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Major review

Advances in molecular phytodiagnostics – new solutions for old problems

Rick Mumford*, Neil Boonham, Jenny Tomlinson, and Ian Barker
*Central Science Laboratory, Sand Hutton, York, YO41 1LZ, UK *Author for Correspondence (Phone: +44 1904462140; Fax: +44 1904462111; E-mail: r.mumford@csl.gov.uk)*

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Abstract

In the last decade, developments in molecular (nucleic acid-based) diagnostic methods have made significant improvements in the detection of plant pathogens. By using methods such as the polymerase chain reaction (PCR), the range of targets that can now be reliably diagnosed has grown to the extent that there are now extremely few, known pathogens that cannot be identified accurately by using laboratory-based diagnostics. However, while the detection of pathogens in individual, infected samples is becoming simpler, there are still many scenarios that present a major challenge to diagnosticians and plant pathologists. Amongst these are the detection of pathogens in soil or viruses in their vectors, high throughput testing and the development of generic methods, that allow samples to be simultaneously screened for large numbers of pathogens. Another major challenge is to develop robust technologies that avoid the reliance on well-equipped central laboratories and making reliable diagnostics available to pathologists in the field or in less-developed countries. In recent years, much of the research carried out on phytodiagnostics has focussed in these areas and as a result many novel, routine diagnostic tests are becoming available. This has been possible due to the introduction of new molecular technologies such real-time PCR and microarrays. These advances have been complemented by the development of new nucleic acid extraction methods, increased automation, reliable internal controls, assay multiplexing and generic amplification methods. With developments in new hardware, field-portable real-time PCR is now also a reality and offers the prospect of ultra-rapid, on-site molecular diagnostics for the first time. In this paper, the development and implementation of new diagnostic methods based upon novel molecular techniques is presented, with specific examples given to demonstrate how these new methods can be used to overcome some long-standing problems.

Historical perspective

The use of molecular (nucleic acid-based) diagnostic methods for the detection of plant pathogens is in itself not new. Plant pathologists have been using such methods since the late 1970s, when the first double-stranded RNA and dot-blot hybridisation protocols were developed for the detection of viruses and viroids (Dodds et al., 1984; Hull, 1986). Despite this, molecular methods failed to make much impact in routine diagnostic laboratories during the 1980s. It was not until the development of the polymerase chain reaction (PCR) (Saiki et al., 1985, 1988) that molecular diagnostics began to develop real momentum. By 1989 the first paper describing the use of PCR for the detection of plant pathogens had appeared (Puchta and Sanger, 1989) and the number of published methods continued to grow rapidly year-after-year (Henson and French, 1993). For

example, the first plant virus PCR paper was published in 1990; by 1994 the total had reached over 40. Yet despite the amount of research being carried out in this area, the overall uptake of PCR-based methods for routine use in diagnostic laboratories was slow. Indeed, by the mid-1990s only the largest diagnostic laboratories had adopted such methods, and even then applications were often limited to niche areas such as the detection of viroids or phytoplasmas. The reasons for the slow uptake were many and varied, but were predominantly related to the practicality of performing molecular tests, especially PCR. Specific problems included cross-contamination, interpretation of results, extraction reliability, and the high labour-requirements of testing large numbers of samples. At this time it became clear that if molecular diagnostics were to become more widely adopted, then much more effort was required to address the practical issues related to performing molecular assays, rather than focussing almost exclusively on the specificity and sensitivity of such tests, as had previously occurred. The main breakthrough in achieving this aim of developing practical, routine methods came at the end of the 1990s with the development of real-time amplification technologies.

The real-time revolution

Beyond PCR: TaqMan®

Throughout much of the 1990s, researchers had grappled with ways to pull PCR out of research laboratories and into routine use. Much of the work focussed on investigating alternatives to gel electrophoresis for post-PCR analysis. One approach, sometimes termed 'PCR-ELISA', was to detect PCR products colorimetrically using enzyme-linked antibodies designed to bind to antigen (such as DIG-labelled nucleotides) incorporated into the products during amplification (Hartung et al., 1996; Rowhani et al., 1998; Weekes et al., 1996). A similar alternative system was DIAPOPS (reverse transcription detection of immobilized, amplified product in a one-phase system; Nicolaisen et al., 2001). While these approaches were successful in terms of offering comparable levels of sensitivity and reliability to gel-based detection, they failed to provide significant practical advantages, simply replacing one multistage detection system (i.e. gel electrophoresis) with another (i.e. plate-based immuno-detection). As a result, this technology failed to make much if any impact on the routine detection of plant pathogens.

The real breakthrough in reliable, simple amplicon detection came in the early 1990s with the development of TaqMan® chemistry by Applied Biosystems (Foster City, CA, USA) (Holland et al., 1991). This system combines an oligonucleotide probe labelled at opposite ends with a reporter and a quencher dye respectively, which is designed to anneal to a sequence internal to the PCR primers, with the 5′ exonuclease activity of Taq polymerase (Figure 1). While the probe is intact, fluorescence emitted by the reporter is absorbed by the quencher (fluorescent resonance energy transfer or FRET). However, during amplification the probe is cleaved by the

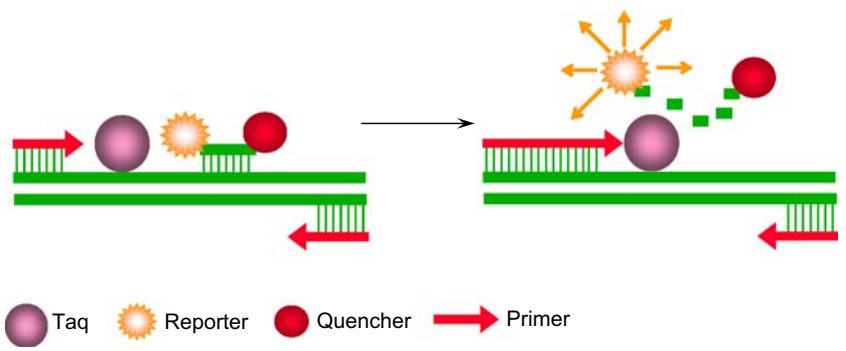

Figure 1. TaqMan chemistry. Step 1: Intact probe anneals to a target sequence internal to the PCR primers; fluorescence emitted by the reporter is absorbed by the quencher. Step 2: during amplification, the probe is cleaved by the 5′-3′ nuclease activity of Taq, separating the dyes resulting in an increase in fluorescence.

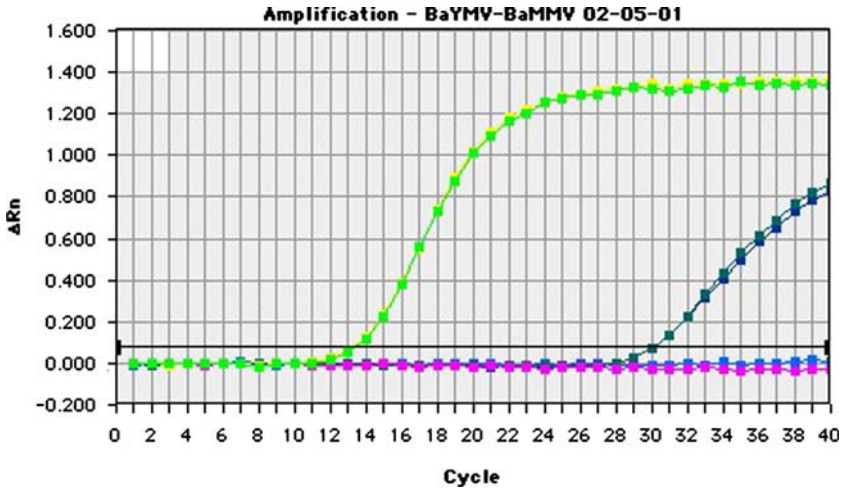

Figure 2. TaqMan results. Fluorescence is plotted vs cycle number: the number of cycles needed to generate a fluorescent signal above a defined threshold (C_t value) is inversely related to the log of the number of target molecules in a sample.

nuclease activity of Taq, separating the dyes and resulting in an increase in fluorescence, which is related to the amount of product amplified. The increase in reporter fluorescence is monitored in real time during amplification using a combined thermal cycler-fluorescence reader system (Figure 2). Ultimately, this means that no post PCR manipulations are required; in particular, the need for gel electrophoresis is removed.

The first published examples of the use of TaqMan chemistry for the detection of plant pathogens were for the detection of potato viruses, initially as a two-step, heterogeneous assay (amplification followed by fluorescent end-point detection; Schoen et al., 1996). Papers were subsequently published using genuine real-time, homogeneous assays, with simultaneous amplification and detection, for the detection of the main plant pathogens including fungi (Bohm et al., 1999; Zhang et al., 1999), bacteria (Schaad et al., 1999; Weller et al., 2000), viruses (Mumford et al., 2000; Eun et al., 2000), viroids (Boonham et al., 2004), and phytoplasmas (Bianco et al., 2004). The technology has also been developed for the specific identification of virus vectors (Walsh et al., 2005). With these and other TaqMan-based assays that have been developed, there are a range of advantages over existing methods. In most cases, the increased sensitivity of TaqMan has been a significant factor; the end-point sensitivity of TaqMan assays is generally greater than conventional PCR and on a par with nested PCR (Mumford et al., 2004a). A good example of where this increased sensitivity has proved invaluable is testing for viruses in insect vectors (Boonham et al., 2002; Fabre et al., 2003; Olmos et al., 2005). It also allows certain testing procedures to be accelerated by providing a reliable, rapid alternative to traditional biological-based techniques. An excellent example of this is seed potato virus indexing. This has traditionally relied upon the growing-on of eye plugs taken from dormant tubers and the subsequent testing of sprouts by serological methods e.g. ELISA. While this method is the standard approach used worldwide, it takes 6–8 weeks to complete; however, by using a testing method based on TaqMan there is sufficient sensitivity to detect the low titres of virus found in dormant tubers, thus circumventing the need for bio-amplification (growing-on) (Barker et al., 2005). In this way testing can be reduced from weeks to days, and in some cases less than 24 h. Another good example is the use of TaqMan for the direct detection of pathogens in soil (Cullen et al., 2001, 2002; Ward et al., 2004), avoiding longer, biological-based assays such as bait testing, which often take several weeks.

Another area where TaqMan-based assays have proved extremely useful is providing a reliable testing alternative where more traditional methods have failed. Examples include the detection of pathogens that lack proteins, e.g. viroids or NM-type strains of *Tobacco rattle virus* (Boonham et al., 2004; Mumford et al., 2000), or where

antisera of sufficient quality (e.g. the barley mosaic viruses; Mumford et al., 2004b) or specificity (e.g. *Beet necrotic yellow vein virus* P-type; Harju et al., 2005) is unavailable.

The final key area where real-time PCR offers a significant advantage over conventional PCR is that it can be used for quantification. By using real-time procedures it is possible to not only detect the presence of a target pathogen but also accurately quantify the amount present in plants (Bohm et al., 1999; Winton et al., 2003). Further examples of real-time PCR applications can be found in the reviews of Schaad and Frederick (2002), Schaad et al. (2003) and Schena et al. (2004).

Other real-time PCR systems

While TaqMan is by far the most widely used system, other real-time PCR methods for the detection of plant pathogens have also been published. Some utilise different amplification systems such as the Ligase Chain Reaction (LCR; Wilson et al., 1994; O'Donnell et al., 1996) or are based on Nucleic Acid Sequence Based Assay (NASBA; Bentsink et al., 2002; Klerks et al., 2001; Leone et al., 1997). In addition there are a range of alternative real-time detection chemistries which are now available (these have been comprehensively reviewed by Wong and Medrano, 2005). Several of these have been employed for the detection of plant pathogens, including methods based on DNA-intercalating dyes such as SYBR Green I (Bates et al., 2001; Fraaije et al., 2001; Nicolaisen, 2003) and others using FRET probes, where spatial separation between the reporter and quencher dyes is achieved not by degradation of the probe (as in TaqMan) but through a loss of complex secondary structure due to probe binding e.g. 'Molecular Beacons' (Bonants et al., 2004; Leone et al., 1998; van Beckhoven et al., 2002) and 'Scorpion primers' (Bates and Taylor, 2001; Schena et al., 2002). While all the real-time different methods have their own specific set of advantages and disadvantages, in reality they are often highly comparable in terms of overall performance.

Using real-time PCR for routine diagnostics

While in many situations real-time PCR offers advantages over other diagnostic techniques including conventional PCR, it does also present some challenges for routine diagnosis, particularly in establishing it as a high-throughput method for testing large numbers of samples. Here again the advantage of using a plate-based assay system means that automation can be used to aid real-time PCR set-up. By employing liquid handling robotics, reaction master mixes and extracts can be automatically dispensed into plates, greatly reducing staff input and time. Throughput can be further dramatically increased by the use of 384-well plates instead of standard 96-well plates: a 4-fold increase in capacity per run. In this way it is possible to run thousands of real-time assays in a week: for example, running a single 384-well real-time machine at five plates per day, 5 days per week, with samples in duplicate and controls, gives you the capacity to test around 4500 samples. While this clearly shows that the capacity provided by an automated real-time PCR system is large, it does not, of course, address the real issue facing molecular phytodiagnosticians: sample processing and nucleic acid extraction.

Since the introduction of PCR in the 1980s, one of the biggest challenges faced by diagnosticians has been to produce high quality, amplifiable nucleic acid extracts from plants. In developing new plant extraction procedures much of the focus has been on finding methods that can deal with the inhibitors (e.g. polyphenolic compounds or acidic polysaccharides) that often abound in many species. By inhibiting the enzymes involved in amplification, these compounds can lead to false negative results and hence significantly reduce the overall reliability of these methods. There is a range of methods based on the use of detergents and/or solvents that can remove many inhibitory compounds; in plant work the use of the detergent CTAB (cetyltrimethyl ammonium bromide; Doyle and Doyle, 1987) has proved particularly effective and is widely used. However, these types of procedures require multiple steps and frequently involve the use of hazardous chemicals (e.g. chloroform). As a result, they are not particularly amenable for high-throughput routine use. In virology, alternative methods can be used where virus particles can be trapped using specific-antibodies ('immunocapture'; Nolasco et al., 1993; Wetzel et al., 1992). This is an extremely simple approach, but it is not generic since it requires that a specific antibody has been produced. Other

methods have been developed involving trapping pathogens directly onto the surface of membranes (e.g. 'Print-capture'; Olmos et al., 1996) or onto plastic tubes or plates (Rowhani et al., 1995), but again, while this can work well with certain pathogens e.g. viroids, it is not suitable for all and hence is not a generic solution. With these issues in mind, much work has focussed on the use of formats that can be automated, in particular, systems that rely on the use of spin/vacuum columns or magnetic particles. Many of the wide range of different kits which are commercially available can be processed in a 96-well format, which greatly increases throughput. With the use of automation such as liquid handling robots, or more dedicated systems such as the Kingfisher magnetic particle processor system (Thermo Electron), this capacity can be increased further while offering significant reductions in staff input time.

The other major advance related to extraction is the development of controls designed to monitor the quality of extracts. Even when using methods that have been demonstrated to be reliable (e.g. that can effectively remove inhibitors), it is still very important to demonstrate that an extract of suitable quality has been made from each test sample. This is even more important when working in situations where extensive extraction validation has not been possible e.g. testing a rare or unusual host species and when most test samples are negative. The simplest and most effective way to monitor extraction performance is through the use of extraction control assays either run in multiplex with a diagnostic assay (an 'internal' control) or in parallel (i.e. a separate assay using the same extract). For the majority of cases these control assays are designed to detect endogenous plant genes that are co-extracted from the sample along with any potential target pathogen nucleic acid. Various target genes have been used including cytochrome oxidase 1 (Weller et al., 2000), ribosomal DNA, (Bates et al., 2001) and phenylanaline lyase (Mumford et al., 2004b). As a further refinement, RNA-specific controls have also been designed for working with viruses with RNA genomes. These utilise the intron-processing mechanisms found in eukaryotic organisms (including plants) to ensure that only the spliced mRNA is detected and not unspliced genomic DNA (Boonham et al., 2002). A different approach has been the development of synthetic control templates that contain the same primer binding sites as a target pathogen but have a distinct internal sequence between the primers, and hence are detected by a different fluorescent-labelled probe (Kox et al., 2005). By spiking test reactions with this construct, the performance of the target primer can also be monitored alongside that of the other reaction components. While this approach does offer advantages over the use of endogenous host genes as controls, it does have a negative effect on assay sensitivity and requires considerable effort to construct and synthesize.

With regards to future challenges for extraction technology, the key developments are going to be aimed towards screening methods, in particular targetting growing media (including soil), water and air, allowing detection of pathogens prior to the onset of disease. For example much effort is now being directed towards developing high-capacity, direct soil extraction. Current techniques often work on relatively small soil samples of less than a gram in total. In the context of screening a whole field this is totally unrepresentative and as a result much effort is going into developing methods that can handle samples in the range of hundreds or even thousands of grams. If these methods can be developed, then the opportunity for reliable pre-planting field testing becomes possible for a whole range of different soil-borne pathogens and pests.

In summary, real-time PCR offers obvious advantages over other testing procedures in many situations. As development work continues, solutions are being found to address the major issues of reliability and high-throughput detection capacity. However, at present real-time PCR is still a relatively expensive technology in terms of capital investment and facilities, and as a result it is only really pertinent to large, centralised laboratories. The next challenge is to take real-time PCR out of this environment, and into the field.

Using real-time PCR in the field

There are obvious advantages to on-site plant pathogen testing methods which allow testing either at the point of sampling, thus dramatically reducing the amount of time between taking samples and obtaining results, or in less well-equipped laboratories. Simple antibody-based

technology, originally developed for home pregnancy testing, has successfully been used for a number of years for plant pathogen testing in the field (www.pocketdiagnostic.com; Danks and Barker, 2000). These single-step immuno-chromatographic devices, known as lateral flow devices (LFDs), are designed for field use: they are relatively inexpensive, require no capital equipment and only minimal training to use, and tests can be performed in as little as two minutes. However, they require that suitable pathogen-specific antibodies are available or can be produced. Antibodies that are specific to given species are relatively easy to produce for many plant viruses but are less so for some bacterial plant pathogens or more complex organisms such as fungi. Antibodies raised to fungi may even be life stage-specific and therefore not suitable for detecting, for example, resting spores. In contrast, it is (at least in theory) possible to design real-time PCR assays that are specific to most plant pathogens including at the race or sub-species level, as has already been described.

The concept of using real-time PCR platforms for the detection of micro-organisms in the field is already a reality, with typical applications for these devices, including the identification of anthrax spores in postal services and monitoring for hospital-acquired infections in patients. Commercial companies have invested heavily in the development of robust, portable real-time machines, to satisfy both the growing demand in counter-terrorism and 'point of care' (POC) clinical applications. Following work in the late 1990s, prototype portable real-time PCR instruments such as the MATCI (Miniature Analytical Thermal Cycling Instrument; Belgrader et al., 1998b) and the ANAA (Advanced Nucleic Acid Analyzer; Belgrader et al., 1998a) were developed, which involved significant increases in the speed of thermal cycling. Reduced size, weight and increased durability (for example, the absence of moving parts) improve the suitability of such platforms for field use and simple, intuitive operating software increases accessibility to non-specialists and first-responders. The development and early deployment of such instruments has mostly been in the areas of clinical and veterinary pathogen detection and biosecurity, although recently a number of plant health applications have been described. The SmartCycler (Cepheid, Sunnyvale, CA, USA; www.cepheid.com) is a portable real-time PCR platform that allows up to 16 samples to be tested simultaneously, each with independently controlled thermal cycling and fluorescence monitoring. The SmartCycler has been widely used for clinical and veterinary applications (e.g. Belanger et al., 2002; Hearps et al., 2002), and has also been used for the detection of plant pathogens, both in the laboratory (Schneider et al., 2004) and in the field (Schaad et al., 2002; Tomlinson et al., 2005). The R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device) developed by Idaho Technologies (Salt Lake City, UT, USA; www.idaho-tech.com), which can test 32 samples simultaneously, has been used for the detection of human pathogens in food (Van Kessel et al., 2003), and its use in a mobile laboratory in response to a bioterrorism attack (Higgins et al., 2002) has been described. The R.A.P.I.D. has also been used for the detection of citrus bacterial canker (*Xanthomonas citri*) in the laboratory (Mavrodieva et al., 2004). The R.A.P.I.D. and the SmartCycler demonstrated similar levels of sensitivity and specificity for the detection of a range of biothreat agents in a direct comparison of performance (Christensen et al., 2006). The Bioseeq is a handheld (3 kg) real-time PCR platform developed by Smiths Industries (Edgewood, MD, USA; http://trace.smithsdetection.com) that has been used to detect a number of pathogenic bacteria (Higgins et al., 2003; Emanuel et al., 2003). The Bioseeq has a smaller capacity than other platforms (6 samples) but can be run on internal batteries without connection to a laptop computer, unlike the SmartCycler or R.A.P.I.D., and weighs 5 to 10 kg less than the larger devices. Other recently developed portable PCR platforms include the battery-powered RAZOR instrument (Idaho Technologies) intended for military and biosecurity applications, and the Evocycler (Evogen), which combines rapid thermal cycling with fluorescent end-point product detection. Pre-mixed real-time PCR (or RT-PCR) reagent kits, which are compatible with portable real-time PCR machines, have also been developed for selected human pathogens such as *E. coli* (Idaho Technologies) and MRSA (Cepheid); animal pathogens such as FMD (VetAlert reagents; Tetracore Inc, Rockville, MD, USA); and biothreat agents such as anthrax (Idaho and Cepheid).

Given the developments in other areas, there is no reason in principle why real-time PCR assays

developed for the laboratory detection of plant pathogens should not be transferred to a portable platform such as the SmartCycler for use at field locations (Bentley et al., 2005). Real-time PCR assays suitable for field use have been described for *Xylella fastidiosa*, the causal agent of Pierce's disease of grape (Schaad et al., 2002) and *Phytophthora ramorum* (Tomlinson et al., 2005), the cause of sudden oak death in California. This last assay allows detection of *P. ramorum* in symptomatic plant material in less than two hours, compared with several days in the laboratory; working alongside inspection services at outbreak sites, molecular plant pathologists have been able to give a reliable and rapid diagnosis to better inform the eradication action being undertaken. The same technique should have merit in monitoring imported plants and plant products for quarantine organisms at ports of entry and points of inspection, particularly for highly perishable produce such as fresh fruit, flowers and vegetables.

While the advantages of field-based PCR are obvious, there are certain technical problems that need to be addressed. A key problem is sample processing and nucleic acid extraction; as in the laboratory, DNA extraction remains the rate-limiting step in any test method, yet for field-based testing the options are more limited due to restrictions such as the type of equipment that is available and practical to use. There is also a need to focus on simplifying sample preparation and nucleic acid extraction with the goal of permitting assays to be performed in the field by non-specialist staff with minimal training. For this reason, these areas form a significant part of a number of projects investigating on-site molecular diagnostics e.g. the EU-funded Port Check project (www.portcheck.eu.com). As a result, methods are being developed which are relatively simple, rely on little powered equipment, and do not use hazardous reagents (Tomlinson et al., 2005). Another key area is the use of stabilised reagents that can be stored at ambient temperature (i.e. do not require chilling or freezing). As noted above, pre-mixed stabilised reagents, which only require rehydration before use, are commercially available for a number of high-profile clinical and biothreat targets; however, this is not yet the case in the area of plant disease diagnostics. Real-time PCR reagents are available in a dry bead format, such as OmniMix HS (Cepheid), which are stable at ambient temperature; however, primers and probe for the required assay must still be added before use. A method has been described for freeze drying complete pre-mixed PCR reagents in-house, allowing long-term storage at ambient temperature without loss of performance (Klatser et al., 1998); a simple freeze-drying approach has been employed for *P. ramorum* detection and found to be both affordable and effective (Tomlinson et al., 2005).

Field testing in the future

In the longer term, new technology is being developed that could have a major influence on the future direction of field-based testing. For example, systems have already been developed in other arenas that combine sample preparation and DNA extraction, such as ultrasonic disruption of bacterial cells and spores (Belgrader et al., 1999b), with the addition of real-time PCR reagents in an integrated cartridge which is then inserted into a dedicated real-time PCR machine. Devices such as the Cepheid GeneXpert system (Raja et al., 2005) effectively offer a rapid single-step real-time PCR process, and small portable devices coupled with integrated sample processing cartridges are the likely future direction for the field application of this technology.

A major advantage of the portable real-time PCR platforms currently available is the greatly increased speed of thermal cycling, potentially allowing results to be obtained within minutes (Belgrader et al., 1999a). The majority of real-time assays described for use on field portable platforms have tended to use either TaqMan or SYBR Green chemistry; however, in some instances increasing the speed of thermal cycling has a detrimental effect on the sensitivity and reproducibility of assays using these chemistries (Hilscher et al., 2005). Scorpion primers allow detection of real-time PCR amplicons by a unimolecular mechanism, allowing faster and more efficient probing and hence faster thermal cycling (Thelwell et al., 2000). Thus, alternative real-time chemistries may allow the fast thermal cycling capabilities of new real-time PCR platforms to be fully exploited without a concurrent decrease in performance.

Recently non-PCR (isothermal) methods of DNA amplification have been used for the detection of plant pathogens, and the simplicity of

methods that do not require thermal cycling makes them particularly attractive for on-site testing. Loop mediated isothermal amplification (LAMP) uses a set of four or six primers and a DNA polymerase with strand displacement activity (*Bst* DNA polymerase) to amplify DNA with high specificity under isothermal conditions in less than 1 h (Nagamine et al., 2001, 2002; Notomi et al., 2000), without the need for a thermal cycler. This amplification method has been used for the detection of plant pathogenic bacteria (Okuda et al., 2005) and viruses, both in host tissue (Fukuta et al., 2003a, 2004; Nie, 2005) and in insect vectors (Fukuta et al., 2003b). LAMP products can be visualised either by gel electrophoresis, as for conventional PCR products, or using a number of other methods which may be suitable for use in the field. The synthesis of large amounts of DNA in a LAMP reaction yields a white precipitate of magnesium pyrophosphate which can be detected either with the naked eye (Fukuta 2003b; Nie, 2005) or using a real-time turbidity reader (Mori et al., 2004; Fukuta et al., 2004; Thai et al., 2004). Alternatively, the addition of an intercalating dye such as SYBR Green I to a positive LAMP reaction produces a colour change which allows detection with the naked eye (Iwamoto et al., 2003): this method may improve sensitivity compared to visual detection of magnesium pyrophosphate turbidity (Sun et al., 2006). Since LAMP does not require expensive thermal cycling and optical detection equipment, this method clearly holds potential for testing in the field or in under-equipped laboratories (Okuda et al., 2005). However, the majority of LAMP assays described to date have been used in conjunction with nucleic acid extraction methods which are not suitable for use in the field and this would need to be addressed in order to fully exploit the simplicity of this amplification method.

Successful future deployment of real-time PCR methods in the field will largely depend on integrating them within crop protection and plant health systems in an appropriate manner. The technique also appears eminently suitable for small regional laboratories and locations where plant products flow through 'choke points' such as packhouses and distribution centres. It is likely that such methods will form part of a hierarchy of complimentary methods, starting with pathogen detection techniques such as visual inspection or even automated methods such as the detection of disease-specific volatiles using biosensors. Plant Health authorities in England and Wales are currently considering a strategy of identifying *Phytophthora* species at the genus level in the field using lateral flow devices and subjecting any positive samples to real-time PCR testing to look for quarantine species. On-site real-time PCR methods will allow rapid diagnosis of any suspect material with the option of sending further samples to central laboratories for confirmation or more detailed characterisation if needed.

Of course, one of the limitations of all PCR-based methods including real-time PCR, be they field- or laboratory-based, is that they are generally designed to detect just a single target. The challenge is to develop systems that can screen effectively for multiple targets.

Generic multi-target detection: arrays and beyond

The development of systems to effectively detect a larger range of targets in a single assay has provided a constant technological challenge; this kind of testing is often referred to as parallel testing. Using PCR, this shortcoming has been partially resolved by the development of multiplex techniques, in which multiple targets are amplified and then resolved in a single assay. In conventional PCR this is done by amplifying targets of different sizes whilst in real-time techniques this is done by using different fluorescent dyes. The approach has significant limitations, however, in that multiplexing PCR primers together, regardless of the platform used, will result in interactions and competition between the primers. Using conventional PCR for plant pathogen detection, up to six or seven targets have been effectively multiplexed (Bertolini et al., 2001; Ito et al., 2002; Ragozzino et al., 2004). Using real-time PCR the number of targets that can be multiplexed together is effectively limited by the number of dyes that can be resolved by the real-time instrumentation; it should currently be possible to multiplex the detection of up to four pathogens effectively (Persson et al., 2005). However, due to competition between the primers this is effectively limited to the detection of just two pathogens if a good dynamic range of detection of each is required (Mumford et al., 2000, 2004b). These examples

illustrate the technical limits of the number of PCR assays that can be performed together in a single reaction using different primer sets. To resolve this problem a number of approaches have been developed that can be termed 'array' techniques, in which a single sample can be tested for a range of different targets in a single assay.

PCR arrays

The first approach to achieve the desired goal of performing multiple assays in a parallel fashion is simply to develop technology that allows the spatial separation of individual PCR or real-time PCR reactions. This method, often referred to as a PCR array, is essentially a technique for spatially separating, running and resolving simplex PCR reactions (reactions based on single primer pairs). These techniques are exemplified by developments from Applied Biosystems (Foster City, CA, USA) utilising TaqMan® low density arrays, in a proprietary micro fluidic card system. This system uses small channels to transport reagents from the sample-loading ports to each of 48 reaction wells where lyophilised primers and probes are resuspended. This enables the generation of real-time PCR data for up to 48 different assays (potentially 48 target pathogens) from eight individual samples in a single run (Gallagher et al., 2005). Another example is Thru-Hole® technology (BioTrove, Woburn, MA, USA), in which individual real-time reactions are performed in 33 nl holes (arranged in 48 sub-arrays of 64 holes) in microscope slide-sized plates. In this case the assays are held within the holes by surface tension created by the hydrophilic surface of the hole and the hydrophobic surface of the plate. This enables a total of 3072 separate assays to be performed simultaneously on a single OpenArray® plate (www.biotrove.com)

Microarrays

The concept for true microarrays was first developed for carrying out multi-analyte immunoassays (Ekins, 1989): the basic principles were then applied to nucleic acids leading to the first DNA microarrays (Schena et al., 1995; Shalon et al., 1996). Since then the use of microarrays has increased dramatically with a plethora of technologies becoming available; however, the concepts remain identical to those first patented by Hyseq (Sunnyvale, CA, USA), Affymetrix (Santa Clara, CA, USA), Oxford Gene Technologies (Oxford, UK) and Stanford University (Stanford, CA, USA) in the early 1990s. These techniques were developed to allow assays to be performed in a parallel fashion, the most significant application being investigations of the transcriptome, i.e. parallel monitoring of gene expression.

DNA microarray techniques in this context could be most simply described as any technique that allows the resolution of specific hybridisation events between nucleic acid in a sample and known nucleic acid probes bound in a solid phase. In the context of phytodiagnostics the simplest analogy that could be drawn is essentially a dot-blot in reverse (Hsu et al., 2005), where the probe rather than the sample is bound to the solid phase. The logical extension of this approach is to immobilise a number of different spatially separated probes to the solid phase such that the sample can be tested for multiple targets. DNA microarray methods are now available in a range of different formats, although the basis of each is identical. DNA capture probes (or spots) for each of the genes/pathogens to be detected are immobilised onto a solid support in a spatially separated and individually addressable fashion. Nucleic acid from the sample to be tested is extracted and labelled, and this labelled nucleic acid (known as the target) is then hybridised to the array. The array is scanned such that the hybridisation events can be identified, and the presence of the gene/pathogen is resolved by the pre-defined position of the DNA capture probe on the array.

Array manufacture

If a DNA microarray (often simply referred to as an array) can most simply be described as DNA immobilised onto a solid support, in this sense it is made up of two component parts (the DNA and the support) and these components, along with the process that is used to link the two, are worthy of discussion.

The most frequently used (and industry standard) solid supports for microarrays are 25×75 mm glass microscope slides, which form very flat regular surfaces with very low fluorescence. The surface of the glass slides are chemically coated (e.g. poly-L-lysine, aldehyde or amino) to facilitate the binding of DNA; the different coatings have

properties that may alter the size, density, orientation, or even the concentration of DNA in each spot. The solid support can also be manufactured from other materials such as charged nylon membranes, and this is especially common for low-density arrays (e.g. Yamakawa et al., 2004). Binding DNA to planar surfaces such as glass slides or nylon membranes is a readily accessible approach ideally suited to the research laboratory; however, other formats are available that may offer advantages in terms of throughput and automation. Liquid phase or bead-based arrays are starting to become available; the Luminex system (www.luminex.com) is one of the best developed of these formats. In this approach DNA is bound to polystyrene beads which, following hybridisation, are interrogated using a modified fluorescence-activated cell sorter (FACS) instrument. The instrument is able to interrogate each bead individually and assess the colour of the bead (different capture probes are bound to different colours) and if the fluorescent labelled target is hybridised to its surface. Since the arrays are supported in the liquid phase, the system allows automation in microtitre plates and 96 'bead arrays' can be interrogated in as little as 30 min.

The second aspect to manufacturing an array is the type of DNA that is deposited on the surface. Traditionally this was either DNA amplified using PCR from cDNA or genomic libraries using vector-specific primers, or known DNA sequences amplified from genomic DNA/RNA using gene-specific primers. These DNA probes could vary from 200 to 1000 bp in size and, since they comprised of double stranded DNA, required denaturing following binding to the solid support. The problem with this approach is the time required to produce all the required capture probes at the required concentration and purity. Thus, more recently capture probes made of single-stranded DNA (oligonucleotides) 20–70 nt in length have been used. These synthetic capture probes can be synthesised to the required concentration and are a considerably more consistent and reproducible reagent. The DNA that makes the probes can be unmodified, or modified by the addition of a moiety (e.g. an amino group linked to the 5′ or 3′ end) in the DNA strand that enables it to bind either more effectively or in an orientated fashion in relation to the solid surface.

The third aspect of microarray manufacture is the methods with which the DNA capture probes are applied to the surface; there are two main competing methodologies. The first approach is to build the oligonucleotides base-by-base on the surface of the support achieved by photolithography (Affymetrix, Santa Clara, CA, USA) or by using standard phosphoramidite chemistry (Agilent, Palo Alto, CA, USA). The second approach, pioneered at Stanford University (CA, USA), and perhaps more commonly used, is the deposition of DNA into pre-defined positions on the solid support, a technique referred to as arraying, printing or spotting. Printing can be achieved either by contact techniques in which the printing pins (a printing head made up of an array of solid or split pins) dip directly into the DNA and transfer it to the solid support by touch, or by non-contact techniques such as piezoelectric dispensing or ink-jet printing of the DNA to the solid support, also pioneered by Agilent (a subsidiary of Hewlett-Packard Company).

Labelling and amplification

In microarray experiments fluorescence is most commonly used to label the nucleic acid extracted from the sample. The incorporation of fluorescence results in a certain amount of signal amplification, as the fluorescent dye in illuminated at its excitation wavelength and the resulting fluorescence detected at the emission wavelength (Figure 3). A further advantage of using fluorescence is that dyes with different excitation and emission spectra can be used allowing on-slide comparisons, for example, of the expression levels of a range of genes in a test and control sample. The two dyes that are most widely used in this respect are Cy3 and Cy5 (GE Healthcare (formerly Amersham Biosciences), Little Chalfont, England), which have excitation wavelengths of 635 nm and 532 nm, respectively. Other labels can also be used; the incorporation of colorimetric substrates is often used when working with arrays on nylon membranes (e.g. Lievens et al., 2003), but can also be used on glass slide arrays (e.g. Lin et al., 2005). In gene expression experiments the fluorescent label is incorporated into cDNA synthesised from sample RNA either directly, using fluorescent labelled primers or nucleotides, or indirectly, by incorporating modifications that can

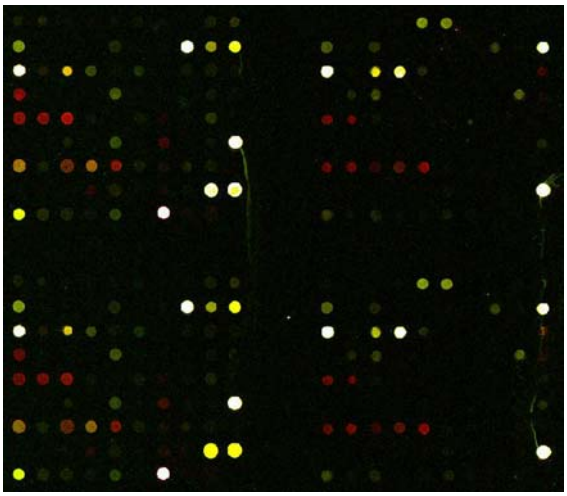

Figure 3. Microarray showing the simultaneous detection of Potato virus X and Potato virus Y. White fluorescent spots indicate specific detection of the viruses, while yellow spots are endogenous plant controls.

be bound to reactive dye following cDNA synthesis. This random labelling approach has been used effectively for the detection of RNA viruses (Boonham et al., 2003; Bystricka et al., 2003, 2005), and can also be used for detection of DNA targets (Vora et al., 2004) using different enzymes (e.g. Klenow). Although both approaches have been used in a diagnostic setting, they offer limited sensitivity. An advantage to using a random labelling technique is that no bias is introduced; targets at low and high abundancy are labelled equally efficiently.

Labels can also be incorporated using other techniques, and PCR amplification combined with labelling using universal primers is perhaps the method of choice for diagnostics. This is especially true for bacteria and fungi where DNA can be amplified with completely universal primers designed, for example, in either the 16S or ITS regions (Bodrossy et al., 2003; Franke-Whittle et al., 2005; Rudi, 2003; Stralis-Pavese et al., 2004). An advantage of using PCR to perform labelling is that it also gives signal amplification, so very small amounts of the target can be amplified from a sample, and the amplified region can then be identified using the microarray. PCR amplification of universal regions of non-complex mixtures of target pathogens gives effective detection. However, when the mixtures become more complex, and where multiplex PCR (mixed primer sets) is used to give good coverage of all target pathogens, PCR bias becomes a significant problem. As competition begins to occur in the PCR amplification, less abundant targets are not amplified efficiently, and only the most abundant targets are amplified and hence detected (Call, 2005).

Other PCR-independent techniques for amplification of a signal have been investigated that should give less bias than PCR-based techniques since they amplify total nucleic acid in a sequence non-specific way and as such are not affected by the abundance of pathogens within the sample. For the complete amplification of genomic DNA a technique referred to as whole genome amplification (WGA) has been used, utilising highly processive polymerase enzymes (e.g. ϕ29) for non-specific copying of genomic DNA (Vora et al., 2004). For the amplification of cDNA (made from RNA targets) methods referred to as Eberwine cDNA amplification techniques can be used (Van Gelder et al., 1990), which involve first synthesising cDNA using primers incorporating phage polymerase binding sites, and subsequent reactions which copy the cDNA into cRNA resulting in signal amplification (t'Hoen et al., 2003; Marko et al., 2005). Although these techniques incorporate much less bias into the assay, amplifying abundant and non-abundant targets effectively, they also usually result in lower levels of amplification than PCR, resulting in less sensitive assays.

Techniques are being developed that combine both universal amplification and increased multiplex capability and which may help with some of the problems associated with bias and sensitivity. One of the more promising approaches is that based on proximity probes or circularizable ligation probes. Initially developed as a highly multiplex method for screening for the presence of genes or single nucleotide polymorphisms (Nilsson et al., 1994), they can also be integrated with microarrays to resolve the products of amplification (Zhang and Liu, 2003). The term 'padlock probes' has been coined for these reagents and their use in multiplex diagnostics has been reviewed recently (Landegren et al., 2004). Briefly, a padlock probe is a long oligonucleotide (approximately 100 bp) containing target complementary regions at the 3′ and 5′ ends; these regions are complementary to adjacent sequences on the target pathogen to be detected. Only when the target nucleic acid and

padlock probe are hybridised together, can the ends of the probe be enzymatically ligated together, creating a circularised molecule. Once circularised the probe can be amplified using either inverted PCR or rolling circle amplification techniques; the presence of amplified probes can then be resolved using an array, either based on the pathogen-specific sequence or on a 'Tag sequence' amplified from the padlock probe (Shoemaker et al., 1996). Array techniques based on the Tag approach could lead to a method that could be used to resolve products of many different types of padlock probes for a range of applications on a single 'universal array'.

Applications in phytodiagnostics

The application of microarrays to phytodiagnostics is potentially far reaching, especially if issues of sensitivity and throughput can be addressed. Clearly, if diagnostics is taken literally as 'finding the causal virus and recognising it' (Bos, 1999) then the ability to test a sample for many different pathogens using a single generic method is very appealing. The first published examples utilised arrays constructed from long fragments of DNA bound to glass slides for the detection of potato viruses (Boonham et al., 2003; Bystricka et al., 2003) and several cucurbit infecting Tobamoviruses (Lee et al., 2003). Each method gave detection of a range of viruses either individually or in mixed infections; with a sensitivity level similar to that of a TAS-ELISA, the arrays were able to detect a range of isolates and strains of each of the viruses and achieve discrimination at the species level. More recently plant virus detection (Bystricka et al., 2005) and discrimination (Deyong et al., 2005) has been achieved using synthetic oligonucleotide-based arrays, which offer a number of advantages over arrays based on longer PCR amplified fragments. The use of oligonucleotides allows greater control over the specificity of the detection, for example it was found to be possible to discriminate serotypes and subgroups of Cucumber mosaic virus that differed by only 8% in an amplified PCR product larger than 700 base pairs (Deyong et al., 2005). Each of these examples for plant virus detection illustrates that the approach taken so far has been on a commodity basis (i.e. potato or cucumber). In the case of viruses though, it appears that this technique could be effectively developed for the detection of a very large range of viruses, across a range of different hosts provided that a universal labelling method is used. There are between 900 and 1000 recognised plant-infecting viruses, which is well within the range of numbers of targets that could effectively be detected and discriminated on a single microarray and work has already started at Central Science Laboratory (York, UK) and by others (Hadidi et al., 2004) to begin to assemble a large virus diagnostic array.

Arrays have also been used for fungal pathogens in a purely diagnostic application, again in a commodity-by-commodity basis. Currently one of the most comprehensive of these arrays allows the detection of a range of fungal pathogens (and some bacterial pathogens) in horticultural crops (www.dnamultiscan.com) and is based on oligonucleotides bound to nylon membranes which are probed with PCR- amplified and colourimetrically labelled DNA (Lievens et al., 2003). However, such approaches based on the ITS sequence regions are not without problems; discrimination of very closely related fungal species can lead to mis-identification. Profiling the presence of fungal pathogens in diseased plant material requires a considerable amount of expertise, in order to give proper interpretation e.g. separating the primary pathogens causing disease from secondary infections. More elaborate systems have also been explored, for example the exploitation of padlock probes and universal tag arrays has been used for the detection and discrimination of 11 fungal pathogens and one nematode (Szemes et al., 2005). This development enabled sensitive detection down to 5 pg of target genomic DNA in a large excess of other target DNA although the dynamic range of detection was similar to systems based on PCR amplification of conserved regions (Szemes et al., 2005). In addition to simply identifying the causal agents of disease, microarray methods have also been developed for the discrimination of closely related species that could cause problems in the food chain, for example the discrimination of mycotoxin-producing species of Fusarium (Nicolaisen et al., 2005). Similar approaches have been used for the detection and identification of bacterial pathogens, with the favoured approach being based on amplification of the 16S region using universal primers. A method has been described for the detection of a range of bacterial

pathogens on potato based on specific oligonucleotides (designed to 16S sequence data) bound to nylon membranes; labelling of the target was achieved by incorporation of digoxigenin-dUTP during PCR amplification using 16S universal primers (Fessehaie et al., 2003). The oligonucleotides used were between 16 and 22 bp in length and could discriminate between species with a sequence difference of as little as 2 bp.

Beyond the basic diagnostic concept (i.e. identifying causal agents of disease) is the whole area of screening plants for the presence of pathogens. This could be screening of material during the vegetative propagation chain, where many tests are carried out in parallel to check for a wide range of pathogens. A similar approach is used for screening imported material for national and international quarantine purposes, where a range of assays are performed in parallel to confirm that any listed pathogens are absent. Again this is an area of work that has received some interest, with developments being made for the detection of quarantine pathogens on a commodity basis. For example the EU-funded project DiagChip (www.diagchip.com) aimed to develop a microarray that could be used to screen for all the EU-listed quarantine potato pathogens (including viruses, viroids, nematodes, bacteria and fungi) in a single array-based assay. The concept of screening could also be extended beyond the testing of plant material into the screening or profiling of communities of disease-causing agents, or even beneficial microbes either in soil or irrigation water. This profiling of populations could prove to be one of the most important applications of array techniques since it is so difficult to achieve using conventional techniques. Arrays have been successfully developed for community profiling of bacterial populations in environmental samples such as soils and composts (Bodrossy et al., 2003; Franke-Whittle et al., 2005; Rudi, 2003; Stralis-Pavese et al., 2004).

A further application that could be envisaged at this point for microarrays in phytodiagnostics is their use for identification purposes, for example identification of pest insects. A similar approach has been shown to be effective for the identification of species of small mammals using oligonucleotide probes designed to detect the cytochrome oxidase I gene (Pfunder et al., 2004).

The future of microarrays

Microarray techniques for phytodiagnostics are currently being developed for a range of applications in a whole plethora of different formats, and at this point there is not one single technique that is becoming more widely used than others. However it seems there are some general conclusions that can be drawn at this time for the future direction of these techniques. From a technology point of view, planar arrays (i.e. DNA bound to a two-dimensional surface) are by their very nature low throughput, due to problems of handling large numbers of membranes or slides and problems with automating the procedures involved. Thus it seems likely that other formats that are more suitable for automation such as liquid phase or bead-based arrays may well start to make an impact. It is hoped, therefore, that the methods and oligonucleotides developed thus far may find a home in a more appropriate format as they become available. Another aspect of array techniques that is constantly debated is the need for methodologies to achieve signal amplification. Some of the techniques developed so far achieve levels of sensitivity without signal amplification that approach or surpass those of other well-established techniques such as ELISA (Boonham et al., 2003). However, many applications require greater levels of sensitivity. This might be achieved most conveniently by exploiting universally amplifiable regions, for example 16S or ITS for bacterial and fungal pathogens respectively, and this has been used to great effect as discussed for profiling microbial populations. However, there are no universally amplifiable regions in plant-infecting viruses, and achieving greater sensitivity will continue to pose a technological challenge. The final observation worthy of discussion from the current literature is that most arrays developed so far have been developed for specific commodities (e.g. potatoes). This is expected as the technology is still in development, but another driving factor is the cost implication in having redundancy in the arrays. For example, if an array was developed that contained spots for all plant-infecting viruses and the array was used for testing potatoes, clearly the spots on the array representing viruses that are unable to infect potato would be redundant. Currently arrays are built on a per-array, per-spot basis and this redundancy will currently increase

the cost per test of any array-based assay. Unless this issue can be resolved one of the greatest powers of arrays, the ability to test for a huge range of pathogens, both expected and unexpected, may be lost. As these problems become resolved it may be possible in the future to combine the detection of broader ranges of different types of pathogens from large host ranges on single arrays. It may also be possible to include the screening of other traits in plants, such as variety, presence of GM events (Germini et al., 2005; Rudi et al., 2003) or even the expression of genes caused by environmental stress that could be confused with pathogen infection (Tamaoki et al., 2004).

So what of the future?

Recent developments have spurred the increased use of molecular diagnostics and this trend will undoubtedly continue with increasing momentum over the years to come. Nucleic acid-based pathogen detection is here to stay and in the short-to-medium term the technologies discussed in this review will be at the forefront of this. However, the manner in which molecular diagnostics will be performed in the more distant future is less clear. The formats used will undoubtedly evolve rapidly, with the evolution driven by the ingenuity of engineers, the commercial imperatives of scientific equipment manufacturers and the R&D strategies of large pharmaceutical companies. Certainly in the case of fledgling technologies such as microarrays, where much of the technology is still geared towards the researcher, there is considerable scope for the development of more diagnostics-friendly formats in the next few years. For in-field testing the drivers are coming from a different area, primarily that of military and counter-terrorism with a need for very rapid testing out in the 'field', be that a battlefield or a mail room. However, from the period of change that confronts us in the future, certain general themes are easy to predict. One of these is the increased use of automation, allowing genuine high-throughput testing (i.e. large numbers of samples) to occur. Given the issues of labour costs, health and safety (e.g. repetitive strain injury), and reproducibility, the increasing use of automation to perform previously labour-intensive procedures will inevitably occur. One obvious repercussion of such developments will be the move towards larger, capital-equipment rich, centralised diagnostic laboratories. The second major trend that can be foreseen is the continued development of generic detection systems, resulting in much less reliance on the kinds of parallel testing often seen in diagnostic laboratories today. The development of array-based technologies, based on either hybridisation, amplification or both, is sure to feature heavily in this. The third significant change that will occur as a direct result of the increased use of molecular techniques will be the replacement of some techniques, especially the slower, more expensive biological methods e.g. grafting or bait testing. While this replacement will initially be gradual, and might possibly never result in the complete extinction of such techniques (especially where generation of live cultures of the pathogen are a legal requirement), it will undoubtedly become more rapid as funding bodies continue to demand cost-saving efficiencies and hard decisions have to be made on what equipment and facilities are bought and maintained. Finally, as centralised laboratory facilities become 'geared-up' for high throughput testing, and as a result become specialised in handling large sample numbers in an automated fashion, there will be a drive away from low sample number, rapid-response diagnosis being performed in these facilities. These diagnoses will be carried out by inspectors at a point of entry (e.g. an airport, port or border crossing) rather than scientists in remote laboratories. At this point, the discipline of plant pathogen molecular diagnostics will have finally come of age.

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