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IMPACTS OF MOLECULAR DIAGNOSTIC TECHNOLOGIES ON PLANT DISEASE MANAGEMENT

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■ **Abstract** Detection and diagnosis of plant viruses has included serological laboratory tests since the 1960s. Relatively little work was done on serological detection of plant pathogenic bacteria and fungi prior to the development of ELISA and monoclonal antibody technologies. Most applications for laboratory-based tests were directed at virus detection with relatively little emphasis on fungal and bacterial pathogens, though there was some good work done with other groups of plant pathogens. With the advent of molecular biology and the ability to compare regions of genomic DNA representing conserved sequences, the development of laboratory tests increased at an amazing rate for all groups of plant pathogens. Comparison of ITS regions of bacteria, fungi, and nematodes has proven useful for taxonomic purposes. Sequencing of conserved genes has been used to develop PCR-based detection with varying levels of specificity for viruses, fungi, and bacteria. Combinations of ELISA and PCR technologies are used to improve sensitivity of detection and to avoid problems with inhibitors or PCR often found in plants. The application of these technologies in plant pathology has greatly improved our ability to detect plant pathogens and is increasing our understanding of, their ecology and epidemiology.

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INTRODUCTION

This review explores how new detection technologies have impacted pathogen detection, identification and classification, disease management, plant production, and quarantine. For the details of the various detection methods, readers are referred to reviews on polymerase chain reaction (PCR) (63), monoclonal antibodies (60, 166) and general molecular techniques for detection of plant pathogens (27, 36, 43, 62, 108). This review uses case studies to illustrate how these methods have impacted plant production under certification programs, plant quarantine, pathogen identification, disease management, and crop production. The case studies could have covered almost any pathogen or cropping system since the objective is to illustrate the principles rather than make a comprehensive list of pathogens that are detected by each method. As an example, the section on viruses covers grapevine viruses. For fungi, *Phytophthora* is used because much work has been done on the molecular characterization and detection of species in this genus, but again other examples could have been used. The section on quarantine covers a broad spectrum of pathogens, but selected examples are used to demonstrate the principles. We do not cover bacterial pathogens because there was an excellent review on bacterial diversity, detection, and diagnosis in 1999 (96). Molecular genetic approaches have also greatly facilitated the detection (80, 81, 153, 154), identification, and classification (57, 147, 155) of plant pathogenic phytoplasmas. Because many of the principles that apply to bacterial pathogens also apply to the phytoplasmas, they also are not covered in this review.

Why Molecular Testing for Pathogens is not Routine

DNA extraction techniques, enzymes and protocols, specificity of primers, labeling and detection systems, and instruments for quantitative PCR are continuously being

improved. Robust and cheap protocols to extract DNA and/or RNA from plant tissue or soil are needed for practical application of these techniques in diagnostic laboratories. Several papers describe such techniques (34, 38, 84, 150, 170). In some situations nested PCR is necessary to achieve consistently high sensitivity and reduce the incidence of false negatives. Contamination, always a concern with PCR, is an even greater risk with nested PCR. The cost of the test in terms of extraction and enzymes is still quite high compared to ELISA. Most plant viruses have RNA genomes, and thus there is the additional cost of reverse transcriptase required to synthesize cDNA to serve as a template for the PCR reaction.

APPLICATIONS FOR VIRUSES: GRAPEVINE AS A CASE STUDY

In this section we review the advances in virus detection from the perspective of their impact on grapevine certification programs. This part of the review could have covered almost any crop since the same principles and techniques have been applied to viruses of most crops. However, with woody, vegetatively propagated crops many viruses have proven recalcitrant to conventional methods of isolation and characterization. The tools of molecular biology have been especially useful for studying these viruses. There are 44 viruses that have been reported to infect grapevine (103). At this time, grafting onto indicator hosts is still required for viruses that cause some of the most important diseases of grapevine. From the perspective of someone responsible for a certification program, there is little advantage in adopting new assays for viruses as they become available until all viruses in the crop can be identified by serological or nucleic acid–based techniques. New serological and nucleic acid–based tests must be "validated" to be as good as, or better than, indexing by grafting. The lengthiest assay sets a limit on the time required to certify a plant free of known viruses. For grapevines this is the time required to carry out the graft test for grapevine leafroll, corky bark, and rupestris stem pitting viruses; this typically takes two to three years for symptom development on indicators. Serological and nucleic acid–based tests have been developed recently for the viruses that cause leafroll, corky bark, and rupestris stem pitting. For this article, we concentrate on three major virus disease complexes of grapevines, degeneration and decline caused by nepoviruses, leafroll caused by closteroviruses, and rugose wood caused by vitiviruses and a foveavirus (Table 1).

Grapevine Degeneration and Decline

The detection of the nepoviruses was greatly improved with the introduction of ELISA technology to plant pathology by Clark & Adams (28). Prior to ELISA these viruses were detected by mechanical transmission to herbaceous hosts or by agar gel diffusion serology (49). The nepoviruses are relatively easy to purify and are quite immunogenic. As a result, high quality antisera specific for the nepoviruses were available prior to the development of ELISA. Thus, these

TABLE 1 Major diseases of grapevine caused by viruses and the development of laboratory-based diagnostic tests

	Methods of detection[a].			
Disease **Causal agent(s)**	**1970**	**1980**	**1988**	**1998**
Vine degeneration and decline				
Nepoviruses[b]	MT, GD	ELISA	ELISA	ELISA/PCR
Grapevine leafroll				
Closteroviruses				
Grapevine leafroll pathogen(s)	Gr-Mission	Gr-CF	Gr-CF/PN	Gr-CF/PN
GLRaV-1			ELISA	ELISA/PCR
GlRaV-2			ELISA	ELISA/PCR
GLRaV-3			ELISA	ELISA/PCR
GLRaV-4			ELISA	ELISA/PCR
GlraV-5			ELISA	ELISA/PCR
GLRaV-6 (14)				ELISA/PCR
GLRaV-7 (26)				ELISA/PCR?
GLRaV-8 (112)				ELISA/WB
Rugose wood				
Vitiviruses				
Corky bark (GVB)	Gr-LN-33	Gr-LN-33	Gr-LN-33	Gr/ELISA/PCR
Kober stem grooving (GVA)			Gr-K5BB	Gr/ELISA/PCR
Grapevine virus C				
Grapevine virus D (2)				ELISA/PCR
Foveaviruses				
Rupstris stem pitting			Gr-SG	Gr-SG/PCR/ ELISA

[a]The detection for the different decades are taken from 1970 (49), 1980 (17), 1988 (127), and 1998 (103).
[b]Nepoviruses infecting grape include *Arabis mosaic, Artichoke Italian latent, Grapevine Bulgarian latent, Grapevine chrome mosaic, Grapevine fanleaf, Peach rosette mosaic, Raspberry ringspot, Strawberry latent ringspot, Tobacco ringspot, Tomato black ring*, and *Tomato ringspot viruses*. MT = mechanical transmission, GD = gel diffusion serology, Gr = grafting, SG = 'Saint George', PN = 'Piont Noir', CF = 'Cabernet Franc', WB = Western Blot, GLRaV = *Grapevine leafroll associated virus*, GVA = *Grapevine virus A*, GVB = *Grapevine virus B*, GVC = *Grapevine virus C*, GVD = *Grapevine virus D*.

viruses have been readily detected by ELISA since the late 1970s. Several of the nepoviruses have been cloned and sequenced, and PCR tests for their detection is possible but this offers few advantages over ELISA testing. Where PCR tests are being carried out for the leafroll and rugose wood pathogens, it may be more convenient to use PCR to detect the nepoviruses as well, because the extracted RNA is already available. Many of the nepoviruses that infect grapevine have been sequenced, but these isolates are from crops other than grapevine (144), and care must be taken to ensure that any oligonucleotide pairs developed for PCR cover conserved regions of the genome to increase the likelihood that the test will be successful for isolates from grapevine.

Grapevine Leafroll

Grapevine leafroll is detected by grafting onto 'Cabernet Franc' or 'Pinot Noir' vines. The disease causes delayed fruit ripening, fruit low in sugar, and smaller clusters of berries. Elucidation of the etiology of the leafroll complex began in 1979 with the demonstration that there were closterovirus-like particles associated with the disease (115). Between 1979 and 1990, five different closteroviruses were identified in symptomatic plants (14, 66, 183), and the terminology grapevine leafroll associated viruses (GLRaV 1-4) was proposed (66). From 1990 to 1998, the number of closteroviruses associated with the grapevine leafroll syndrome increased to eight (103, 112) (see Table 1). During the 1980s, efforts concentrated on making polyclonal and monoclonal antibodies (McAbs) suitable to detect the grapevine leafroll associated closteroviruses. Polyclonal antisera were developed in several laboratories but they required cross absorption of host antibodies before they could be used in ELISA (26, 66, 112, 183). Monoclonal antibodies were developed to GLRaV-3 (65, 184), GLRaV-1, (Bioreba), and GLRaV-8 (112).

Even though there are antisera available for each of the eight closteroviruses associated with grapevine leafroll disease, they are not used routinely for detection with the exception of GLRaV-1 and GLRaV-3. Even with the best monoclonal antibodies available, testing needs to be done late in the season when virus titers are at their peak to get a reliable result (58). For most of these tests, ELISA results are not reproducible even under the best conditions (47, 136). During the 1990s, much effort went into sequencing the viruses associated with leafroll disease of grapevine [GLRaV-1 (58, 143); GLRaV-2 (1, 182); GLRaV-3 (95, 141); GLRaV-4 and GLRaV-5 (47, 136); and GLRaV-7 (143)]. In addition to the published papers describing the closterovirus and their sequences, there are entries for GLRaV-1 to -7 in Genebank. Following is a list of Genebank ID numbers for grapevine leafroll associated viruses: GLRaV-1, Y15890, U58335; GLRaV-2, AF039204, Y14131; GLRaV-3, Y15891, AF037268, U22158; GLRaV-4, AF030168, AF039553; GLRaV-5, AF039552; GLRaV-6, U22170; GLRaV-7, Y15987.

During the 1990s, reverse transcription (RT) and immunocapture (IC) RT-PCR tests were designed to detect many of these viruses (58, 136, 139, 143). Because the viruses associated with leafroll of grapevine are all in the *Closteroviridae*, it should be possible to design primers that react with several or all of these viruses. Primers representing conserved sequences of the heat shock protein (163), a signature feature of members of the *Closteroviridae*, appear to be a good target for developing a test to detect all grapevine leafroll–associated closteroviruses (143). These primers amplified a fragment of the conserved HSP70 homologue gene of GLRaV-1, -3, -4, -5, -7 from infected grapevine tissue as well as the expected sized fragment from *Nicotiana benthamiana* infected with GLRaV-2 (143). GLRaV-6 and -8 were not included in this study. Further work is needed to confirm the utility of this test for GLRaV-6 and GLRaV-8. Development of a universal detection for the leafroll closteroviruses together with virus specific tests will also aid in identifying other viruses that may be involved in the leafroll syndrome.

Grapevine Rugose Wood

Rugose wood is a disease complex characterized by pitting and grooving of the trunk wood of grapevine. Several viruses have been implicated as the causal agents. These viruses belong to two newly recognized genera, the *Vitiviruses*, that include *grapevine virus A* (GVA), *grapevine virus B* (GVB), *grapevine virus C* (GVC), and *grapevine virus D* (GVD) (102), and the *Foveaviruses* grapevine rupestris stem pitting associated virus (GRSPaV) (101). Corky bark was recognized as a disease of grapevine prior to 1970 (49) and the elucidation of the different viruses involved in rugose wood has been ongoing since (Table 1). *Grapevine virus A* has been associated with Kober stem grooving and GVB with corky bark. A specific virus has not been associated with LN-33 stem grooving, another recognized component of the rugose wood complex.

GVA was first isolated in 1980 (29), and a comparison between graft indexing and ELISA showed a close correlation between GVA and Kober stem grooving (50). Closterovirus-like particles were purified from corky bark–infected grapevines in 1991 (114), and GVB was isolated in 1993 (15). In 1996, GVB was found to be associated with corky bark disease symptoms in Italy (13). The complete nucleotide sequence of GVA and GVB is known (109, 142). GVD was isolated from a vine showing rugose wood symptoms and the genome partially sequenced (2). The coat protein of GVD has 75% sequence identity with GVA and a distant serological relationship between GVA, GVB, and GVD has been reported (25). Degenerate primers based on conserved sequences of the RNA-dependent RNA polymerases of GVA and GVB *vitiviruses* and *apple chlorotic leafspot* (ACLSV) *trichovirus* have been used in RT-PCR to amplify fragments from GVA, GVB, GVD, and *heraculum latent* vitiviruses, and from two strains of ACLSV and *potato virus T* trichoviruses (143).

These vitivirus-specific oligonucleotides will be useful in the cloning and sequencing of GVC and in the development of oligonucleotide pairs that should help to differentiate between the vitiviruses. A universal primer can be used for reverse transcription, and then by employing the same primer together with a virus specific primer in the PCR reaction, the individual vitiviruses can be identified from grapevines with multiple infections in a multiplex PCR. This combination of primers together with the degenerate universal primers will be effective in identifying new members of the *Vitivirus* genus.

Rupestris stem pitting was first reported from Italy in 1961 and later from South Africa, Greece, and the USA (100). The virus is detected by indexing on grapevine cultivar St. George and it may take 2–3 years for symptom development. A virus has been cloned and sequenced (107, 181) from plants infected with rupestris stem pitting, and the virus has similarities to *apple stem pitting virus*. A new plant virus genus, *Foveavirus*, has been established with *apple stem pitting* as the type member, *grapevine rupestris stem pitting associated virus* (GRSPaV) as a member, and *sour cherry green ring mottle* as a tentative member (101). An excellent correspondence of the GRSPaV with rupestris stem pitting was found

when two pairs of oligonucleotide primers were used in RT-PCR assays. Sixty of 62 sources that were positive by grafting were positive in the RT-PCR test (181). In a similar study that included 115 graft indexed and 14 nonindexed dormant grapevine cuttings collected in the USA, Canada, Italy, and Portugal, there was also an excellent correspondence between results from graft indexing and RT-PCR testing (106). In both studies, differences were observed in the number of samples testing positive by RT-PCR, depending on primer pairs used in the test. This suggests that there may be multiple strains of the GRSPaV or multiple foveaviruses infecting grapevines. Credi (33) observed differences in symptoms with different isolates of rupestris stem pitting, suggesting the possibility of multiple strains or viruses associated with the disease.

The characterization of viruses involved in the leafroll or the rugose wood complexes has been difficult because of multiple infections that occur in many affected vines (13). As a result, the literature on viruses associated with these diseases has been confusing. The development of oligonucleotide pairs that are effective for detecting a broad range of viruses in a group such as has been done with the closteroviruses (163) and the vitiviruses (143) will enhance the characterization and detection of recalcitrant viruses of woody crops.

Comparison of Detection Methods

Laboratory tests, especially RT-PCR and ELISA, can generally be done in 2 days or less compared to the 2 years required for the biological indexing for grapevine leafroll or rugose wood. In a study where ELISA was compared to biological indexing for detection of GLRaV-1 to -4, there was a perfect relationship between symptom development on indicator plants and the ELISA results for GLRaV-1, -2, and -4. With GRLaV-3, only 49 of 57 infected plants gave positive results in the biological indexing, whereas all 57 were positive by ELISA (139). The eight samples with varying results were then tested by IC-RT-PCR and all were positive. The discrepancy between the tests may have been due to uneven distribution of the virus in grapevines. In a study on distribution of GLRaV-1 to -4 in grapevines, 20 to 40 subsamples were collected from each of 36 vines and tested by ELISA. The distribution of the different GLRaVs varied considerably. GLRaV was detected in over 75% of the subsamples from 20 of the 36 vines, in 50-75% of the subsamples collected from 2 vines, from 25-50% of the subsamples collected from one vine, from 2.5-25% of the subsamples collected from 6 vines, and in none of the subsamples collected from 7 vines (139). With the increased ease with which large numbers of samples can be assayed with RT-PCR or ELISA, it is now possible to carefully study virus distribution and develop statistically sound sampling procedures to be used in certification and quarantine programs.

The application of serological and nucleic acid-based tests are increasing our understanding of the etiology of the leafroll and rugose wood complexes in grapevine.

Another benefit from applying these newer detection methods for the individual viruses is that studies can now be carried out to identify interactions between the different viruses of grapevine. Many grapevine certification programs do not regulate rupestris stem pitting. The tools are now available to study interactions between GRSPaV and each of the GLRaVs and vitiviruses. This is especially important for a crop like grapevines where most plants are grafted and graft compatibility is often influenced by viruses. If a state or country desires to maintain a quarantine for a specific virus such as GRSPaV, which most countries do not consider an economically significant pathogen, it may be necessary to demonstrate that the quarantine is based on disease potential and is not designed as an artificial trade barrier. With grapevines there is an incredibly wide range of hosts since each rootstock-scion combination can be considered as a unique host. If a virus is present in either the rootstock or scion wood, it will be present in the grafted plant. For each new rootstock or scion cultivar developed there will be many new grafted combinations that could be affected by one or more of these viruses. Little is known about the transmission of the viruses that cause the grapevine leafroll and rugose wood diseases. Mealy bugs have been shown to transmit GLRaV-1 and -3 (128, 134). The new detection methods will be useful in the identification of vectors of the other closteroviruses, the vitiviruses, and foveaviruses of grapevine and allow for the identification of any helper/dependent transmission interactions that might occur between these viruses. Proper control measures can only be implemented once the causal virus and vectors in an area have been identified.

Development of Acceptable Tests

What is required before these test can be used in certification schemes and in plant quarantine laboratories? The nucleic acid and ELISA tests must be validated as equivalent to, or better than, graft indexing. Some of this work has been started, as described above for GRSPaV, GVA, and GVB (106, 143, 181). An internationally approved protocol is needed for validation of the laboratory tests. For serology-based tests, this requires the evaluation of each of the monoclonal and polyclonal antibodies in various serological formats. Each oligonucleotide pair that has been reported to be suitable for detection of a virus must be compared for its utility in PCR–based formats. The comparisons must involve a wide range of isolates, and the robustness of the assay must be demonstrated unequivocally in blind tests in several different laboratories. After the testing is finished, a recognized body with international representation should determine which test or tests, if any, are suitable alternatives to graft indexing. This will necessitate that specific oligonucleotide pairs or monoclonal and polyclonal antibodies be identified to give an internationally acceptable test and that test protocols be described in detail, including how leaf sap should be prepared for ELISA or how the RNA should be extracted for PCR tests. The test parameters must also be defined. Time of year and number of samples per vine must also be specified because virus titers range widely during the year, and uneven distribution has been reported.

APPLICATIONS FOR FUNGI

Recent advances in molecular systematics of fungi provide extensive DNA sequence information that is of great benefit in molecular detection and diagnostics. The publication of several sets of universal primers for fungal amplification by White et al (176) was important for the growth of the fungal database. Molecular analyses are now commonly used for fungal identification, especially for large genera whose species have overlapping morphological characteristics (e.g. *Fusarium* and *Pythium*). Molecular analysis techniques with resolution higher than the species level have fueled a rapid expansion of population genetics studies, which were reviewed recently (3, 104). Normally, molecular analyses are performed using pure cultures and are used to identify species or particular genotypes within a population. When fungal isolation from the host is required to produce a pure culture, very little time is gained compared to more traditional diagnostic techniques. For many disease management applications, direct molecular detection or diagnosis from host tissue would be more useful. The topic of molecular detection of fungi was reviewed recently (4, 37, 124, 148). The first part of this section on filamentous fungi focuses on *Phytophthora*, an economically important genus for which molecular tools were developed in the early days of molecular biology, and includes a case study on *Phytophthora fragariae*. Oomycetes such as *Pythium* and *Phytophthora* are included in the working definition of fungi (111). After the case study, additional applications pertinent to the issues of multiplexing, support for decision making in disease management, soil testing, and detection of beneficial microorganisms are covered.

Phytophthora Detection Methods

Chemotaxis of Zoospores and Antibodies Many of the advances in integrated pest management of insects were made possible by the routine use of pheromone traps. Similarly, the ability of zoospores to move toward their host can be exploited to monitor a pathogen like *Phytophthora* for which baiting has been used extensively (46). The fungal detection techniques most comparable to synthetic pheromone trap monitoring are probably the capillary root model (88) or dipsticks coated with attractants (21). Zoospores of *Pythium* and *Phytophthora* species, including species with narrow host ranges, are attracted by a wide range of chemicals that are released from roots (35). Therefore, synthetic or natural baits are likely to be nonspecific and assays that include such baits will require further sample processing to obtain meaningful monitoring results. In this regard, dipsticks coated with chemoattractants that are used directly with a species-specific immunoassay can both attract and detect *P. cinnamomi* (21) or *P. nicotianae* (133). This elegant and fast procedure can be used to monitor these species in soils (20, 51). Note that zoospore chemotaxis can be an easy way to concentrate the inoculum on a small surface before running a molecular assay.

DNA Probes Plasmid libraries can be made with random DNA fragments of fungal pathogens. Similar to the screening of monoclonal antibody cell lines, individual fungal DNA fragments in the plasmids of bacterial colonies can be tested for their specificity as probes. Three random chromosomal DNA clones hybridized only to *P. parasitica* when tested against ten other *Phytophthora* species (55) and two other clones were species specific to *P. citrophthora* (56). DNA hybridization was used to detect *P. parasitica* directly from leaf disk baits (54). A species-specific randomly cloned DNA fragment of *P. cinnamomi* was estimated to be present at 12,000 copies per haploid genome and gave quantitative hybridization signals related to days after inoculation or increased inoculum level (76).

DNA sequences have been generated for phylogenetic analyses of *Phytophthora* species (18, 31, 32, 89). Instead of developing probes from randomly produced libraries, it is possible to select a specific sequence directly from an alignment. Oligonucleotide hybridization probes were designed for the genus *Phytophthora* and for *P. cinnamomi, P. palmivora, P. megakarya* and for *P. capsici* from alignments of ITS sequences, the first such public sequence data set for *Phytophthora* species (89, 90). Some of the same oligonucleotides have been used in other types of *Phytophthora* assays described below.

PCR—Random Sequences PCR has been a major breakthrough in the field of molecular taxonomy and detection (63). Primers have been designed from either random genomic fragments that have been partially sequenced or from gene sequences used in phylogenetic studies. After partial sequencing of plasmid clones that were developed as specific probes for *P. parasitica* (54, 55) and *P. citrophthora* (56), specific PCR primers were designed for these two species and used successfully on tomato stems (45). PCR primers were designed from the partial sequence of a tandem repeat satellite DNA and were used to detect *P. infestans* in potato leaves or tuber slices 2 days after infection (122). The cross reactivity of these primers against other *Phytophthora* species is not well known since only *P. erythroseptica* was checked. PCR primers were designed from RAPD fragments amplified from *P. cambivora* and *P. quercina* and were used to detect these fungi in inoculated plants (149).

PCR-rDNA Internal Transcribed Spacer The region of the *Phytophthora* genome coding for ribosomal DNA has been the most extensively sequenced for phylogenies, and within the rDNA region, the Internal Transcribed Spacer (ITS) region has been used preferentially for studies targeting species differences. A primer pair species-specific for *P. citricola* was designed within the ITS1 and -2 regions and was used to detect this fungus in inoculated plants (149). The *P. capsici* oligonucleotide probe (90) used in combination with the universal ITS1 primer (176) gave a PCR amplicon for only *P. capsici, P. citricola*, and *P. citrophthora* of the 16 *Phytophthora* species tested (132). Positive reactions with the three species were further resolved by digestion with *Msp* I or by comparing the size of the amplicons. The technique was used successfully with infected pepper plants

from the field (132). A primer pair from the ITS1 and -2 regions was designed to amplify both *Peronospora sparsa* and *P. cactorum* directly from *Rubus arcticus* (94). Differentiation between the two species was accomplished by using another primer pair specific only to *Pe. sparsa*.

Recent introductions of the A2 strain of *Phytophthora infestans* into Europe and North America and the appearance of strains tolerant to metalaxyl have literally re-instated potato late blight as front page news. Better tools to detect latent infections and monitor inoculum would help in the management of this re-emerging disease. Trout et al (167) designed a primer from the ITS2 region that, used in combination with the universal ITS5 primer (176), could detect *P. infestans* from potato and tomato field samples. Among the 13 *Phytophthora* species tested, cross reactivity was observed with *P. cactorum* and *P. mirabilis* (167). Tooley et al (164) designed three sets of ITS primers that could detect *P. infestans, P. erythroseptica*, and *P. nicotianae* directly from potato leaves or tubers. Only the *P. nicotianae* primer did not show some level of cross reactivity against any of the nine *Phytophthora* species tested. *P. infestans* was detected 3 days after inoculation, which was the first day of sampling for the detection assay (165).

PCR-Other Genes or Spacers The intergenic region between the 5S gene and the small ribosomal subunit of *P. medicaginis* was sequenced and specific primers were designed and successfully tested with infected alfalfa from both the glasshouse and field (93). This region is more variable than the ITS region and could be useful in differentiating varieties such as *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi*, which have the same ITS sequence. Gene families such as elicitins (125) are also being used in phylogenetic studies. Species specific primers were designed from the elicitin ParA1 gene sequence to detect *P. nicotianae* directly from inoculated tomato or tobacco plants (86). Targeting new genes will become easier as more genes are studied for phylogenetic purposes (24) and as more data become available through projects such as the *Phytophthora* Genome Initiative (5).

PCR-Differentiating Amplicons by Electrophoresis Maes et al (98) used the universal ITS1-4 primers (176) to separate a limited number of oomycete species from ascomycetes based on the size of the PCR amplicon. To further separate *Pythium* species from *Phytophthora* species that had the same size of amplicon, they used as PCR primers the oligonucleotide that was specific to the genus *Phytophthora* in hybridization assays (90), in combination with the universal ITS1 primer. PCR amplification products of DNA extracted from pure cultures have also been differentiated by restriction digests and gel electrophoresis. Three restriction enzymes can diffentiate 27 different *Phytophthora* species after digestion of the amplicon generated by primers that amplify the ITS region of *Phytophthora* species directly from infected plant material (A Drenth, personal communication). An asymetric PCR was done with a primer pair that could amplify *Phytophthora* species found on potato. Species were differentiated by running a non-denaturing

gel for single-strand conformation polymorphism analysis (152). This has not been tried with infected tissue.

PCR-Differentiating Amplicons by Hybridization When probes can be generated directly by PCR, it is possible to use DNA hybridization to characterize an unknown PCR product. A cloned amplicon from RAPD-PCR was found to be *P. cinnamomi*-specific in a dot blot hybridization assay (40). Although not describing *Phytophthora* species, the following two examples illustrate an approach that might be useful with certain gene regions amplified by PCR. A 5S tandem repeat spacer amplified with conserved primers was used to develop species-specific probes for several *Pythium* species (82). Similarly, the amplified ITS1 region of *Pythium ultimum* was used as a species specific probe (92). PCR amplicons of the ITS 1 region from well-characterized isolates were bound to nylon membranes and used in a reverse dot blot hybridization (RDBH) assay for *Pythium* species. The PCR product of the ITS1 region from samples were used as probes (91). This approach produced patterns that could identify pure cultures but cross reactivity with some of the bound amplicons was observed for several *Pythium* species (91) and *Phytophthora* species (CA Lvesque, unpublished data). Similar results with this approach were obtained with *Trichoderma* and *Gliocladium* species (19). Cross reactivity with membrane-bound amplicons made this approach unsuitable for direct detection from environmental samples. The RDBH approach gave much better results when membrane-bound oligonucleotides were used instead of entire ITS1 amplicons (91). A group of specific ITS oligonucleotides including one for *P. cinnamomi* from Lee & Taylor (90) were bound to nylon membranes and used successfully in a RDBH assay for *P. cinnamomi, Pe. ultimum, Py. aphanidermatum*, and *Py. acanthicum* (91). Oligonucleotides specific to species or to clusters of species of *Phytophthora* and *Pythium* have been designed, synthesized, and immobilized in a macroarray format (Figure 1, see color plate) (CA Lvesque, unpublished). After a PCR-labeling amplification with primers specific for the ITS region of oomycetes (CA Lvesque, unpublished data), the PCR product is hybridized to the DNA macroarray. In the example given here, nested PCR was used to amplify the ITS region of oomycetes directly from a soil sample (Figure 1). The species producing the strongest reactions in RDBH were the same ones that were isolated from the roots (M Mazzola & CA Lvesque, unpublished data).

Detection of *Phytophthora fragariae*—A Case Study

Phytophthora fragariae is a well recognized pathogen of strawberry. Only recently has this pathogen been phylogenetically linked to another *Phytophthora* species that has caused an increasing number of outbreaks of root rot in raspberry plantings in North America and Europe (177). RFLP analysis with random clones from *P. fragariae* var. *rubi* confirmed the differences as well as the close association between these two pathogens and *P. cambivora* (158). The proper nomenclature is now *P. fragariae* var. *fragariae* for the strawberry pathogen and *P. fragariae*

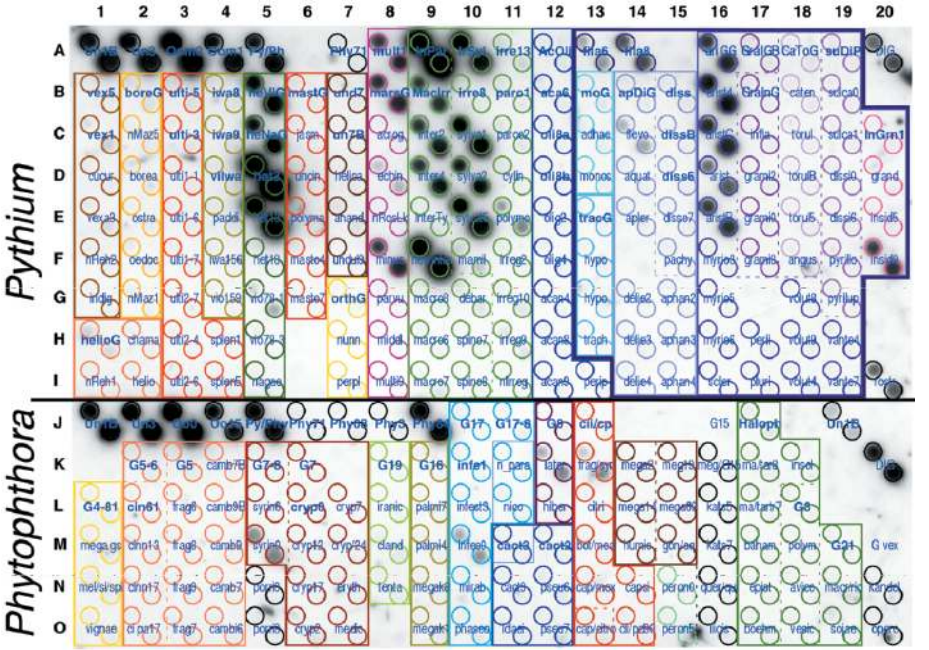

Figure 1 Results of a reverse dot blot hybridization (RDBH) test done after amplification and labeling by nested PCR of DNA extracted from soil known to have apple replant problem (CA Lévesque, unpublished). Each oligonucleotide is dotted in duplicate. The membrane is dotted with DIG labeled control oligos for detection (20A & K) as well as with more universal oligonucleotides (dots of rows A and J not within color blocks). Each block of color represents a phylogenetic cluster to which the oligonucleotide(s) in bold react. *P. intermedium* and *P. heterothallicum* were recovered in isolations from apple tree roots and apple seedling baits (M Mazzola; unpublished). *Pythium* species detected in this RDBH assay include one of the genotypes of *P. heterothallicum* (5E), *P. minus* (8F), a genotype (9F) of *P. intermedium* (9C & D) different from the genotype of the type strain (9E), *P. sylvaticum* (10C–E), *P. aristosporum* (16B–E) and a weak reaction with *P. flevoense* (14C). Some positive dots are known cross reactions with one or more of the species detected, as determined by RDBH tests done with DNA of pure cultures. These are as follows: *P. intermedium* (5G & M, 12K, 20F & I), *P. sylvaticum* (5G & M, 10M, 11E, 12K, 13K), and *P. heterothallicum* (5G, 20F & I).

var. *rubi* for the raspberry pathogen (177). This fungus is easily transmitted via vegetative propagation since strawberry and raspberry plants do not show visible symptoms at early stages of infection. *P. fragariae* is currently on the A2 Quarantine list of the European and Mediterranean Plant Protection Organization (EPPO). Unlike the A1 list, the A2 list allows member countries the freedom to include or exclude species from their country's quarantine list. This pathogen is present in Europe but its dispersal must be minimized as much as possible. A baiting technique is being used to detect *P. fragariae* (42), and there is wide interest in complementing this approach with faster as well as more specific and sensitive techniques.

PCR primers have been designed to amplify the small rDNA subunit (159) of *P. fragariae* directly from plant tissue. The forward primer was located within the intergenic spacer and the reverse primer was located at the 5′ end of the large rDNA subunit (Figure 2) (159). These primers lacked sensitivity in a single round PCR and some cross reactivity was found with *P. citrophthora, P. capsici*, and *P. nicotianae* (12), three species that had not been tested originally. Cooke & Duncan (30) provided additional sequence data to generate primers with better specificity. *Phytophthora fragariae* was detected directly from strawberry roots, raspberry roots, and zoospore dilutions with a high sensitivity using a nested PCR approach (12). The primers of Stammler & Seemüller (159) were used for an initial PCR, and a nested PCR reaction was done with the new forward (DC1) and reverse (B5) primers located within the ITS1 and ITS2 regions, respectively (Figure 2) (12). Lacourt et al (85) obtained the same results with strawberry roots using the same primers.

Baudry (8) confirmed Bonant's results but detected cross reactivity with the previously untested *P. cambivora*. By mixing various ratios of healthy and infected strawberry roots, Baudry determined that direct PCR could detect the equivalent of a single infected root from one plant, whereas nested PCR increased this sensitivity by at least 100-fold (8). She wants to develop a certification scheme that would test lots of 100 to 300 plants. Increasing plant tissue volume for PCR testing will be challenging with respect to the total amount that can be accommodated by a single PCR reaction. This issue will become even more important with the predicted further scaling down of PCR assays.

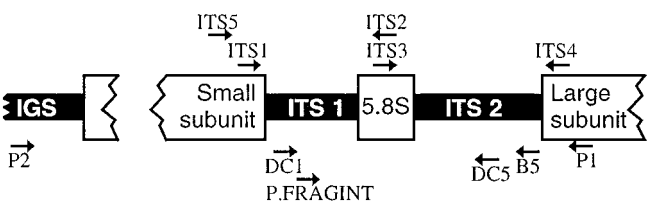

Figure 2 Diagrammatic representation of a eukaryotic ribosomal cistron showing the location of PCR primers that are universal for fungi (*top*) or specific for *Phytophthora fragariae (bottom)*.

Hughes et al (67) also developed a unique primer within the ITS1 region (P.FRAGINT, Figure 2) that when used in combination with the universal ITS4 primer gave the same specificity as the DC1-B5 pair by Bonants et al (12). Five raspberry plants were destructively sampled at various intervals after inoculation. All plants but one tested positive at 1, 2, 3, and 5 days after inoculation, after which the number of plants testing positive declined (67). Inoculated plants were all negative 50 days after inoculation. The increased incidence of negative results coincided with the appearance of oospores and the disappearance of coenocytic mycelium in the roots. The researchers concluded that the negative results were not due to PCR inhibition and postulated that DNA could not be readily extracted from oospores. DNA from coenocytic mycelium must have been rapidly degraded. A second round of PCR, a proven technique for higher sensitivity, might have improved the detection of *P. fragariae* a few days after inoculation. In a similar study, the percentage of strawberry root samples testing positive by a single round of PCR with the DC1-ITS4 primers (Figure 2) dropped from 100% to 60% after 5 days but rose again to 100% by day 12 after inoculation (85). However, all samples from 2 days onward tested positive when nested PCR was done with primers DC1-ITS4 followed by DC1-DC5 (85).

Races have been characterized for *P. fragariae* var. *fragariae* (121, 169) and for *P. fragariae* var. *rubi* (79). A molecular test that could determine the race profile in a field directly from soil samples or from baits would help in the selection of cultivars. Sensitivity of such tests will be very important since a race present at very low density will be selected for rapidly if a susceptible cultivar is planted. Monoclonal antibodies were produced in an attempt to detect *P. fragariae* and several races of *P. fragariae* var. *fragariae*. None of these was specific enough to pass cross reactivity screening against other *Phytophthora* root colonists of raspberry and strawberry (HS Pepin, unpublished data). It has been proposed that races of *P. fragariae* var. *fragariae* have avirulence genes that can be recognized by corresponding resistance genes in the host (169). Once such genes are characterized, it will be possible to design a PCR assay to determine the race profile in a field.

Additional Applications of Direct Detection or Diagnosis of Fungi

Fungal pathogens compete for ecological niches and are often present in plants as complexes. Therefore, it is important to be able to detect more than one pathogen at a time. A nested multiplex PCR assay was used to simultaneously detect *Cylindrocladium floridanum* and *C. destructans* in nursery seedlings. Specific nested primers were used after a first round of PCR with fungal specific ITS primers (61). By sequencing selected RAPD markers, PCR primers were developed to detect the cereal pathogens *Fusarium culmorum* (120), *F. avenaceum* (168), *F. graminearum* (120), *F. poae* (126), *Rhizoctonia cerealis* (118), *Tapesia yallunda* (119), and *T. acuformis* (119). In fungicide trials for *Fusarium* ear blight, the *Fusarium* assay was found to give better correlation with yield loss than visual

disease assessment (41). Based on all these different assays, a multiplex PCR test was developed that includes, in addition to the preceding species, *Microdochium nivale* var. *nivale* and *M. nivale* var. *majus* (PCR multiscreen; Adgen, Auchincruive, UK). Using this molecular analysis service, winter wheat was surveyed in the UK during 1997 and 1998 and quantitative results were obtained within 7 days after sampling (7). The speed and ability of the test to detect early infections were considered beneficial enough to use this test to assist in making decisions about fungicide applications (7). How extensive can multiplexing become? Six PCR reactions with approximately 92 different primer pairs in each tube amplified 85% of the 558 single nucleotide polymorphisms from various loci of the human genome that were targeted within the amplified regions (171).

The issue of affordability is very important if molecular testing is to be used routinely to assist in making plant disease management decisions. The cost of the test must be balanced with the cost and time of development, the ease and rapidity of the test, its portability (on-site versus laboratory), sensitivity, and specificity. Two separate primer pairs were designed from the ITS region to detect and differentiate the two causal agents of eyespot of small grain cereals, *Tapesia yallunda* and *T. acuformis* (9). *T. yallunda* is pathogenic on wheat, rye, and barley, whereas *T. acuformis* is more pathogenic on wheat than on other cereals. Symptoms caused by these two pathogens can be easily confused with other cereal diseases, especially during early stages of plant development. An ELISA assay was developed to detect *Tapesia* species in cereals. It did not differentiate between the two species but correlated well with pooled PCR results for the two species (9). Specificity of the assay was considered important in decision making (JJ Beck, personal communication), and the PCR test was used in a survey to detect and quantify these two pathogens in winter wheat (175). A pathogen detection service to assist growers is currently available (Eyespot PCR, Novartis, Basel, Switzerland). *Septoria tritici* and *Stagonospora nodorum* both cause a leaf blotch of wheat. S. nodorum also causes glume blotch. Like the previous example, detection of the pathogen in its latent phase is important for optimal fungicide efficacy. Antibody-based assays have been developed for this purpose (87, 110, 161). PCR assays targeting the ITS region (10) and the β-tubulin gene (48) have also been developed. For *S. tritici*, a PCR assay (48) and an immunological assay (78) gave very similar response curves when the detection signal was plotted against days after inoculation. Currently, an antibody-based assay is being used for routine testing over large areas and a PCR assay is also available (Septoria Watch & Septoria PCR, Novartis, Basel, Switzerland).

Direct detection in soil and quantification by PCR of a genetically modified strain of the disease biocontrol agent *Trichoderma virens* was found to be 10 to1000 times more sensitive than dilution plating (6). A rapid and cost-efficient method for extraction of DNA from soil has been developed, and the ITS region of *Verticillium dahliae* was successfully amplified with nested primers (170). *V. dahliae, V. albo-atrum*, and *V. tricorpus* were detected individually in soil by PCR (99). Interestingly, the relative detection incidence among the three pathogens

in a given field differed between the soil and the in planta testing, showing the complex competition for ecological niches that occurs. Soil is a complex environment that will require multiplex assays to better understand microbe-microbe interactions. The very wide diversity in texture and chemical content represents a difficult challenge for DNA extraction and subsequent enzymatic assays. Some of these challenges were reviewed recently (83, 116). It will be important to monitor both beneficial organisms and pathogens in soils in order to improve root disease prediction capabilities. Bacterial and fungal DNA from soil were successfully amplified by PCR using primers specific to broad phylogenetic groups (84). DNA microarrays could be used to identify the PCR products in a similar manner to what is currently being done in human genetics, where the PCR products from hundreds of different primer pairs are pooled, labeled, and sorted out by a single hybridization on a DNA chip (171).

PLANT QUARANTINE POLICIES AND REGULATIONS

International and trans-border movements of plant germplasm are necessary to satisfy increasing demands for food, access to germplasm with desirable traits, and access to newly developed or identified varieties for evaluation or inclusion in commercial production systems. These are all normal and desirable components of a sustainable and developing agricultural industry. Plant quarantine policies and regulations exist in many countries to prevent the movement of undesirable pests and/or the introduction of exotic pests when plant germplasm is moved from one location to another (174). Unregulated movement of plant germplasm could result in devastation of crops and even large-scale loss of human life (174). To limit or prevent pest movement, some complex control systems have been designed (64, 140, 162, 174), and these often include guidelines for pest detection or diagnosis. The target pests include bacteria, fungi, nematodes, viruses, phytoplasma, and viroids. Viruses and virus-like organisms (phytoplasma and viroids) are not easily detected in the vascular tissue where they reside (151, 174) and so control of their movement presents a greater challenge. In this section, other organisms are described where necessary, but emphasis is given to viruses and virus-like organisms. The principles, considerations, and benefits identified will have significance and relevanceto all pests.

Recent developments in molecular biology have helped to alleviate some of the challenges associated with the detection, control, and regulation of the movement of these pests. Molecular biology–based techniques for pest detection in quarantine and certification programs are not yet universally accepted, because of the need to validate techniques and determine their limitations, especially as related to specificity. Also, tests used for pest management and regulation must be suitable for large-scale indexing and applicable in remote areas or in laboratories lacking sophisticated equipment (39). The major considerations associated with pest detection technologies applied for the enforcement of plant quarantine regulations

and policies include cost, duration of testing, sensitivity, reproducibility (reliability), and utilization to screen large numbers of samples. Although molecular diagnostic techniques satisfy some of these needs, several barriers preventthe use of molecular techniques in routine diagnostics for plant quarantine regulation. Recent innovations and approaches for standardization and ease of implementation are making significant contributions to possible application and use of molecular techniques for routine diagnosis in implementing plant quarantine regulations.

Issues of Cost

Cost is perhaps the most important consideration for the routine use of any test in large-scale pest diagnosis in plants. This is especially important where molecular techniques are concerned since key elements of these technologies are often patented and include complex protocols requiring expensive reagents. Molecular-based techniques that are simplified or facilitate simultaneous detection of several target pests (multiplexing) may reduce cost and save time. PCR is a very powerful technique for pest detection and disease diagnosis (59) but the reaction is invariably preceded by complex and costly nucleic acid extraction protocols (97, 180). Immunocapture RT-PCR (IC/RT-PCR), which uses antibody binding to trap and enrich the target, eliminates any need for nucleic acid extraction. IC/RT-PCR detection is simpler, less costly, and more sensitive (23, 68, 73). This approach is limited because some pathogens are weak or inefficient immunogens and suitable antiserum is not always available. Use of a nonspecific direct binding or immobilization system allows PCR detection (DB-PCR) without use of an antibody (138) and this system has been used to detect several viruses. Optimal conditions to detect each virus or pathogen may differ and it may be necessary to evaluate and identify the optimal conditions to obtain maximum levels of sensitivity (75).

Simultaneous detection of several target pests (multiplexing) also reduces the cost of testing. Multiplexing systems are often PCR-based and may involve primer mixtures consisting of primer pairs specific for each target (68, 75), or single primer pairs that allow differential amplification of several targets (74, 156). *Cymbidium mosaic virus* (CymMV) (genus *Potexvirus*) and *odontoglossum ringspot virus* (ORSV) (genus *Tobamovirus*) are the most common and economically important of orchid viruses (179). A touch-down PCR technique using a single primer pair was used for simultaneous detection of both CymMV and ORSV (156). In this test 18-mer degenerate primers with nine complementary bases at the 3′ ends were used in a procedure whereby varying temperatures were used to facilitate annealing of the primers with different target sequences. Nondegenerate single primer pairs with 3′ end complementarity at variable sites have also been used in PCR analysis to simultaneously detect and differentiate *cherry mottle leaf virus* and *peach mosaic virus* (74). These are serologically related viruses with common hosts and that cause distinct diseases (70). Both approaches took advantage of the fact that DNA extension is always in the 5′ to 3′ direction and that 3′ end primer homology, 5–10 nucleotides, is all that is required for amplification to occur. Since

no virus-specific antiserum is used in DB-PCR, this system can be easily modified for multiplex PCR detection of several target pathogens or viruses (75); however, it is also possible to do multiplex PCR detection using antibody mixtures in a multiplex IC/RT-PCR technique (68). A 10- to 100-fold loss in sensitivity was observed when simultaneous detection of *tobacco mosaic virus* and *tomato mosaic virus* was carried out using multiplex IC/RT-PCR. In simultaneous PCR detection, conditions often favor a particular target, possibly due to GC content (156). Modulating the temperature as in touch-down PCR may allow efficient binding where variable primer T_M exists. Reducing the number of cycles, optimizing $MgCl_2$ concentration, and careful selection of sample preparation buffer also influence the sensitivity of the test (75, 156).

Designing specific oligonucleotide primers for PCR detection of each regulated pathogen is costly because it entails sequencing the genome of each target pathogen. Degenerate primer-mediated PCR has been used to detect several members of a particular group of viruses. A single pair of degenerate primers targeted to conserved regions in the cellular heat-shock protein (HSP) 70 gene of the *Closterovirus* genus has been used to detect several of its members (77). A conserved region extending from the virion protein gene to the Nib gene was the target of degenerate primers for detecting members of the *Potyviridae* family (52). The 3′ region of the S RNA was used as a target for *Tospovirus* genus-specific degenerate primers (113). Databases such as GeneBank provide an invaluable resource, and nucleotide sequences of members of a particular group of viruses may be analyzed and conserved regions identified and group-specific primers designed.

Sensitivity and Duration of Testing

Important benefits resulting from the introduction of molecular-based techniques to control and regulate the movement of pests include significant reduction of time required for testing and the increased sensitivity gained. Ironically, this increased sensitivity also represents one of the major disadvantages of the technology since stringent conditions and strict controls are necessary to avoid contamination that would give false positive results (75). Correspondingly and more importantly, the number of false negatives should decrease, a factor of much greater significance from the perspective of a quarantine program. The increased sensitivity allows greater confidence in the results of testing for both certification and quarantine applications, and also allows greater flexibility in the timing of testing and in the type of tissue that may be used to detect the pathogen of interest. Detection of some pathogens using traditional techniques that are serology-based is often limited to certain months of the year and to certain parts of the plant (72). PCR detection facilitates reliable testing at any time of year since the sensitivity of the test allows detection of the pathogen in most tissue including dormant woody material (71, 173). This ability to reliably detect pathogens in dormant budwood should contribute significantly to preventing the spread of pathogens because of the increased sensitivity and reduction of time imported germplasm spends in quarantine

(173). Increased sensitivity has also been achieved by taking advantage of the synergies derived from combining technologies. Immunocapture PCR and colorimetric detection methods such as RT-PCR-ELISA combine serology with nucleic acid-based techniques to obtain increased sensitivity (23, 68, 129, 137, 157). Immunocapture PCR was even more sensitive than regular RT-PCR and 2000-fold more sensitive than a standard polyclonal-antibody ELISA assay to detect *plum pox virus* (PPV) (23). This pattern has been consistent for viruses belonging to other genera such as *Tobamovirus* (68). In RT-PCR-ELISA, biotinylated DNA probes are hybridized to digoxigenin (DIG)-11-dUTP labeled PCR products, which are then detected with enzyme-conjugated anti-digoxigenin antibody. This procedure may be carried out in a microtiter plate format using colorimetric detection. RT-PCR-ELISA techniques have been used for reliable and sensitive detection of a range of viruses, viroids, and phytoplasmas, and the ELISA format facilitates testing of a larger number of samples.

Specificity

Associated with the application of molecular diagnostic techniques for quarantine program regulation is the issue of specificity. Some novel techniques may be too specific for quarantine testing where broad-spectrum tests are required (39). The incredible power and versatility of molecular based technologies allow the design of highly specific techniques where strains of pathogens or very closely related pathogens can be distinguished from each other (23, 68, 74, 123, 130, 156), or their detection capability can be very broad in scope where, instead of being strain- or virus-specific, they recognize an entire genus (52, 77, 113). If the concept of a universal tool for pathogen detection is ever realized it will very likely be a molecular diagnostic technique, or a combination of molecular techniques based on highly conserved genomic sequences. However, disparity in results has been observed when the results of serology-based techniques were compared with novel nucleic acid-based techniques (69). Lack of suitable specificity in some traditional techniques may produce false positive results. Different serotypes may exist with antibodies that are too specific for broad-spectrum detection (22, 117), which could conceivably lead to false negative results. There are also times when highly specific diagnostic tools are desirable. Very closely related pests may have vastly different pathogenicity, different host range, different vectors, and hence require different management systems. *Plum pox virus* causes plum pox or sharka disease, which is considered the most important disease of stone fruit trees (44). There are different strains of the virus with different levels of pathogenicity, different host range, and different geographic distribution (22, 117, 123, 130). Broad-spectrum detection is important, but specific identification is essential for effective management and techniques such as dot blot hybridization and PCR allow specific PPV strain identification (22, 117, 123, 130). Some traditional techniques based on serology, even when monoclonal antibodies are utilized, may be nonspecific, and techniques such as slot blot hybridization and PCR may be required for accurate diagnosis (69).

Specific diagnostic tests are also essential for bacteria such as *Erwinia*, which has many species and strains with varying host range and pathogenicity. Digoxigenin-labeled DNA probes and the Ligase Chain Reaction (LCR) assay have been used for highly specific diagnosis of *Erwinia* (172, 178). LCR, which uses a thermostable ligase enzyme for assembling segments of a target sequence, was used to develop a technique for the specific identification of the plant pathogenic bacterium *Erwinia stewartii* (178). The 16S rRNA gene of *E. stewartii* and the closely related *E. herbicola* were sequenced and primers were designed that allowed the specific detection of *E. stewartii* when 24 different species and strains of *Erwinia* were tested. This LCR technique can discriminate between *Erwinia* species on the basis of single-base-pair differences of the 16S rRNA gene. In the case of specific detection of *Erwinia carotovora* subsp. *atroseptica*, regions of repetitive DNA were identified and labeled to produce very specific probes (173). This level of specificity is essential for proper management and control of the related diseases.

Large-Scale Testing

Another requirement of diagnostic techniques used in a quarantine program is adaptability for screening a large number of samples. The techniques should be relatively simple and robust, with minimal risk of cross contamination or false positive results. Although limited, serology-based techniques such as ELISA have met these needs very successfully in the past; consequently, some molecular diagnostic techniques have been modified into an ELISA-type format. Serology has been combined with nucleic acid–based technologies in techniques such as immunocapture PCR. The protein coat found in most plant viruses has allowed the development of direct solid support binding techniques (75, 138) that facilitate reliable detection and multiplex systems. Solution hybridization using oligonucleotide probes is a nucleic acid-based ELISA-like approach to diagnosis that facilitates testing of large numbers of samples (135). This system was used to reliably detect potato virus X. The probes were complementary to target RNA sequences and may be ^{32}P-labeled or biotinylated. The probes were allowed to form hybrids with the target viral nucleic acid in solution and captured using avidin-coated polystyrene beads. Alternatively, the hybrids from the poly(A) tail of the virus could be captured using oligo (dT) cellulose. Sample preparation using this approach is simple but the sensitivity achieved is limited. A combination of colorimetric detection and immunocapture PCR provides a relatively simple but sensitive technique for the analysis of large numbers of samples and was used successfully for the detection of a range of viruses (137). In this approach, 96 well microtiter plates were coated with streptavidin, then incubated with 5′-biotin-labeled oligonucleotide detection probe. PCR was carried out using digoxygenin-11-UTP as a component of the added nucleotides. The denatured PCR products were added to the wells, followed by alkaline phosphatase conjugated anti-digoxigenin f(ab)$_2$ fragments, and eventually *p*-nitrophenyl phosphate in diethanolamine was added

as the substrate. The samples were then analyzed using a microtiter plate reader. Colorimetric detection appears to be more sensitive than gel detection of PCR products, and since most quarantine facilities use microtiter plates and plate readers, technology transfer should be relatively easy. Colorimetric detection of PCR products in a microtiter plate has also been used effectively to detect several viroids, using either biotin-labeled cDNA or cRNA capture probes (157). This approach was more sensitive than gel electrophoresis detection using either agarose or polyacrylamide. Apart from sensitivity and suitability for screening a large number of samples, microtiter well hybridization is also adaptable for automation of sample processing (157).

FUTURE TRENDS AND CONCLUSIONS

Biochip and Miniaturization

Recently, DNA biochips composed of densely packed probe arrays have brought dramatic changes and excitement to genetic analyses (53, 146). The chip-based format is very flexible and robust and has the potential to increase diagnostic throughput while simultaneously reducing unit cost. Both nucleic acid-based and antibody-based microarrays are being developed. Microarray-based techniques such as high-throughput multiplexed ELISA are being adapted for disease diagnosis (105), thereby providing a platform to modernize and enhance tried and tested serological assay systems. The technology can be used to specifically detect numerous antigens simultaneously. Biochips consisting of optically flat glass plates containing 96 wells have been used for high-throughput microarray ELISA. Each well contains four identical 36-element arrays (144 elements per well) and provides tremendous capacity for accurate reliable large-scale testing. The format is compatible with automated robotic systems.

With the further miniaturization and scaling down of molecular procedures, the probability of having the contents of even one cell of the pathogen in the aliquot to be processed will be very low (11). Concentrating the pathogen inoculum or DNA will become an essential step for most samples. Using chemotactic behavior of the pathogen for sampling will be of value whenever possible. Growth of the pathogen in a semiselective environment before processing as in BIO-PCR (145), to increase the pathogen's biomass, will probably still be necessary for many pathogens. Even with high-titer viruses, as sample size gets smaller, the likelihood of getting false negatives will increase due to uneven distribution in the host. With the miniaturization of assay volumes as tests are adapted to microchips, multiple sampling of a specimen will become very important. If sample preparation can be simplified and automated, then multiple sampling to account for uneven distribution in a host will be feasible. At the present time, collecting larger samples or pooling multiple small samples from a plant into a single extraction is more feasible due to the tedium and expense of the nucleic acid extraction methods.

Sample preparation for ELISA testing has been partially automated and testing multiple small samples from a single specimen is quite feasible. Also, using modified ELISA protocols samples can be assayed directly in the field. Nucleic acid preparation will be improved and nucleic acid–based assays will progress toward the ease with which serological assays are now done.

The future of disease diagnosis and its application to disease management is exciting. New technologies are being developed with increased sensitivities, traditional techniques are being enhanced or combined with novel nucleic acid-based techniques for significant gains in sensitivity, and innovative modifications are making complex procedures simpler and amenable to testing a large number of samples. As private companies have increasingly become involved in research and technology development, their need for cost recovery and profit has resulted in the patenting of some key technologies. The added costs and complexities associated with the utilization of the patented techniques often prevent their use in routine testing. This environment will continue to drive the development of alternate technologies and/or innovative technique modifications that reduce cost, to ensure access and relevance to quarantine programs for the management and control of pest movement and spread.

Development of Internationally Acceptable Tests

In many cases, diagnostic reagents are developed as tools used in research programs on studies of the biology, ecology or epidemiology of a pathogen. Once the research is finished, the diagnostic tools are often set aside or put in storage. These reagents are generally evaluated for specificity to local isolates of the pathogen under study or in a few cases to a broader range of isolates. The steps required to evaluate the diagnostic reagents for their usefulness in certification programs or plant quarantine are rarely taken. The evaluation process must be monitored by an internationally recognized organization that ensures suitable expertise for the crop(s) and pathogen(s) in question involved in evaluating the test. Once standards are agreed upon, protocols must be revised regularly to avoid becoming obsolete; cost and efficiency must be maintained at optimal levels and new tests evaluated. The WTO agreements will likely ensure that refusal to accept a test be based on proven science and not be used as an artificial trade barrier.

In the medical field, there is no gold standard to detect common and important fungal diseases such as candidiasis (16, 131). A project with this goal for plant pathology is entitled "Development of Diagnostic Protocols for Red Core Disease of Strawberry (*Phytophthora fragariae*)". It is funded by the European Union under the Standards Materials & Testing program. Standard protocols are being established to test strawberry plants in transit between European countries (P Bonants, personal communication). The participants are from Scotland, Germany, Sweden, and the Netherlands. The project will provide all the details and protocols (e.g. identifying specific monoclonal antibodies or oligonucleotide sequences) to minimize false positives while maintaining maximum sensitivity.

In a recent symposium titled "Moving Plant Disease Detection from the Ivory Towers to the Real World," a ring test process was described to validate and standardize new diagnostic procedures (160). Once the techniques have been validated and their limits established, certification and quarantine regulations and directives must then be modified to recognize and include molecular-based techniques as valid for determining the disease status of plants. There is already some movement in this direction. The Tree Fruit Virus and Small Fruit Virus Working Groups of the ISHS make recommendations on acceptable tests for viruses of their respective crops and the last set of recommendations were published in *Acta Horticulturae*, Numbers 471 and 472 Volume 2. These recommendations are reviewed and updated every three years by these ISHS working groups. In their recommendations for pathogen detection, PCR and ELISA are singled out for detection of a number of viruses and phytoplasmas. As more and more techniques are validated by the scientific community and their reliability and cost-effectiveness demonstrated, molecular biology-based techniques will play a greater and more important role in the detection of all classes of plant pathogens and management of the diseases they cause.

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