratory analysis is very difficult, as sampling errors increase with increased populations of plants or plant parts. In practice, European Union (EU) protocols for *Clavibacter michiganensis* pv. *sepedonicus* and *Ralstonia solanacearum* [2, 3] assume that 95% probability gives good protection, whereby 0.1% infection can be detected using a completely randomised sampling frequency. In this case, sampling error is more important than test sensitivity [26], and both statistical probability and the sampling factor determine that a zero occurrence can never be completely guaranteed. Furthermore, when discussing zero infection levels in pathogenic bacteria it should be taken into account that viable, or at least culturable, bacterial populations usually decline at low temperatures [26, 64], such as those in winter and in cold storage. Fortunately, serological and molecular techniques can also detect non-viable and/or non-culturable bacterial cells in plant tissues and should be used in monitoring and eradication of pathogenic bacteria [9].

In spite of the great advances in sensitivity and specificity of the available techniques and protocols, there is an evident lack of information on the epidemiology of most diseases, the sources of inoculum, and on the hidden life of pathogens in soil and other reservoirs. Furthermore, the distribution of most bacteria and viruses is not homogeneous in the plant and even less in the plot, orchard or nursery, and there is an urgent need for studies on sampling methodology and sample processing. The battery of available techniques and probes for detection of plant pathogens has increased considerably over the last few years. In addition to time benefits, there is a great advantage in terms of specificity when using serological techniques with specific monoclonal or recombinant antibodies, or PCR with specific primers, as they allow the detection of plant pathogenic bacteria and viruses even camouflaged by a high number of other microorganisms. This paper reviews some of the relatively recent advances in plant pathogen detection that provide high enough sensitivity and specificity to be used in routine analysis.

Detection methods in the past

Historically, it was necessary to perform time-consuming indexing for virus detection or to cultivate the microorganisms for one or more days at a certain temperature on the appropriate medium in order to identify bacterial colonies using appropriate stains as well as their biochemical and physiological characteristics. This process was obviously not suited to routine analysis of a large number of samples. Unfortunately, the use of new detection techniques in phytopathology has traditionally lagged behind developments in other fields. In the past, it has taken several years from the development of a new technique for clinical diagnosis before it has also been applied to detect phytopathogenic viruses and bacteria. Three decades ago, detection and diagnosis techniques available for bacteria were based on microscopy, isolation, biochemical characterisation, serological techniques (mainly IF), biossays and pathogenicity tests, and for viruses on biological indexing, electrophoresis, electron microscopy and on some serological techniques based on precipitation. Among the major developments that have taken place over the past 25 years it is obvious that the enzyme linked immunosorbent assay (ELISA) [15] is the most significant advance, especially in virus detection. This is not only with respect to sensitivity, which is among the highest in protocols for virus detection, but also to the productivity regarding the number of analyses per year. As an example, the use of ELISA for detection of Citrus tristeza virus (CTV) has allowed more than 3 million tests to be performed in Spain from 1997 to 2003.

During the course of those (nearly) two decades, two Nobel prizes were awarded, to J.F. Köhler and C. Milstein in 1984 and to K. Mullis in 1993, for the development of monoclonal antibodies and for the amplification of nucleic acid sequences by polymerase chain reaction (PCR), respectively. Both prizes reward advancements that have revolutionised the detection of pathogens and represent just the most visible tip of the iceberg in a period of many innovative improvements. Without these techniques, many immunoassays and protocols for molecular testing would simply never have been developed. In addition, the last 25 years have been characterised by constant advances in molecular knowledge, sequencing, nanotechnology and computer sciences [5, 35].

Detection methods today

Currently, the detection of phytopathogenic viruses and bacteria responsible for plant diseases is a changing, dynamic and evolving world where established protocols can be modified or optimised only months after having been developed. In the past, protocols were established and used routinely for many years, which is

not the case today. There is a tendency to use polyphasic approaches to detection including conventional, serological and molecular techniques and to validate them in ring tests. Diagnostic protocols for detection of the 23 EU quarantine viruses, bacteria, fungi, nematodes and insects listed in Table 1 have recently been set up and validated by ring tests in the DIAGPRO project financed by the "Standard, Measurements and Testing" programme of the EU. They are intended to form the basis of improved detection of pathogens in plant material and are available through the web page of the Central Science Laboratory (http://www.csl.gov.uk/science/organ/ph/diagpro/). The European and Mediterranean Plant Protection Organization (EPPO) is also publishing diagnostic protocols for the most important quarantine and non-quarantine organisms (http://www.eppo.org).

Currently, automation and electronic data management are vital to increase the productivity and efficiency of routine analysis for detection of plant pathogenic bacteria and viruses. Furthermore, rigorous quality-control systems have had to be introduced into the laboratory because standards of accreditation and certification are increasing to control not only the outcome of laboratory testing but also the actual process of carrying out the tests.

Several genomes from causal agents of plant diseases, both viral and bacterial, have been completely sequenced and more are underway (wit.integratedgenomics.com/GOLD). Based on their analysis, new specific sequences could be used to design detection probes for different pathogens [65]. The sequences of complete genomes in GenBank are available through NCBI (www.ncbi.nlm.nih.gov/Entrez/), and other databases. Table 2 lists some of the viruses and Table 3 the bacteria responsible for plant diseases that have been or are being sequenced. Note that of the 108 completed bacterial genomes published in public databases, only 4 are of plant pathogenic bacteria, and of the 114 bacterial genomes whose sequencing is in progress, only 6 are phytopathogenic bacteria.

In the following sections a number of important developments and improvements in serological and molecular techniques are described.

Table 1 Quarantine organisms for which a diagnosis protocol has been set up in the DIAGPRO project[a]

Insects and nematodes	*Bemisia tabaci*
	Liriomyza spp. (*L. bryoniae*, *L. trifolii*, *L. huidobrensis*, *L. sativae*)
	Thrips palmi
	Meliodogyne chitwoodii and *M. fallax*
	Globodera rostochiensis and *G. pallida*
Bacteria	*Clavibacter michiganensis*subsp. *sepedonicus*
	Xanthomonas fragariae
	Erwinia amylovora
Fungi	*Tilletia indica*
Viruses and viroids	Potato tuber spindle viroid (PSTVd)
	Tomato spotted wilt virus (TSWV)
	Impatiens necrotic spot virus (INSV)
	Watermelon silver mottle virus (WMSM)
	Tomato mottle virus (ToMV)
	Tomato yellow leaf curl virus (TYLCV)
	Plum pox virus (PPV)
	Citrus tristeza virus (CTV)
	Beet necrotic yellow vein virus (BNYVV)

[a]Protocols available at www.csl.gov.uk/prodserv/know/diagpro

Table 2 Available genome sequences from some plant pathogenic viruses[a]

Organism	Size (bp)	Year
Alfalfa mosaic virus	3,644	1993
Apple chlorotic leaf spot virus	7,555	1993
Apple mosaic virus	3,476	1999
Barley yellow dwarf virus-GAV	5,685	2003
Barley yellow dwarf virus-MAV	5,273	2002
Barley yellow dwarf virus-PAV	5,697	2002
Barley yellow virus	7,637	2002
Bean common mosaic necrosis virus	9,612	1995
Bean common mosaic virus	9,992	2002
Bean dwarf mosaic virus	2,576	1993
Broad bean mottle virus	2,293	1993
Broad bean necrosis virus	5,600	2002
Broad bean wilt virus 2	5,951	2001
Carnation ringspot virus	3,840	1993
Citrus tristeza virus	19,296	2002
Cucumber mosaic virus	2,216	1993
Grapevine fanleaf virus	7,342	1993
Grapevine fleck virus	7,564	2002
Grapevine leafroll-associated virus 3	17,919	2003
Lettuce mosaic virus	10,080	1996
Olive latent virus 1	3,699	1996
Olive latent virus 2	2,438	1994
Pelargonium zonate spot virus	3,383	2000
Plum pox virus	9,741	1993
Potato leafroll virus	5,987	1992
Potato virus A	9,585	2000
Potato virus V	9,848	2000
Potato virus X	6,435	1987
Potato virus Y	9,704	2002
Potato yellow mosaic virus	2,593	1993
Strawberry latent ringspot virus satellite RNA	1,118	1993
Strawberry mottle virus	7,036	2002
Strawberry vein banding virus	7,876	1996
Tobacco bushy top virus	4,152	2002
Tobacco curly shoot virus	2,743	2002
Tobacco etch virus	9,494	1993
Tomato bushy stunt virus	4,776	1993
Tomato leaf curl virus	2,766	2002
Tomato mottle virus	2,601	1993
Tomato ringspot virus	7,273	2002
Tomato spotted wilt virus	4,821	1993
Tomato yellow leaf curl virus	2,781	2002
Watermelon spotted wilt virus	8,917	2001
Wheat streak mosaic virus	9,384	1998

[a]From GNN Genomes (www.genomenewsnetwork.org), TIGR's Comprehensive Microbial Resource (www.tigr.org), NCBI Entrez Genome (www.ncbi.nlm.nih.gov/Entrez/), Gold Genomes OnLine Database (wit.integratedgenomics.com/GOLD/)

Serological detection techniques

Monoclonal and recombinant antibodies

The specificity of detection of viruses and bacteria by well known serological techniques such as IF and ELI-

Table 3 Available genome sequences from some plant pathogenic bacteria[a]

Organism	Size (Mb)	Year of discovery
Agrobacterium tumefaciens	5.6	2002
Clavibacter michiganensis subsp. *sepedonicus*		2003[b]
Xanthomonas campestris pv. *campestris*	5.1	2002
Ralstonia solanacearum	5.8	2002
Erwinia chrysanthemi	~3.7	2003[b]
Pseudomonas syringae pv.*Tomato*	~6.0	2003[b]
*Xanthomonas axonopodis*pv. *citri*	5.2	2002
Xylella fastidiosa (*Citrus variegated chlorosis* strain)	2.7	2000
Xylella fastidiosa (Pierce's disease strain)	2.7	2003
Xylella fastidiosa (strain*dixon*)	~2.6	2003[b]

[a]Sources as in Table 2
[b]Annotation in progress

SA has improved greatly with the use of specific monoclonal and recombinant antibodies. Both allow the selection of specific target epitopes to avoid false positives. Specificity problems are frequent when analysing bacteria in plant material, soil, water, etc., due to the large quantities of other microorganisms present in the sample and to the difficulties in obtaining polyclonal antibodies specific for the target pathogen without cross-reaction with other microorganisms. Commercial monoclonal antibodies for detection of plant pathogenic viruses are now available from several companies. Among them, Agdia (Elkhart, Ind.), Adgen (Ayr, UK) and Agritest (Valenzano, Italy), have available specific monoclonal antibodies. Some Spanish companies, including Ingenasa (Madrid), Durviz (Valencia), and Plant-Print Diagnostics (Valencia), commercialise monoclonal antibodies produced in Spain and kits based on serological methods using specific antibodies. Several companies have developed a wide range of kits for rapid and/or in situ bacterial detection using polyclonal or monoclonal antibodies, based on slide agglutination, IF, and ELISA. The sensitivity of those based on IF allows detection of latent bacterial infections. The kits based on agglutination or ELISA are appropriate only for diagnosing plants already showing symptoms.

Although the use of recombinant antibodies for diagnosis is only at the research level it has a promising future. Single chain Fv (scFv) technology [10] allows the cloning of variable (V) antibody genes, linking them to a flexible peptide as a single chain Fv. These constructs are of great interest in plant pathology because they can be expressed in bacteria [53] as soluble proteins, fused with the capsid proteins of filamentous phages [70], and expressed in plants [18]. In addition, antibody genes can be expressed fused with other proteins such as alkaline phosphatase or amphipathic helices [28, 29]. The expression of these antigen-binding proteins in bacterial cultures provides standardised diagnostic reagents that are theoretically able to replace conventional monoclonal or polyclonal antibodies and conjugates, providing significant advantages in time and cost. However, their applications for diagnostic purposes are still scarce. In plant pathology, serological detection has been widely used, and some recombinant constructs have been produced [62] but only a few of them have been applied to routine ELISA tests.

Enrichment-ELISA protocols

Combining ELISA, which uses specific monoclonal antibodies, with a prior enrichment step greatly improves the sensitivity of detection of bacterial pathogens [20, 34]. This methodology has been developed because of the low sensitivity of ELISA for bacterial detection using specific monoclonal antibodies (maximally 10^5–10^6 cfu/ml) and the need to improve this sensitivity, especially to detect latent infections of quarantine bacteria [12, 21]. The use of an optimised enrichment for each plant pathogenic bacterium allows its specific multiplication in the sample before detection. Specific protocols are needed for each target that take into account their respective requirements. The medium, temperature, duration and incubation conditions of the enrichment are crucial for optimising it and improve the detection sensitivity [20, 33].

On-site-testing: tissue print-ELISA and lateral flow devices

Simple commercial methods for rapid detection are required for testing large numbers of samples by non-experienced technicians. For this purpose, tissue print-ELISA and lateral flow devices have been designed for several plant viruses and bacteria. Although detection specificity is very high when using the appropriate monoclonal antibodies, the sensitivity of these methods, although good for virus detection, is relatively low for bacteria, and they are more appropriate for analysing plants with symptoms. In some cases tissue print-ELISA has demonstrated potential in, for example, CTV detection in plant tissues imprinted on nitrocellulose membranes (without the need for extract preparation). This method, using monoclonal or recombinant antibodies, is the most reliable and inexpensive when compared with molecular and other serological techniques [11] and it is officially recommended in EU protocols for CTV detection. Lateral flow devices kits are based on existing technology similar to a pregnancy test kit. They use specific antibodies and the tests give results in a couple of minutes. but such kits are only available for a few viruses and bacteria.

Flow cytometry

Flow cytometry is a technique for rapid identification of cells or other particles as they pass individually through a

sensor in a liquid stream. Bacterial cells are identified by fluorescent dyes conjugated to specific antibodies and detected electronically using a fluorescence-activated cell sorter, which measures several cellular parameters based on light scatter and fluorescence. Multiparameter analysis includes cell sizing, fluorescence imaging, and gating out, or elimination of unwanted background associated with dead cells and debris [1, 17]. Flow cytometry has excellent potential as a research tool and possibly for routine use in seed health testing and other fields. Several parameters can be analysed simultaneously, including total particle count, distinction between living and dead cells, and differentiation of target and non-target bacterial populations associated with seeds or other plant material. Optimal conditions for antibody conjugation to fluorochromes are determined, and the assay sensitivity is comparable to current methods. Direct screening of plant extracts by flow cytometry would permit early release of negative lots without waiting for colony development on culture plates. This technique has also been adapted to the analysis of viability, metabolic state and antigenic markers of bacteria [17]. Fluorescent markers based on membrane integrity can be selected to assess the viability of cells by staining dead and live cells in different colours. This procedure can be combined with specific antibody staining using antibodies labelled with R-phycoerytrine. The cost for instrumentation is currently a major disadvantage that will be resolved when less expensive models become available.

Molecular detection techniques

Molecular techniques based on hybridisation or amplification, and especially on PCR, have been developed for the most important plant pathogenic viruses and bacteria. Although PCR can reach high sensitivity and specificity, its introduction for routine detection has been hampered by a lack of robustness [63]. The failure of PCR amplification to correctly diagnose infected and non-infected plant material has been reported in different comparative assays. Carry-over contamination of amplicons can be responsible for false-positive results and inhibitor components in sample extracts is the main reason for false negatives [63]. PCR, in different formats, is the most widely used molecular technique for detection of bacteria and viruses. Less employed techniques based on hybridisation are of interest for specific requirements. Their main advantages are specificity and rapidity. Specificity is directly related both to the design of the primers or probes and to the amplification or hybridisation protocols.

For PCR, the sequences of most published primers for phytobacteria have been compiled by various authors [35, 59], but these lists do not include those most recently published. The time required until the final result is usually less than 24 h whereas that required for conventional microbiological detection of bacteria was of the order of several days in the case of a negative result and 5–10 days in the case of a positive result due to the tests necessary to confirm it. Furthermore, the possibility of designing a multiplex PCR saves time and reagent costs compared with monospecific PCR, which requires several reactions for the same number of tests [5, 61]. Colorimetric detection of PCR products, on membranes or in microtitre plates, has been employed successfully, increasing sensitivity and facilitating interpretation of results for the use of the technique in routine analyses [6, 14, 37, 46]. Although amplicon-hybridisation requires more time and manipulation of samples than gel electrophoresis using ethidium bromide staining [30], its great advantages favour its application for indexing programs. In addition, the procedure avoids the use of the toxic ethidium bromide, and it is possible to immobilise hundreds of samples in a single membrane. The design of other internal probes would allow subtyping of isolates where necessary.

Fluorescence in situ hybridisation

Fluorescence in situ hybridisation (FISH) is a technique applied for bacterial detection that combines the simplicity of microscopy observation and the specificity of hybridisation [66]. Its use in detection of plant pathogenic bacteria is recent [71] and is dependent on the hybridisation of DNA probes to species-specific regions of bacterial ribosomes. They are particularly suitable as diagnostic targets because ribosomal RNA contains functional sequences that are common to all species but also sequences that are very specific to individual species, and FISH only needs to recognise this specific information. The probes hybridise with a three-dimensional protein/RNA structure not only with a linear sequence of RNA. The sensitivity of the FISH technique is equivalent to that of amplification technologies and, in theory, FISH can detect single cells. This high sensitivity is the result of the high affinity and selectivity of DNA probes because FISH takes place under very stringent hybridisation conditions, where a difference of one nucleotide in a 15–20 oligonucleotide probe is sufficient to discriminate binding. Furthermore, FISH maintains the structural integrity of the microorganism, confining the reagents in one small vessel and one probe will bind to each of the $1–5\times10^4$ ribosomes inside. This extremely high signal is responsible for the theoretical ability of the technique to achieve single-cell sensitivity. In practice, however, the detection level is 10^3 cells/ml.

Polymerase chain reaction

Detection of bacteria or viruses in a given sample by PCR is not only dependent on the performance on the PCR assay itself, but also on the efficiency of the procedure employed to extract the nucleic acids from the plant material. The sensitivity of detection can be reduced by inhibitors that may be present in the extract of

nucleic acids. To check for substances that may interfere with the amplification process, internal controls can be designed for each pair of primers [16], or real-time PCR can be employed. Another possibility to decrease the negative effects of inhibitors is the use of enrichment-PCR, also called bio-PCR, for several plant pathogenic bacteria. The enrichment step can be performed in solid or liquid medium, and even in planta [33, 34, 52, 58] and has to be optimised for each pathogen and amplification protocol. Liquid enrichment also allows the detection of *R. solanacearum* in a viable but non culturable state (VBNC) from water samples at low temperature [9].

PCR efficiency is controlled by many parameters, such as polymerase type, buffer composition and stability, purity and concentration of dNTPs, cycling parameters as well as the characteristics of the starting template. In addition, the quality of the DNA to be amplified is critical. The very long, complicated and time consuming protocols developed for DNA extraction in the 1990s have often been replaced by rapid, simple DNA extraction protocols [31] or by commercially available DNA-extraction kits. Among them, the RNeasy and DNeasy Plant System (Qiagen, Hilden, Germany) and the Easy-DNA-Extraction kit (Invitrogen, Carlsbad, California, USA) have been used successfully for different types of plant material. Several expensive commercial integrated systems allow for the automated extraction and analysis of nucleic acids from microorganisms, but they are not efficient with all types of plant material and need to be evaluated before they can be adopted for routine detection.

Due to its high sensitivity, reverse transcription coupled to PCR in a single step (RT-PCR) is the molecular method most frequently used for the detection of plant viruses. Different RT-PCR variants have been developed, including immunocapture RT-PCR, which has been used with plant extracts [43, 68] or with immobilised targets on paper print/squash-capture (PC/SC) RT-PCR [45, 46] allowing the detection of minimal quantities of RNA targets from plant material or insect vectors without extract preparation. PCR has also been frequently utilised for the detection of bacterial plant pathogens [23, 35]; for the detection of most plant pathogenic bacteria, specific primers have been designed based on either the amplification of specific genes from the chromosome or plasmids, or on different approaches such as sequences selected from RAPD differential bands obtained by subtractive hybridisation [35].

When the sensitivity of detection is not good enough, a nested PCR can be helpful, but it requires two rounds of amplification in different tubes, resulting in a high contamination risk [54]. Several interesting alternatives with single closed tubes have been developed in order to avoid this problem [7, 32, 50, 72]. The development of nested-PCR in a single closed tube using a single compartmentalised Eppendorf tube with a pipette tip is another interesting alternative [47, 49]. This method, however, requires the use of 0.5 ml Eppendorf tubes and cannot be employed in rapid reaction capillary tubes.

Co-operational PCR

A new PCR concept of high sensitivity for the amplification of viral RNA or bacterial targets from plant material has recently been described [48]. The method has been patented as Co-PCR (Spanish patent P20002613; 31 October 2000). The Co-PCR (co-operational amplification) technique can be performed easily in a simple reaction based on the simultaneous action of four or three primers. The reaction process consists of the simultaneous reverse transcription of two different fragments from the same target, one internal to the other, the production of four amplicons by the combination of the two pairs of primers, one pair external to the other, and the cooperational action of amplicons for the production of the largest fragment (Fig. 1).

The Co-PCR technique has been used successfully, both in metal block and capillary air thermal cyclers, for the detection of plant RNA viruses [*Cherry leaf roll virus* (CLRV), strawberry latent ringspot virus (SLRSV), *Cucumber mosaic virus* (CMV), *Plum pox virus* (PPV) and *Citrus tristeza virus* CTV]. Coupled with colorimetric detection, the sensitivity observed is at least 100 times higher than that achieved with RT-PCR and is similar to that of nested RT-PCR. Co-PCR usually produces the largest amplicon, in contrast to nested-PCR, which requires two sequential reactions and obtains the smallest fragment. Metal block and capillary air thermal cyclers have been employed for the detection of some plant RNA viruses from different genera, and to a bacterium, but by using only three primers [13], which shows the possibilities of this new approach. The low amount of reagents (ten times less than in conventional PCR) probably increases susceptibility to inhibitors, requiring prior RNA extraction for sensitive virus detection. However, this step was not necessary when analysing the presence of *R. solanacearum* in water [13].

Multiplex PCR

Multiplex PCR allows the simultaneous and sensitive detection of different DNA or RNA targets in a single reaction [25, 39, 51, 55, 69]. On the other hand, PCR detection protocols can be designed to verify the presence of more than one pathogen in plant material by looking for common specific sequences in two or more of them, or to detect related viruses or bacteria on multiple hosts [35]. Multiplex PCR is useful in plant pathology because different bacteria and/or RNA viruses frequently infect a single host and consequently sensitive detection is needed for the propagation of pathogen-free plant material. There are several examples in plant pathology of simultaneous detection of several targets and the amplification by multiplex PCR of two or three plant viruses has been reported [22, 24, 38, 40, 56, 57, 60, 61]. Nevertheless, there are still only a few examples in which more than three plant viruses were amplified in a single PCR-based assay [4, 39, 41, 44],

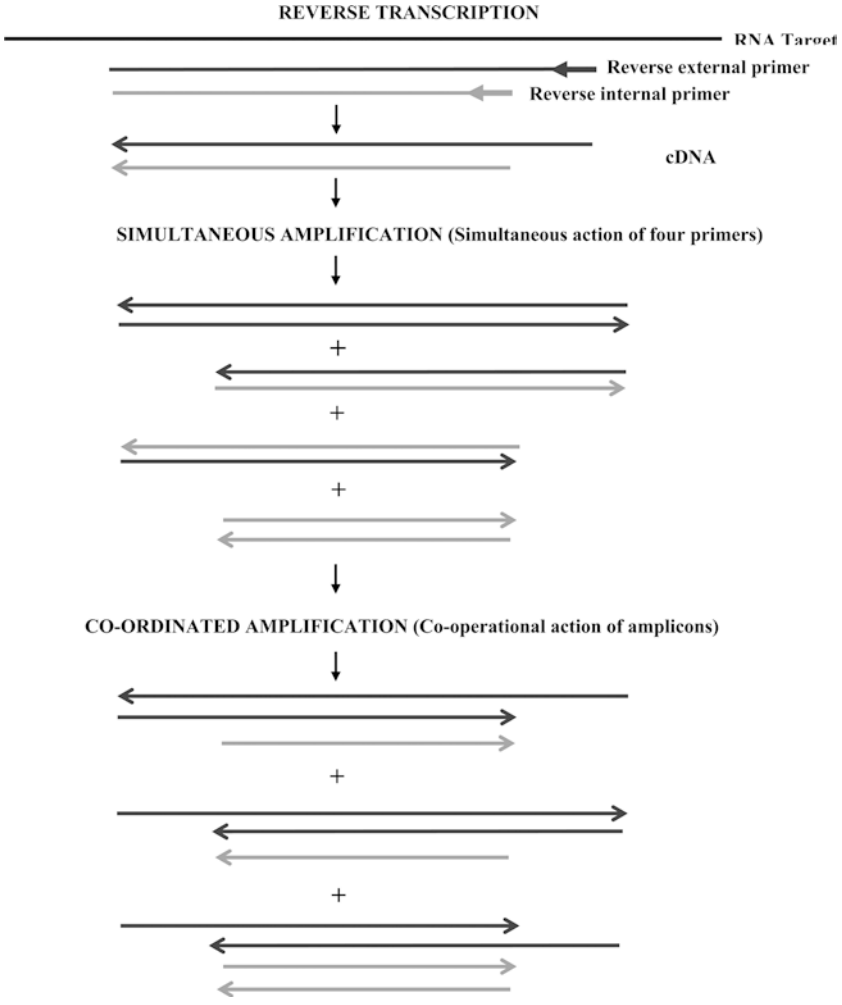

Fig. 1 Scheme of the Co-operational amplification (Co-PCR) procedure [48]

probably due to the technical difficulties of designing a reaction involving many compatible primers. One of them is the simultaneous detection of the six major characterised viruses described in olive trees, which belong to four different genera: *Cucumovirus* (CMV), *Nepovirus* [CLRV, SLRSV and Arabis mosaic virus (ArMV)], Necrovirus [*Olive latent virus*-1 (OLV-1)] and Oleavirus (Olive latent virus-2) [6]. This includes accurate design of six primer pairs for one-step RT-PCR amplification in a single closed tube and specific probes, enabling the detection of all major viruses described in olive trees, which are problematic for RNA extraction.

Multiplex nested RT-PCR

A multiplex nested RT-PCR in a single closed tube has been developed for simultaneous and sensitive detection of the viruses CMV, CLRV, SLRSV, and ArMV, and the bacterium *Pseudomonas savastanoi* pv. *savastanoi* from olive plants [7] using 20 compatible primers in a compartmentalised tube. This newly developed method combines the advantages of multiplex RT-PCR with the sensitivity and reliability of nested RT-PCR carried out in a single closed tube. It enables the simultaneous detection of several viral RNA and bacterial DNA targets in a single analysis, performed with woody plants. It also saves time and reagent costs because it can be performed in a single reaction, although accurate design of compatible primers is needed. The compartmentalisation of a single Eppendorf tube with a pipette tip [47, 49] allowed multiplex-PCR and nested PCR to be combined effectively. During the first amplification reaction there is no interference of the external with internal primers because they are physically separated from the initial reaction cocktail. Once the multiplex RT-PCR ends, the internal primers are mixed with the products of the first reaction before proceeding to the nested multiplex. Because the concentration of internal primers is very high compared with that of the external primers (which will also have been consumed by the first amplification), the nested multiplex can be performed with minimal interference. Consequently, sensitivity is increased at least 100-fold over that of multiplex RT-PCR for the detection of viruses. Furthermore, the sensitivity achieved by multiplex nested RT-PCR for the bacterium *P. savastanoi* pv. *savastanoi* is similar to that reached by applying monospecific nested PCR after an enrichment step, which was demonstrated to be 100-fold

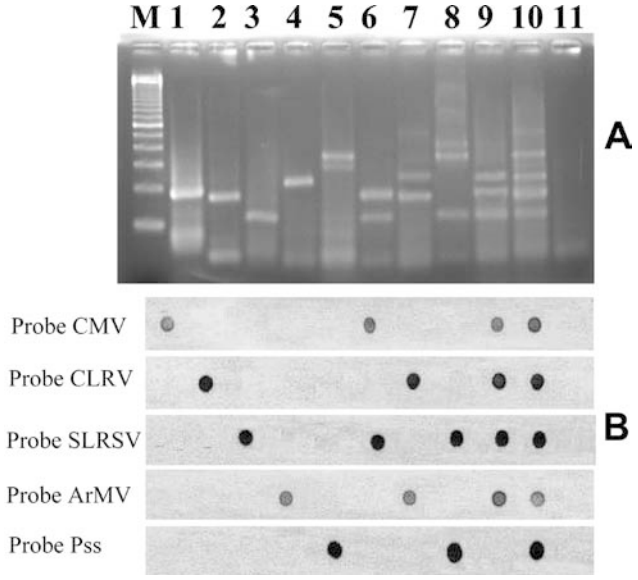

Fig. 2A, B Detection of four olive viruses and *Pseudomonas savastanoi* pv. *savastanoi* by multiplex nested RT-PCR. **A** Multiplex nested RT-PCR products separated on a 3% agarose gel, stained with ethidium bromide and visualised under UV light. **B** Colorimetric detection using 3′ digoxigenin (DIG)-labelled probes. Lanes: *M* 100 bp molecular marker (Gibco BRL), 1 *Cucumber mosaic virus* (CMV) (172 bp), 2 *Cherry leaf roll virus* (CLRV) (171 bp), 3 strawberry latent ringspot virus (SLRSV) (109 bp), 4 *Arabis mosaic virus* (ArMV) (203 bp), 5 *P. savastanoi* pv. *savastanoi* (338 bp), 6–10 mixtures of CMV + SLRSV (*6*), CLRV + ArMV (*7*), SLRSV + *P. savastanoi* pv. *savastanoi* (*8*), CMV + CLRV + SLRSV + ArMV (*9*), CMV + CLRV + SLRSV + ArMV + *P. savastanoi* pv. *savastanoi* (*10*), *11* PCR control. Data from [8]

more sensitive than conventional PCR. This multiplex nested RT-PCR has been coupled with colorimetric detection, allowing the discrimination of amplicons of similar size, which would require additional monospecific analysis if gel visualisation only was employed (Fig. 2).

Real-time or quantitative PCR

The exponential nature of PCR in theory allows the amount of starting material to be calculated from the amount of product at any point in the reaction. In practice, however, reaction conditions can interfere with exponential amplification and affect product concentration. Standard PCR works best, therefore, as a qualitative technique. Early attempts at quantitation involved stopping the PCR reaction at various points to generate standard curves, which resulted in a laborious, low-throughput process. Real-time reaction monitoring with specific instruments and fluorescent probes combines amplification, detection and quantitation in a single step and has been applied to the detection of several pathogens [67]. TaqMan probes, developed by Applied Biosystems (Foster City, California, USA), consist of single-stranded oligonucleotides that are complementary to one of the target strands. A fluorescent dye adorns the 5′ end and a quencher is bound to the 3′ end. As an alternative approach, Idaho Technology (Salt Lake City, Utah) developed hybridisation probes. Other probes are based on stem-loop hairpin structures, molecular beacons being the simplest hairpin probes. Scorpion probes covalently couple the stem-loop structure to a PCR primer and, because hybridisation of probe sequence to amplicon is intramolecular, Scorpion probes are more efficient than binary systems such as molecular beacons. The Amplifluor Universal Detection System, developed by Serologicals Corporation (Norcross, Ga.), also uses the paired fluorophore-quencher hairpin structure. Invitrogen recently developed a new class of real-time probes called LUX (light upon extension) fluorogenic primers. Like hairpin probes, LUX primers adopt a stem-loop structure in solution and, like Scorpion probes, are intended for use as PCR primers. A recent comparison of TaqMan and molecular beacon chemistries found them to be more or less equivalent in sensitivity, accuracy, and reproducibility [19]. The fluorescent probes are expensive, but that price pales in comparison to the thermal cycler itself. These instruments offer a variety of options and support a range of chemistries, but they are all expensive, and not every laboratory can afford to upgrade to real-time quantitative capabilities.

A less expensive alternative are the portable rapid cycling real-time PCR platforms such as the RAPID (Idaho Technologies) and Smart Cycler (Cepheid, Charleston, USA) that can be used for rapid on-site diagnosis. However, only few data are available to compare the sensitivity level reached by their protocols with other currently available molecular protocols.

DNA microarrays

DNA microarrays or biochips are made of a surface on which are linked multiple capture probes, each one being specific for a DNA or RNA sequence of the targets. Their purpose is the detection of numerous sequences in a single assay. Various supports are currently in use for the elaboration of microarrays, including glass, nylon and different polymers. Up to 30,000 DNA probes (gene sequences) can be arrayed onto a single chip. The probes arrayed can be PCR products amplified to high concentrations or relatively short (30–50 bp) oligonucleotide probes. Once arrayed, the chip can be exposed to fluorescently labelled DNA/RNA from the sample to be tested. The detection system uses one or several fluorophores, that can be read with laser technology to reveal the targets present in the sample. Extraction of nucleic acids from the sample, labelling and hybridisation can be achieved with standard laboratory facilities. Another possibility is to use the nanochip technology developed by Nanogen (San Diego,USA), based on the combination of microelectronics with microarray technology in a solid support covered with streptavidin to increase the power of union with biotin-labelled DNA. The theo-

retically possible detection of multiple targets is problematic in practice, especially when trying to simultaneously detect bacteria and viruses. Until now, the protocols used in the different laboratories involved in developing microarrays for detection of phytopathogens require a prior step of PCR amplification and reach low levels of sensitivity. Consequently, their use for routine detection is still far from being common, in contrast with their widespread use for functional genomics studies. Microarrays can generate fast results for several pathogens but their cost is still very high. They also generate significant amounts of data requiring expert interpretation. It is likely that microarrays will follow a path similar to that of PCR, which spent several years as a research tool before being used in diagnostics.

Concluding remarks

The panel of techniques for rapid, specific, and sensitive detection of plant pathogenic viruses and bacteria has improved and increased in the last few years. To reach higher sensitivity in serological or molecular detection, two complementary strategies can be applied: amplification of the target and/or amplification of the signal. The pathogen or target amplification or enrichment can be carried out in vivo (in planta) or in vitro, preferentially with selective media and at the optimum growing conditions for each pathogen, or by molecular techniques including PCR. Signal amplification can be obtained by chemical, molecular or electronic methods. Among molecular techniques, multiplex PCR is increasingly used because it improves the efficiency of diagnostic PCR [27]. In the near future multiplex PCR will probably be adapted for the simultaneous detection of viruses and bacteria of one particular crop and for the simultaneous detection of other major plant pathogens such as viruses, viroids, bacteria, and fungi in the same reaction, as already demonstrated for viruses and viroids [42] or viruses and bacteria [7].

The future will bring more novel tests for detecting plant pathogens and novel demands will be introduced. However, only some of these will be accepted by phytopathologists, with not only the analytical quality of test results but also their sensitivity and specificity contributing to selection of the best cost-effective diagnostic strategies. The objective is real-time analysis or immediate detection of microorganisms in their natural environment without culturing or amplification. Qualitative and quantitative detection data generated with new technologies should provide a more complete picture of the life cycle of plant pathogens. Consequently, more appropriate sampling methodologies will be set up for efficient detection of latent infections and pathogen reservoirs. The sequencing of more genomes of plant pathogenic bacteria and viruses will ensure advances in genomic research. Thereafter, the era of proteomics will be gradually translated into new developments. Overall, there will be a major shift towards innovative methods based on genetic profiles of the different pathogens, probably including more automation and a greater variety of instruments.

New detection tools will be used not only for rapid, sensitive and specific diagnosis but also to help to understand plant-pathogen relationships and the structure and function of pathogens and their communities. They will yield exciting results but will also demand large commitments of capital and expert human resources in the principle and practice of the different approaches. The risk is that laboratories trying to exploit innovative diagnostic methods become so caught up in the tools themselves that they lose sight of the enormous possibilities of their application. Only when new technologies become fully integrated with other conventional tools, which they should complement, not substitute, will they provide useful information in the understanding and prevention of plant diseases.

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