

# ADVANCES IN MOLECULAR-BASED DIAGNOSTICS IN MEETING CROP BIOSECURITY AND PHYTOSANITARY ISSUES\*

---

Norman W. Schaad,<sup>1</sup> Reid. D. Frederick,<sup>1</sup> Joe Shaw,<sup>2</sup>  
William L. Schneider,<sup>1</sup> Robert Hickson,<sup>3</sup>  
Michael D. Petrillo,<sup>4</sup> and Douglas G. Luster<sup>1</sup>

<sup>1</sup>USDA/ARS, Foreign Disease-Weed Science Research Unit, Ft. Detrick, Maryland 21702; email: nschaad@fdwsr.ars.usda.gov; rfrederick@fdwsr.ars.usda.gov; wschneider@fdwsr.ars.usda.gov; dluster@fdwsr.ars.usda.gov; <sup>2</sup>Lexicon Genetics, The Woodlands, Texas 77381; email: jshaw@lexgen.com; <sup>3</sup>Joint Special Operations University, Hulburt Field, Florida 32544; email: hicksonrd.dfg@usafa.mil; and <sup>4</sup>USDA/APHIS, Plant Inspection Station, South San Francisco, California 94080; email: michael.d.petrillo@aphis.usda.gov

**Key Words** polymerase chain reaction (PCR), real-time PCR, bacteria, viruses, fungi, and nematodes

■ **Abstract** Awareness of crop biosecurity and phytosanitation has been heightened since 9/11 and the unresolved anthrax releases in October 2001. Crops are highly vulnerable to accidental or deliberate introductions of crop pathogens from outside U.S. borders. Strategic thinking about protection against deliberate or accidental release of a plant pathogen is an urgent priority. Rapid detection will be the key to success. This review summarizes recent progress in the development of rapid real-time PCR protocols and evaluates their effectiveness in a proposed nationwide network of diagnostic laboratories that will facilitate rapid diagnostics and improved communication.

## INTRODUCTION

### New, Emerging, and Introduced Pathogens

The greatest threats to agriculture have always come from nature: freezing temperatures, hail, high winds, flooding, droughts, and pests and pathogens. New and emerging diseases are an ever-increasing reality for phytopathologists. The ease, speed, and growth in international travel over the past century have ended the United States' isolation. Travelers entering the United States often attempt

---

\*The US Government has the right to retain a nonexclusive, royalty-free license in and to any copyright covering this paper.

to bring along live plants or plant materials that may harbor exotic pests and pathogens.

The deliberate release of a crop pathogen is not a new threat. Deliberate releases of plant pathogens onto crops have been reported since biblical times (64). The Former Soviet Union (FSU), the United States, Canada, Germany, Japan, China, South Africa, and Britain are known to have had major crop biowarfare programs (104).

Despite the historical record of biological agents as weapons (2, 4, 69, 104, 105), the potential scope of the deliberate release of a crop pest or pathogen has been realized only relatively recently (57, 81, 102). The likelihood of a major deliberate release in the United States was deemed remote before the anthrax releases in October 2001 dramatically brought home the reality and need for increased biosecurity.

An accidental or intentional introduction of a regulated plant pathogen could easily cause losses to the economy in the tens of billions of dollars (37). The California legislature, mindful of the threat to the state's agriculture, enacted the Hertzberg-Alarcon California Prevention of Terrorism Act in 1999 (Article 4.6 commencing with Section 11415 of Chapter 3 of Title 1 of part 4 of the Penal Code). This legislation includes specific language regarding intentional release of any animal or crop pathogen.

The protection of animals and crops from accidental or deliberate introduction of pathogens is an important national security priority for the United States (28, 38). APS/Net Feature articles in 1999 (81) and 2002 (56) characterized crop biosecurity threat issues, and the former contained numerous links to related web sites. Recent technological advances that allow for the genetic manipulation of pathogenic virulence and other characters (49) have significantly raised the threat of a deliberately released foreign pathogen to become established. The number of high-risk pathogens identified today far outstrips those that were known during WWII and the Cold War.

## Crops and Crop Products as Soft Targets

The food production and distribution network is susceptible to contamination with human pathogens such as *Escherichia coli* and *Salmonella* species. *E. coli* 0517:H7 increases in numbers very rapidly on small fruits and vegetables following infection with plant pathogens (23). If no plant pathogens are present, numbers of *E. coli* remain below thresholds needed for human infection. Also, plant pathogens themselves can pose real threats. Several plant pathogenic fungi (1, 12, 14, 58) and bacteria (7, 43, 58) produce animal and human toxins in plants. Hybrid vegetable seed, which is mostly produced abroad by local contract growers, can serve a major avenue for accidental or deliberate introduction of seedborne pathogens. Watermelon, for example, is an important fresh market vegetable threatened by a devastating seedborne bacterial disease—watermelon fruit blotch caused by *Acidovorax avenae* subsp. *citrulli* (48). Although there are only 250,000 acres, the wholesale crop value is estimated at approximately \$450 million, with hybrid seed adding a wholesale market value of \$75 million (A. Abbott, personal communication).

How do we protect our crops and crop products against introductions that may manifest as subtle, even latent, disease outbreaks? The phytopathologist has much to contribute, in well thought out collaboration with other professionals, to the strategic protection of crops against the accidental or deliberate introduction of foreign pathogens. Soft targets such as agriculture do have consequences, especially when one considers the economic impact in potential losses in trade (37).

## PLANT PATHOGEN DETECTION AND DIAGNOSTIC TECHNOLOGIES

### Identifying High-Risk Pathogens

One of the best defenses against the introduction of new plant diseases, by either accidental or deliberate means, is rapid detection. One mission of the plant pathology research program of the USDA/ARS Foreign Disease-Weed Science Research Unit (FDWSRU) at Ft. Detrick is development of rapid molecular-based systems for detection of naturally introduced foreign pathogens for use by Animal Plant Health Inspection Service (APHIS), DOD, other federal and state agencies, and universities. A top priority in accomplishing that goal is to identify those pathogens posing the greatest threat to U.S. crops (71). There are over 500 pathogens that can cause major disease losses; a reliable methodology for rating and prioritizing those pathogens of the highest risk is therefore essential. Madden et al. (57) have discussed the importance of epidemiological data in establishing lists of high-threat pathogens. An official list of select foreign pathogens of high risk has been compiled independently by APHIS (<http://www.aphis.usda.gov/ppq/permits/bioterrorism/>). Work with any of those on the list requires the laboratory and personal to be registered.

### Diagnosing Diseases Under Field Conditions

Presumptive diagnosis of plant diseases in plants showing symptoms can be relatively simple when typical, definitive symptoms are evident. However, symptoms are not always unique and can be confused with other diseases. Typical examples are halo blight of beans, caused by the regulated *Pseudomonas syringae* pv. *phaseolicola*, and brown spot of beans, caused by the unregulated organism *P. syringae* pv. *syringae*. The lesions can normally be differentiated on green leaves because of the yellow halo produced by *P. syringae* pv. *phaseolicola*. However, no halos are visible on dried pods; both pathogens produce indistinguishable brown spots (Figure 1). Diagnosis of plant diseases can be even more difficult with infected seeds or asymptomatic infected propagative materials such as tree-grafting stocks or potato tubers (70). Traditional isolation and pathogenicity tests require 10 to 20 days or longer, enough time for bacteria and aerial fungi to spread dramatically, causing severe epidemics.

## Serological Assays

Serological assays were originally developed to detect viruses, since they could not be cultured. Formats for immunodiagnostic techniques include enzyme-linked immunosorbence assays (ELISA), immunofluorescence (IF), immunofluorescence colony staining (96), and immuno-strip tests (33). ELISA, by far the most common immunodiagnostic technique, has been consistently used for virus and bacteria detection since the 1970s (15), long before DNA-based techniques were available. There are over 800 different antisera available for plant viruses through the American Type Culture Collection (<http://www.atcc.org/SearchCatalogs/PlantVirology.cfm>). Polyclonal and monoclonal antisera for many viruses and bacteria have been developed for commercial use or in individual labs. These antibodies have been used in numerous protocols to identify viruses, including immunodiffusion assays, western blots, dot-blot immunobinding assays, immuno-strip assays, and serologically specific electron microscopy (SSEM). However, ELISA remains the consistent protocol of choice for viruses and bacteria in diagnostic labs due to its high throughput capability (33, 70, 78).

The sensitivity of an ELISA assay varies depending on the organism. Commercial ELISA kits are available for many viruses, bacteria, and fungi (Agdia, Elkhart, IN and ADGEN, Ayr, Scotland). The typical sensitivity of ELISA for bacteria is  $10^5$  CFU/ml. The technique works best for diagnosis when samples consist of fresh lesions containing very high pathogen titers. IF techniques use fluorescent-labeled antibodies that react either directly with cell antigens or indirectly with anti-rabbit globulin and allow for direct visualization of cells with a fluorescent microscope. The technique has an advantage in that cell morphology is observed. IF is especially useful for detecting seedborne and tuber-borne bacteria (70), but is reliant on human judgment to determine whether a cell is fluorescent. IF can also be used to identify colonies of bacteria in agar media (96), which can be advantageous since it is based on viable cells. For rapid presumptive identification, immuno-strips can be very useful. The technique is inexpensive and requires little labor or knowledge. Specificity of all immunoassays can be improved by using monoclonal antibodies. However, increased specificity means that some target strains may be missed (false negative). Bacteria, which are very complex and heterogeneous with respect to surface antigens, can vary with the environment in which the cells are growing. Serological techniques can greatly reduce the time needed for diagnosis; however, the results should only be considered as presumptive since both false positives and negatives are possible.

## DNA-BASED POLYMERASE CHAIN REACTION (PCR) TECHNIQUES

PCR (59) has revolutionized molecular biology and diagnostics. A rapid serological dip-stick technique followed by PCR has been the preferred method for detection of spores of anthrax. However, currently such results are only considered presumptive, as the official gold standard assay certified by the Centers for Disease

Control (CDC) for identification of anthrax is isolation of the organism. With the development of PCR, DNA-based techniques have rapidly become the preferred tool for identification of plant pathogenic bacteria. A large number of classical PCR primers for identification of important plant pathogenic bacteria are listed in the Laboratory Guide for Identification of Plant Pathogenic Bacteria (78) and many more are becoming available. PCR-based assays offer many advantages over traditional isolation and immunological methods; most important are specificity and time.

Specificity of PCR depends upon the uniqueness of the sequences selected for primers and probes. Improvements in sequencing technologies are making the selection of reliable PCR primers routine (75).

## Real-time Fluorescent-based PCR

Real-time PCR can use TaqMan<sup>TM</sup> probes, fluorescent resonance energy transfer (FRET) probes, or molecular beacons to detect the production of amplicons. These methods are based upon the hybridization of fluorescently labeled oligonucleotide probe sequences to a specific region within the target amplicon that is amplified using traditional forward and reverse PCR primers. In the TaqMan<sup>TM</sup> system, an oligonucleotide probe sequence of approximately 25–30 nucleotides is labeled at the 5' end (39) with a fluorochrome (50), usually 6-carboxyfluorescein (6-FAM), and a quencher fluorochrome, usually 6-carboxy-tetramethyl-rhodamine (TAMRA), at the 3' end. The TaqMan<sup>TM</sup> probe is degraded by the 5' to 3' exonuclease activity of the *Taq* polymerase as it extends the primer during each PCR amplification cycle and the fluorescent chromophore is released (Figure 2). The amount of fluorescence is monitored during each amplification cycle and is proportional to the amount of PCR product generated.

FRET probes require labeling of two adjacent oligonucleotide probe sequences within the PCR target fragment (16, 20). Probe 1 contains a fluorescein label at its 3' end, whereas the second probe is labeled at its 5' end with another label such as Light Cycler Red 640. The two probes must be designed so that when they hybridize to the amplified PCR product they are aligned head-to-tail to bring the two fluorescent dyes in close proximity to each other (20, 26). The fluorescein dye attached to the first probe is excited by the light source of the appropriate wavelength, and it emits a green fluorescent light at a slightly longer wavelength. When the second probe is in close proximity, the energy emitted by the first probe excites the Light Cycler Red 640 dye attached to the second probe, and red fluorescent light at longer wavelength is now emitted that can be detected at 640 nm. Fluorescence is measured during the annealing step of each of the amplification cycles when both probes hybridize to the PCR amplicon (16, 20). Molecular beacons are hairpin-shaped fluorescent oligonucleotide probes. The loop portion of the molecule contains nucleotide sequences that are complementary to the target amplicon. A fluorescent chromophore is attached at the 5' end of the probe and a quencher molecule is attached at the 3' end. A stem structure is formed by annealing of the complementary arm sequences that are added on both sides

of the probe sequence. When a stem structure is formed, the fluorophore transfer energy to the quencher. However, when the probe hybridizes to the target amplicon during PCR amplification, the fluorophore and quencher become separated from each other and fluorescence can be detected (16, 20).

The first platform designed for real-time PCR, ABI7700 Sequence Detection System® from Applied Biosystems (Foster City, CA), allows up to 96 samples to be run simultaneously and provide endpoint data analysis within 2 to 3 h. Its high throughput capacity makes the ABI7700 well suited for use in the pharmaceutical industry, but its very high cost (over \$US 80,000) is a deterrent to routine diagnosis of plant diseases. By using real-time TaqMan PCR and a Perkin Elmer 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), the time for PCR can be reduced from 2 days to 2–3 hours (29, 55, 72–74, 79, 98). Due to its very high throughput, the 7700 is very cost-effective when used routinely for large numbers of samples.

Perhaps the most significant improvement in real-time PCR for diagnostics has been the development of relatively inexpensive, ultra-fast portable thermocyclers. Several such instruments are available for performing rapid-cycle real-time PCR, including the LightCycler™ from Roche Diagnostics Corporation (Indianapolis, IN), the R.A.P.I.D. from Idaho Technologies (Salt Lake City, UT), the SmartCycler® from Cepheid, Inc. (Sunnyvale, CA), the iCycler iQ™ from Bio-Rad (Hercules, CA), the MX4000™ from Stratagene (La Jolla, CA), the Rotor Gene, from Corbett Research (Sydney, Australia), and the Gene Amp 5700® from Applied Biosystems (Foster City, CA). Except for the GeneAmp 5700® instrument, these units are capable of data collection (monitoring fluorescence) during each cycle of PCR amplification. The R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device) and SmartCycler units are portable instruments that cost under \$US 30,000. These units were designed for military personnel to rapidly detect biological agents in the field. Watermelon fruit blotch (76, 82) (Figure 3) and Pierce's disease (PD) of grape (79) could be diagnosed using real-time PCR on-site in one hour or less. The PD assay is even easier and more reliable with the use of dry bead formulations (Cepheid) containing all PCR ingredients, including *Xylella*-specific primers and probe (N.W. Schaad, unpublished). Real-time PCR has many important advantages over classical PCR: it yields data in real-time, is quantitative, and does not require a separate step to detect amplified products (agarose gel electrophoresis); results are based on hybridization of the probe to the target amplicon, i.e., a time-consuming Southern blot is not needed for maximum sensitivity and for confirming the identity of the amplified product.

## PCR PROTOCOLS TO DETECT PLANT PATHOGENS

### Bacteria

Classical and real-time PCR protocols are available for many different bacteria (35, 53, 78). Although real-time PCR is fast and can be very specific, the technique is often not as sensitive as agar media-based assays to detect pathogens present in plant extracts, as inhibitors (74, 98) and small sample volumes can

reduce sensitivity. However, PCR can be used in combination with isolation on media, a technique termed BIO-PCR (74, 80) whereby viable cells of the target bacterium can be enriched in liquid or solid media and detected in extremely low levels in seeds and other propagative materials. In a BIO-PCR assay, the plant extract is plated onto agar or added to liquid media and enriched for 15 to 72 h, depending upon the organism, and the resulting cell growth used for direct PCR. No DNA extraction is needed for bacteria since the cells will lyse during the initial denaturing step of the amplification (74, 80). BIO-PCR protocols have been developed for several bacteria including *P. syringae* pv. *phaseolicola* (72, 74), *Clavibacter michiganensis* subsp. *sepedonicus* (73), *Ralstonia solanacearum* (42), *R. solanacearum* bv-2 (98, 99), *Xanthomonas albilineans* (97), *Acidovorax avenae* subsp. *avenae* (76, 82), *A. tumefaciens* (100), and *E. coli* (87). When time is more important than sensitivity, BIO-PCR is not recommended. For ultra-high sensitivity, BIO-PCR can be used with membranes (77). Using surfactant-free membranes (Sartorius, Edgewood, NY, no. 12587) made especially for BIO-PCR, the sample is filtered to retain the target organism and the membrane is placed on semiselective agar media. After incubation for 1 to 3 days, the filter is removed and placed into a microfuge tube with 50- $\mu$ l water and vortexed to suspend the bacteria. The sample is then used for direct PCR. This works well for samples such as leaf washings or pond water. With *P. syringae* pv. *phaseolicola*, 1 to 3 cfu/ml water are routinely detected (72, 77). Membranes can also be used for standard PCR without waiting for BIO-PCR. An added advantage of membrane PCR is that the sample is freed from PCR inhibitors. Membranes can also be used successfully with direct PCR (without enrichment) by placing membranes of smaller diameter directly into the reaction tube. The main disadvantage of using membranes in PCR is that the technique introduces a step for possible cross-contamination. An alternative to BIO-PCR for increasing the sensitivity of PCR is to use immunocapture PCR (27, 66).

Real-time BIO-PCR protocols have detected *P. syringae* pv. *phaseolicola* in seed extracts (72) and *C. sepedonicus* in potato tuber extract at a concentration of 2 cfu/ml (73). Of 30 naturally infected potato tubers assayed by agar plating and conventional real-time PCR, 4 and 8 were positive, respectively. In contrast, with BIO-PCR 26 of the 30 tubers were positive (73). Similarly, Weller et al. (98, 99) found that conventional real-time PCR worked poorly for detecting *R. solanacearum* in potato tubers whereas BIO-PCR worked well. In addition, multiplex PCR can be employed to detect more than one species of bacterium in the same reaction tube using probes labeled with different fluorescent dyes such as FAM, TET, TAM, and ROX. To avoid cross-absorption, the wavelength of each dye must be well separated. We have used FAM and ROX (Texas Red) to detect the Pierce's disease bacterium and bacterial blight of grape bacterium in a single reaction tube (N.W. Schaad, unpublished).

## Viruses

A number of techniques, including hundreds of electron microscopy, nucleic acid hybridization, ELISA, and PCR-based protocols have been used to detect viruses in

crops. The most commonly used survey and indexing protocols now take advantage of ELISA or PCR techniques. ELISA detection kits are readily available for a large number of viruses, and are often favored by diagnostic labs for their speed and relative cheapness, despite their lack of sensitivity. Many viruses exist at low or variable titer levels that are hard to consistently detect using ELISA. In addition, the time involved in good antibody production, the possibilities for false positives, and the inability to differentiate between closely related viruses also detract from the effectiveness of ELISA detection protocols. Finally, ELISA cannot detect some of the important pathogens that exist solely as nucleic acids, such as viroids.

PCR and reverse transcription-PCR (RT-PCR) have long been used to detect viruses. The vast wealth of available sequence data from viral genomes combined with the rapidly advancing technologies in the field make viruses ideal candidates for PCR detection technology. Sufficient sequence data for diagnostic primer and/or primer/probe sets are available for thousands of plant viruses, and the list grows longer every year. The volume of available sequence data makes it possible to develop primers for detection at several different levels. Alignments of groups of viral sequences are valuable for identifying conserved regions for primers that will amplify numerous viruses as well as variable regions that can be used to distinguish between viral species and strains. Such alignments have been used to develop generalized primers for the detection of entire plant virus families (21, 32), for accurate identification of individual virus species (too many to mention), and for differentiation of strains and isolates (92). Of the many advantages of PCR-based detection of viruses, the most significant for combating a deliberately released virus is the increased sensitivity. PCR is also better to detect emerging viruses because PCR primers can be made with only a little a priori knowledge of the sequence, whereas antibody production requires purification of the virus, animal injection, and purification of the resulting antibody. Most plant viruses have RNA genomes, but the development of consistently sensitive one-step RT-PCR protocols has eliminated any extra time or labor that might be associated with RNA detection using PCR protocols.

As we face challenges to crops from intentional or unintentional introductions of exotic plant viruses, speed and accuracy of detection become paramount. PCR-based technologies will undoubtedly figure strongly in any preventative detection plans. Many researchers have taken advantage recently of the speed, sensitivity, and quantitative nature of real-time PCR to develop assays (9, 25, 45, 53, 84). These techniques are so sensitive that they can detect *Tomato spotted wilt virus* in single thrips vectors (10), and *Plum pox potyvirus* (PPV) in aphid vectors (W.L. Schneider, unpublished data). Multiplex PCR, where several viruses are diagnosed in a single reaction, has been used to detect a number of plant viruses (44, 60, 92). Array technology, which is typically used to study the expression of multiple genes simultaneously, has also been used for viral strain diagnosis (47, 52). Viral detection strategies could also be developed for plant viruses where multiple viruses are detected simultaneously on arrays.

## Fungi

Over the past decade, the number of PCR assays, including real-time assays, to detect fungal plant pathogens has burgeoned. Some of these assays have allowed for the differentiation between closely related species that have been difficult to separate based upon subtle morphological characteristics or differences in host range.

For example, real-time assays using TaqMan™ probes have been described for *Phytophthora infestans* and *Phytophthora citricola* from potato and citrus, respectively (8); *Diaporthe phaseolorum* and *Phomopsis longicola* from soybean seeds (106); *Helminthosporium solani* (18), *Colletotrichum coccodes* (19), and *Rhizoctonia solani* (51) from soil and on potato tubers; and *Aphanomyces euteiches* from alfalfa (95). In addition, a real-time PCR assay using molecular beacons was developed for *Rosellinia necatrix*, white root rot of fruit tress (83) and SYBR Green was utilized in assays to detect *Pyrenophora* species in barley seeds (6, 93); *Blumeria graminis* f.sp. *tritici* on wheat (26); rice blast caused by *Magnaporthe grisea* (68); and *Cladosporium* sp., *Ramularia* sp., and *Microsphaera alphitoides* on oak (36). At the FDWSRU at Ft. Detrick, several real-time PCR assays have been developed using TaqMan™ probes for *Tilletia indica*, which causes Karnal bunt of wheat (29), and *Phakopsora pachyrhizi*, which causes rust on soybean (Figure 4) (31). Initially developed using the ABI 7700 Sequence Detection System, these assays have been adapted for use with both the SmartCycler® (30) and the R.A.P.I.D. (R.D. Frederick & D.J. Sherman, unpublished results) for rapid identification at remote locations or at field sites.

An effective rapid diagnosis of a regulated foreign disease in the United States is illustrated by Karnal bunt disease of wheat, caused by the fungus *Tilletia indica*. The disease was discovered in India in 1930 and remained limited to India, Pakistan, Iraq, Nepal, and Afghanistan (11) until it was found in Mexico in 1972. The United States established a zero tolerance on Karnal bunt to protect U.S. wheat. In 1996, an observant pathologist (Mr. Ron Yakima) at the Arizona State Department of Agriculture found what appeared to be bunted grain and teliospores (resting stage of the fungus) of Karnal bunt in wheat seed in Arizona (11). Warnings from Dr. Morris Bonde, a pathologist at USDA/ARS FDWSRU, had alerted state pathologists to the danger of the disease in wheat seeds (11). Suspect spore samples sent to Dr. Bonde for identification were germinated and within 10 days were confirmed by two separate PCR assays to be *T. indica* (11). The affected fields were immediately deep plowed, quarantines were established in the contaminated sites in Arizona and New Mexico, and the region was surveyed for additional Karnal bunt. The subsequent discovery of the organism in a small area along the Arizona border extended the quarantined area to a three-state area. This discovery caused immediate alarm in the wheat export market and threatened the entire \$6 billion/year U.S. wheat crop.

The U.S. Karnal bunt outbreak, although loss to disease was relatively minor, illustrates the wider economic consequences that the introduction of a regulated pathogen can cause. It also demonstrates the efficacy of rapid quarantine procedures

in containing a slow-moving soilborne pathogen such as Karnal bunt, even though dormant spores can spread via seeds to unaffected areas. Quarantine measures to contain an established bacterial infection in which the pathogen readily spreads during rains have a much lower probability of success than that of Karnal bunt.

Several challenges must be overcome in developing successful real-time PCR assays for fungal plant pathogens. The assays must have absolute specificity for the fungal pathogen of interest and should not detect amplicons from other closely related species or *formae specialis*. PCR primers and probe sequences must be designed to unique DNA sequences, and, therefore, nucleotide sequence information must be available from the target organism or it must be generated. Unique or characteristic DNA sequences need to be identified for the pathogen of interest. Once PCR primers and probes have been developed, the assays must be optimized and rigorously tested against other closely related species to prevent false positives. In addition to having the necessary specificity, assays for fungal plant pathogens must detect the target pathogen at an acceptable level of sensitivity. Furthermore, since direct PCR does not work for fungi, efficient DNA extraction protocols need to be developed from infected plant material, and, in some instances, from isolated spores. *T. indica* is an example of the latter, in which assays are required to correctly identify suspect spores lest entire wheat shipments be refused entry into an importing country. Unlike bacteria, fungal spores tend to be difficult to break open. Methods such as grinding spores with sand or glass beads or sonication show promise for breaking fungal spores.

Few, if any, PCR assays are used to identify fungal pathogens in disease diagnostic laboratories. This reluctance may be attributable to lack of funds to purchase the necessary equipment, lack of adequately trained personnel, or the lack of a system or mechanism to validate assays for general use.

## Nematodes

PCR-based assays have been performed on several genera of nematodes, although most have not focused on the development of diagnostic assays using such techniques. Many of these nematodes are not necessarily of federal quarantine significance nor are they likely candidates for intentional introduction because epidemics of nematodes develop slowly. Interestingly, four of the ten nematode species listed for priority genome sequencing in the white paper, *Plant-Associated Microbe Initiative*, produced from a workshop on "Genomic Analysis of Plant-Associated Microorganisms" in Washington, DC, are of federal quarantine significance. The lack of published molecular research on nematodes with utility for diagnostic identification of quarantine-significant nematodes may be attributable to quarantine restrictions on research and the lack of information regarding economic repercussions if introduced. Current PCR-based protocols of several non-quarantine-significant nematodes provide a strong foundation for developing diagnostic assays, however.

Utilization of PCR-based assays measuring intraspecific, interspecific, and intergeneric genetic variability has revealed numerous polymorphic nucleotide sites specific to a nematode genus or species. Mixtures of *Meloidogyne arenaria*,

*M. incognita*, *M. javanica*, and *M. hispanica* were differentiated using a multiplexed PCR-based assay that simultaneously amplifies two small regions of mtDNA that are then digested with restriction enzymes (89). Using the same technique, *M. hapla* and *M. chitwoodi* were identified from mixed populations. Similarly, Bates et al. (5) developed a quantitative real-time PCR assay for determining as little as 2% of a species in mixed populations of *Globodera pallida* and *G. rostochiensis* using the Light Cycler. Numerous studies are available for developing diagnostic species-specific tests for *Ditylenchus* (101), *Aphelenchoides* (41), *Pratylenchus* (63), *Meloidogyne* (22), *Anguina* (67), *Globodera* (90), and *Heterodera* (13, 40). Such tests would have utility in confirming the presence or absence of known nematodes based on rapid initial identification to the genus level using morphological characters. In the future, it may be possible to rapidly screen for many of the most commonly encountered nematodes solely using multiplexed real-time PCR but several complications must be overcome.

Sampling nematodes requires extraction from soil or roots, which can be time consuming, though maceration of endoparasitic nematodes within roots may be considered. Fortunately, only a single juvenile (91) or cyst (5) is required for acceptable detection, although the resilient nature of the cuticle hinders rupturing of vermiform types. Many of the same challenges in developing PCR-based assays for fungi occur with plant-parasitic nematodes. Development of unique PCR primers and probe sequences, optimizing assay conditions, and testing against closely related species will be required. Species-specificity of primers to the target nematode must be achieved though this may be difficult for all populations of a species. Petrillo (65) observed phenotypic variation among progeny of an isofemale lineage of *M. incognita*, a mitotic parthenogen. Race shifts as a result of host selection in populations of *H. glycines* (17) stress the need for careful selection of primer specificity. Therefore, collection and extensive cataloging of geographically diverse nematode populations is needed to ensure the success of rapid species-specific molecular identification.

## FUTURE TECHNOLOGIES

DNA arrays have become standard for certain diagnostic methods in medicine and are being implemented variously (3, 24, 103). These tools are valuable because they can look for many genes and (hence) many organisms at once, and also because the technology is well understood and commercially available. Other technologies for more rapid disease diagnosis are foreseen. The direction of such detection technologies will likely be driven by military imperatives, clinical needs, as well as food safety. Clearly, work will proceed on the many immunology-based and nucleic acid hybridization- or aptamer-based detection methods (85). Other discernible trends include remote or noninvasive sampling, real-time sampling, and miniaturization.

Electronic noses are an example of a remote sensing technology that occurs in real-time. Electronic noses have been designed to look for markers of food

quality, impurity, or infection and are already in industrial use (34). Electronic noses employ an array of sensors, each sensor devoted to a single compound or class of compounds. There are two basic sensor types used in the commonly available electronic noses: (a) polymers that absorb certain vapors or (b) metal oxides that interact with volatiles. In either case, as the sensors interact with target molecules their electrical resistance is changed: The change in resistance signals a sensing event. Sensors may overlap in sensitivity, be affected by humidity, size of molecules, or have other qualities and traits. The best electronic noses employ multiple sensors in arrays and use pattern recognition algorithms to sort out responses. The technology has been reviewed recently and it clearly has relevance for multiple medical applications (94). Electronic noses have been used to identify pathogens that might be found in respiratory tract infections (46) and also to detect mycotoxins and other compounds in grain (62).

The use of living cells or whole organisms as sensors will continue to grow in the near future. This area of technology is based on the exquisite responses the living cells and organisms have developed to measure and respond appropriately to their environments. The United States Defense Advanced Research Projects Agency has several research programs designed to employ living systems as biosensors. One research area involves the development of tissue-based biosensors (<http://www.darpa.mil/dso/TextOnly/thrust/sp/bwad.2.htm>). Conceptually, the idea is simply to find cells that can be deployed in two or three dimensions (layers or sponges), which can detect trace amounts of substances and appropriately transmit a detection signal. There is no limit to this approach: Cells could come from animals or plants: they could be immunological cells, leaf cells, or microorganisms. Practical implementation of these ideas will not occur soon. Whole organisms coupled with reporter genes have been proposed to detect specific situations (e.g., lack of oxygen) or molecules (e.g., antibiotics). For instance, constitutive production of bioluminescence in cyanobacteria is disrupted by certain herbicides: Bioluminescent cyanobacteria can be used to detect the herbicides and other possible pollutants because they respond to them with decreased growth and thus decreased bioluminescence (86). Clearly, multiple organisms can be engineered to express reporter genes, constitutively or conditionally, and could serve as biosensors depending upon the conditions of induction.

## NEED FOR DIAGNOSTICS IN PHYTOSANITARY PROTOCOLS

### Quarantine and Regulation

Although traditional diagnostic methods such as isolation on agar media and pathogenicity tests work well when time is of less importance such as in routine diagnosis, detection of a deliberately or accidentally released pathogen of high-risk potential is time sensitive. Currently, federal port inspectors employ only visual inspections for detection of plant pathogens. Since seeds are highly

vulnerable to infection and/or contamination without showing any visible symptom (61), thought should be given to the feasibility of incorporating molecular diagnostic testing protocols at ports of entry. This is important since most hybrid vegetable seed is produced abroad in such countries as India, China, Chile, Turkey, Vietnam, and Mexico (H. Bolkan, personal communication).

## Protecting Avenues of Entry

Nearly all federal inspections for cargo, maritime, and mail employ visual identification of fungal pathogens; most bacterial, viral, and nematode pathogens are ignored. Trace-back capabilities can allow likely sources for products found harboring pathogens to be determined. Perhaps the greatest risk lies in the deliberate entry of pathogens through personal mail or in passenger baggage. Addressing this risk presents a challenge to the effectiveness in the interception of pathogen cultures. Although beagle dogs are employed productively to sniff out food products, there is some question as to their effectiveness for detecting cultures of pathogens. Airport passenger inspections typically involve less than 20% of total passenger volume. Diagnostic molecular tools are not yet available to federal inspectors, who must rely on verbal questioning, X-ray technology, and physical inspection to prevent introductions. Such rudimentary techniques allow a high probability of success for deliberate smuggling of a pathogen culture. Suspected materials are submitted for microscopic observation and taxonomic identification based on morphological characters and not molecular markers. Training for detection of signs and symptoms of plant pathogens is port dependent and variable. Improved communication among APHIS plant pathology identifiers and federal inspectors in the field is increasing through sharing of digital images of disease interceptions at ports of entry.

Significant gaps remain in field and laboratory diagnoses due to lack of cost-effective molecular diagnostic tools. Collaborations between plant pathology identifiers and others involved in research plant pathology could provide opportunities to move forward on use of field samples acquired for detection and diagnosis. Such collaboration will add to collections of microbial germplasm and for use in developing rapid molecular diagnostics and understanding better the ecology of the disease agent.

## Changes in the Diagnostics Infrastructure

Currently, there is a grave shortage of both field pathologists to identify and respond to accidental or deliberately released plant pathogens (26) and diagnostic laboratories equipped to perform the assays that are being developed for rapid, same-day detection of plant pathogens (75, 76, 79). Funding by Congress in 2002 to create five regional Plant Surveillance and Detection Centers at Cornell University, Kansas State University, Michigan State University, University of Florida, and University of California, Davis for improving plant diagnostics is a good start. Formal procedures are lacking for systematically surveying, reporting, or tracking new and

emerging plant diseases nationwide (26). Although many reliable assays are available, no official program such as the Centers for Disease Control have for human assays is available to certify their reliability. The European Union (EU) has recently implemented an accreditation program, Diagnostic Protocols for Organisms Harmful to Plants (DIAGPRO) (<http://www.csl.gov.uk/prodserv/know/diadpro>), to validate methods for routine detection of regulated pathogens. A committee of expert scientists is charged with coordinating comparative tests at a number of official laboratories to identify organisms of interest. This program has no counterpart to date in the United States. Diagnostic tools are advancing with highly reliable classical agar plating, serology, and molecular-based assays already available to detect and identify key plant pathogens (70, 78) and many more in the pipeline. However, no diagnostic lab, whether USDA, state-run, or university, is yet set up for routine use of advanced molecular technologies such as real-time PCR. One private diagnostic laboratory, California Plant and Seed Laboratories (Elvira, CA), has introduced real-time PCR (P. Randhawa, personal communication) and Seed Testing of America (Longmont, CO.) and Agdia (Elkhart, IN) are working toward the same capability.

Many fungal diseases, such as rusts, are amenable to rapid chemical controls once a positive identification has been made. However, with bacteria and viruses, rapid eradication and containment are the only available control measures. Successful disease eradication requires quick reaction such as occurred with the discovery of Karnal bunt in Arizona in 1996, where initial presumptive diagnosis based on spore morphology could be confirmed by PCR (11). The PCR confirmation (29) clearly contributed to the quick and successful containment of this highly regulated pathogen to within the infested area of the Southwestern United States.

## CONCLUSIONS

With increased air travel and free-trade agreements, the borders of many countries have become more open to unintentional and deliberate introduction of foreign crop pathogens. Only five years ago, few would have believed that a plant disease, and especially a bacterial disease, could be diagnosed by PCR on-site. Although PCR was discovered nearly 20 years ago (59), PCR technology has not yet been applied for routine plant disease diagnosis in the United States. Only recently with available affordable, portable real-time PCR instruments and protocols has molecular-based diagnosis of crop diseases become a reality. Routine diagnosis of many crop diseases can now be made in one day or less by real-time PCR. The availability of this technology could not have been better timed. The urgency in developing same-day, on-site protocols for identification of plant pathogens was emphasized by the deliberate release of anthrax in the United States. Although PCR-based detection protocols can be developed, unbiased rating models are needed to identify the highest risk pathogens from among the more than 500 economically important crop pathogens. Once protocols have been developed, standardized unbiased procedures, similar to those used in the EU, are needed to

“validate and certify” protocols for diagnosis of regulated crop diseases. Useful protocols should not be left buried in the literature.

## ACKNOWLEDGMENTS

We acknowledge the contributions on real-time PCR made by our technical assistants at Ft. Detrick, including Phil Gauth, Elena Postnikova, Aaron Sechler, Christine Stone, and Diana Sherman. We thank Mark Urlaub and Hank Parker for their critical review of the manuscript. We also gratefully acknowledge support from our CRADA partners, Applied Biosystems, Foster City, CA and Cepheid, Sunnyvale, CA for their assistance with TaqMan PCR and 7700 Sequence Detection System and the Smart Cycler, respectively.

**The Annual Review of Phytopathology is online at <http://phyto.annualreviews.org>**

## LITERATURE CITED

1. Abarca ML, Accensi F, Bragulat MR, Cabanes FJ. 2001. Current importance of ochratoxin A-producing *Aspergillus* spp. *J. Food Prot.* 64:903–6
2. Alibek K, Hanelman S. 1999. *Biohazard: The Chilling True Story of the Largest Covert Biological Weapons Program in the World—Told from the Inside by the Man Who Ran It*. New York: Random House
3. Al-Khalidi SF, Martin SA, Rasooly A, Evans JD. 2002. DNA microarray technology used for studying foodborne pathogens and microbial habitats: mini review. *J. Assoc. Offic. Anal. Chem. Int.* 85: 906–10
4. Ban J. 2000. *Agricultural Biological Warfare: An Overview*. Alexandria, VA/Washington, DC: Chem. Biol. Arms Control Inst. No. 9, June
5. Bates JA, Taylor EJA, Gans PT, Thomas JE. 2002. Determination of relative proportions of *Globodera* species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. *Mol. Plant Pathol.* 3:153–61
6. Bates JA, Taylor EJA, Kenyon DM, Thomas JE. 2001. The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. *Mol. Plant Pathol.* 2:49–57
7. Bender CL, Alarcon-Chaidez F, Gross DC. 1999. *Pseudomonas syringae* phytoalexins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* 63:266–92
8. Bohm J, Hahn A, Schubert R, Bahnweg G, Adler N, et al. 1999. Real-time quantitative PCR: DNA determination in spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. *J. Phytopathol.* 147:409–16
9. Boonham N, Walsh K, Preston S, North J, Smith P, et al. 2002. The detection of tuber necrotic isolates of *Potato virus Y*, and the accurate discrimination of PVY(O), PVY(N) and PVY(C) strains using RT-PCR. *J. Virol. Methods* 102:103–12
10. Boonham N, Smith P, Walsh K, Tame J, Morris J, et al. 2002. The detection of *Tomato spotted wilt virus* (TSWV) in individual thrips vectors using real-time fluorescent RT-PCR (TaqMan). *J. Virol. Methods* 101:37–48
11. Bonde M, Peterson GL, Schaad NW,

- Smilanick JL. 1997. Karnal bunt of wheat. *Plant Dis.* 81:1370–77
12. Branton SL, Deaton JW, Hagler WM Jr, Maslin WR, Hardin JM. 1989. Decreased egg production in commercial laying hens fed zearalenone- and, deoxynivalenol-contaminated grain sorghum. *Avian Dis.* 33:804–8
  13. Caswell-Chen EP, Wiliamson VM, Wu FF. 1992. Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *H. schachtii* populations. *J. Nematol.* 24:343–51
  14. Cheeke PR. 1995. Endogenous toxins and mycotoxins in forage grasses and their effects on livestock. *J. Anim. Sci.* 73:909–18
  15. Clark MF, Adams AN. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 334:475–83
  16. Cockerill FR, Smith TF. 2002. Rapid-cycle real-time PCR: a revolution for clinical microbiology. *Am. Soc. Microbiol. News* 68:77–83
  17. Colgrove AL, Smith GS, Wrather JA, Heinz RD, Niblack TL. 2002. Lack of predictable race shift in *Heterodera glycines*: infested field plots. *Plant Dis.* 86:1101–8
  18. Cullen DW, Lees AK, Toth IK, Duncan JM. 2001. Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. *Eur. J. Plant Pathol.* 107:387–98
  19. Cullen DW, Lees AK, Toth IK, Duncan JM. 2002. Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. *Plant Pathol.* 51:281–92
  20. Didenko VD. 2001. DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. *BioTechniques* 31:1106–20
  21. Ding SW, Howe J, Keese P, Mackenzie A, Meek D, et al. 1990. The tymobox, a sequence shared by most tymoviruses: its use in molecular studies of tymoviruses. *Nucleic Acids Res.* 18:1181–87
  22. Dong K, Dean RA, Fortnum BA, Lewis SA. 2001. Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. *Nematropica* 31:271–80
  23. Duffy B, Raaijmakers JM. 2002. Compromised plant health increases risks of opportunistic colonization by human pathogenic *Salmonella* and *E. coli* O157:H7. *Phytopathology* 92:S21. (Abst.)
  24. Epstein JR, Biran Isreal, Walt D.R. 2002. Fluorescence-based nucleic acid detection and microarrays. *Anal. Chim. Acta* 469:3–6
  25. Eun AJC, Seoh ML, Wong SW. 2000. Simultaneous quantitation of two orchid viruses by the TaqMan® real-time RT-PCR. *J. Virol. Methods* 87:151–60
  26. Forster RL. 1999. *Ground surveillance*. <http://www.apsnet.org/online/feature/biosecurity/abstracts.htm#forster>
  27. Fratamico PM, Schultz FJ, Buchanan RL. 1992. Rapid isolation of *Escherichia coli* O157:H7 from enrichment cultures of foods using an immunomagnetic separation method. *Food Microbiol.* 9:105–13
  28. Frazier TW, Richardson DC, eds. 1999. *Food and Agricultural Security: Guarding Against Natural Threats and Terrorist Attacks affecting Health, National Food Supplies, and Agricultural Economics*. New York: NY Acad. Sci. 233 pp.
  29. Frederick RD, Snyder KE, Tooley P, Berthier-Schaad Y, Peterson GL, et al. 2000. Identification and differentiation of *Tilletia indica* and *Tilletia walkeri* using PCR. *Phytopathology* 90:951–60
  30. Frederick RD, Snyder CL. 2001. Real-time fluorescent PCR detection of fungal plant pathogens using the Smart Cycler. *Phytopathology* 91:S29. (Abstr.)
  31. Frederick RD, Snyder CL, Peterson GL, Bonde MR. 2002. Polymerase chain reaction assays for the detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomia*. *Phytopathology* 92:217–27. (Abstr.)

32. Gibbs A, Mackenzie A 1997. A primer pair for amplifying part of the genome of all potyvirids by RT-PCR. *J. Virol. Methods* 63:9–16
33. Hampton RO, Ball EM, De Boer SH, eds. 1990. *Serological Tests for Detection of Viral and Bacterial Pathogens*. St. Paul, MN: APS Press
34. Harper WJ. 2001. The strengths and weaknesses of the electronic nose. *Adv. Exp. Med. Biol.* 488:59–71
35. Henson JM, French R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* 31:81–109
36. Heuser T, Zimmer W. 2002. Quantitative analysis of phytopathogenic ascomycota on leaves of pedunculate oaks (*Quercus robur* L.) by real-time PCR. *FEMS Microbiol. Lett.* 209:295–99
37. Hickson R. 1999. *Subtle form of strategic indirect warfare: infecting "soft" targets; some psychological, economic, and cultural consequences*. <http://www.apsnet.org/online/feature/biosecurity/abstracts.htm#hickson>
38. Horn FP, Breeze RG. 1999. Agriculture and food security. See Ref. 28, pp. 9–17
39. Holland PM, Abramson RD, Watson R, Gelfand DH. 1991. Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* 88:7276–80
40. Ibrahim SK, Minnis ST, Barker ADP, Russell MD, Haydock PPJ, et al. 2001. Evaluation of PCR, IEF and ELISA techniques for the detection and identification of potato cyst nematodes from field soil samples in England and Wales. *Pest Manag. Sci.* 57:1068–74
41. Ibrahim SK, Perry RN, Burrows PR, Hooper DJ. 1994. Differentiation of species and populations of *Aphelenchoides* and of *Ditylenchus angustus* using a fragment of ribosomal DNA. *J. Nematol.* 26:412–21
42. Ito S, Ushijima Y, Fujii T, Tanaka S, Kameya-Iwaki M, et al. 1998. Detection of viable cells of *Ralstonia solanacearum* in soil using semiselective medium and a PCR technique. *J. Phytopathology* 146: 379–84
43. Jago MV, Culvenor CC. 1987. Tunicamycin and corynetoxin poisoning in sheep. *Aust. Vet. J.* 64:232–35
44. Klerks MM, Leone GO, Verbeek M, van den Heuvel J.F, Schoen CD. 2001. Development of a multiplex AmpliDet RNA for the simultaneous detection of *Potato leafroll virus* and *Potato virus Y* in potato tubers. *J. Virol. Methods* 93:115–25
45. Korimbocus J, Coates D, Barker I, Boonham N. 2002. Improved detection of *Sugarcane yellow leaf virus* using a real-time fluorescent (TaqMan) RT-PCR assay. *J. Virol. Methods* 103:109–20
46. Lai SY, Deffenderfer OF, Hanson W, Phillips MP, Thaler ER. 2002. Identification of upper respiratory bacterial pathogens with the electronic nose. *Laryngoscope* 112:975–79
47. Lapa S, Mikheev M, Shcheelkunov S, Mikhailovich V, Sobolev A, et al. 2002. Species-level identification of orthopoxviruses with an oligonucleotide microchip. *J. Clin. Microbiol.* 40:753–57
48. Latin R, Hopkins DL. 1995. Bacterial fruit blotch of watermelon. The hypothetical exam question becomes reality. *Plant Dis.* 79:761–65
49. Leach JE. 1999. *Assuring food security: detecting and controlling modified pathogens*. <http://www.apsnet.org/online/feature/biosecurity/top.htm#leach>
50. Lee LG, Connell CR, Bloch W. 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Res.* 21:3761–66
51. Lees AK, Cullen DW, Sullivan L, Nicolson MJ. 2002. Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. *Plant Pathol.* 51:293–302
52. Li J, Chen S, Evans DH. 2001. Typing

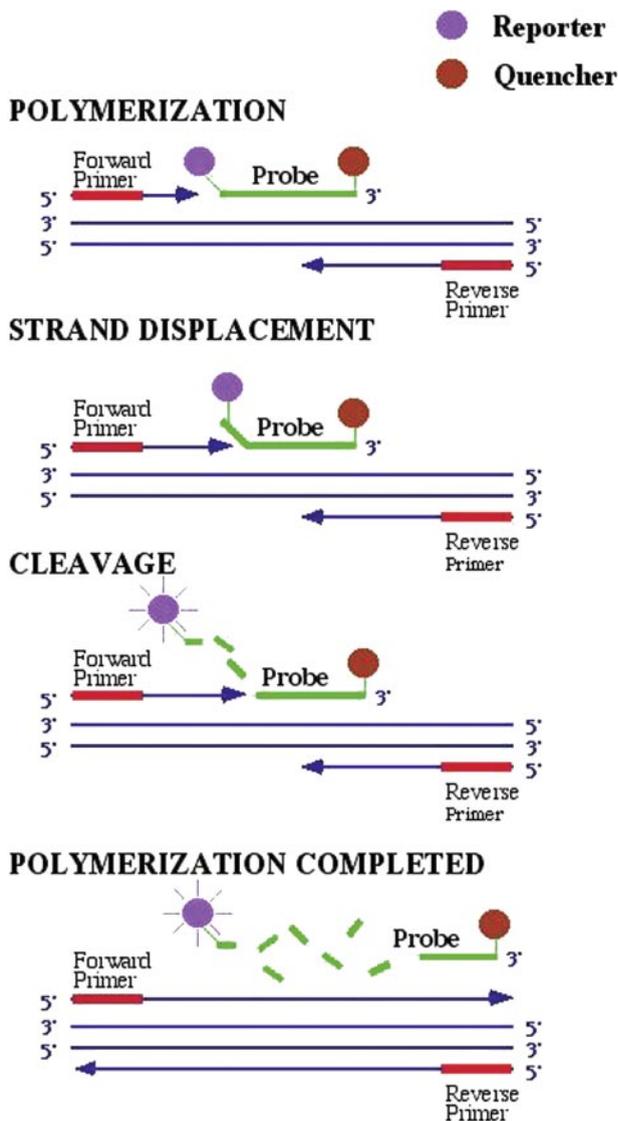
- and subtyping influenza virus using DNA microarrays and multiplex reverse transcriptase PCR. *J. Clin. Microbiol.* 39:696–704
53. Louws FJ, Couples D. 2001. Molecular detection. See Ref. 106, pp. 321–33
  54. MacKay IM, Arden KE, Nitsche A. 2002. Survey and summary of real-time PCR in virology. *Nucleic Acids Res.* 30:1292–305
  55. Madden LV, 2001. *What are the non-indigenous plant pathogens that threaten U.S. crops and forests?* <http://www.apsnet.org/online/feature/exotic.htm>
  56. Madden LV, Beale J, Brown B, Cline M, Cook J, et al. 2002. *Crop biosecurity and countering agricultural bioterrorism: responses of American Phytopathological Society.* <http://www.apsnet.org/online/feature/bioterrorism.htm>
  57. Madden LV, van den Bosch F. 2002. A population-dynamic approach to assess the threat of plant pathogens as biological weapons against crops. *BioScience* 52: 65–74
  58. McKay AC, Ophel KM. 1993. Toxigenic *Clavibacter/Anguina* associations infecting grass seedheads. *Annu. Rev. Phytopathol.* 31:151–67
  59. Mullis K. 1987. Process for amplifying nucleic acid sequences. *U.S. Patent No. 4683202*
  60. Mumford RA, Barker I, Wood KR. 1996. An improved method for the detection of Tosspoviruses using the polymerase chain reaction. *J. Virol. Methods* 57:109–15
  61. Neergaard P. 1977. *Seed Pathology.* London: Macmillan. 839 pp.
  62. Olsson J, Borjesson T, Lundstedt T, Schnurer J. 2002. Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. *Int. J. Food Microbiol.* 72:203–14
  63. Orui Y, Mizudubo T. 1999. Discrimination of seven *Pratylenchus* species (Nematoda: *Pratylenchidae*) in Japan by PCR-RFLP analysis. *Appl. Entomol. Zool.* 34:205–11
  64. Paupard JA, Miller LA. 1992. History of biological warfare: catapults to cap-somers. *Ann. NY Acad. Sci.* 666:9–19
  65. Petrillo MD. 2001. *Virulence and fitness of Meloidogyne incognita in response to susceptible and resistant cowpea.* PhD thesis. Univ. Calif., Riverside
  66. Pooler MR, Myung IS, Bentz J, Sherald J, Hartung J. 1997. Detection of *Xylella fastidiosa* in potential insect vectors by immunomagnetic separation and nested polymerase chain reaction. *Lett. Appl. Microbiol.* 25:123–26
  67. Powers TO, Szalanski AL, Mullin PG, Harris TS, Bertozzi T, et al. 2001. Identification of seed gall nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *J. Nematol.* 33:191–94
  68. Qi M, Yang Y. 2002. Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and Northern blot/phosphoimaging analyses. *Phytopathology* 92:870–76
  69. Rogers PS, Whitby SM, Dando M. 1999. Biological warfare against crops. *Sci. Am.* 280:70–75
  70. Saettler AW, Schaad NW, Roth DA, eds. 1989. *Detection of Bacteria in Seed and Other Planting Material.* St. Paul, MN: APS Press
  71. Schaad NW. 1999. *What is an effective pathogen?* <http://www.apsnet.org/online/feature/bioSecurity/abstracts.htm#schaad>
  72. Schaad NW, Berthier-Schaad Y, Hatziloukas E, Knorr D. 1997. Development and comparison of ABI Prism 7700 sequence detection system to BIO-PCR for sensitive detection of *Pseudomonas syringae* pv. *phaseolicola*. *ASM Conf. Mol. Diagnostics Ther., Kananaskis, Alberta, Can.* Publ. 40.
  73. Schaad NW, Berthier-Schaad Y, Sechler A, Knorr D. 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and automated real-time fluorescence detection system. *Plant Dis.* 83:1095–100

74. Schaad NW, Cheong S, Tamaki S, Hatziloukas E, Panopoulos NJ. 1995. A combined biological amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. *Phytopathology* 85:243–48
75. Schaad NW, Frederick RD. 2002. Real-time PCR and its application for rapid plant disease diagnostics. *Can. J. Plant Pathol.* 24:250–58
76. Schaad NW, Gaush P, Postnikova E, Frederick R. 2001. On-site one hour PCR diagnosis of bacterial diseases. *Phytopathology* 91:S79–89 (Abstr.)
77. Schaad NW, Hatziloukas E, Henrickson G. 1996. Development of membrane BIO-PCR for ultra-sensitive detection of pathogens in environmental samples. *Am. Soc. Microbiol. Annu. Meet.*, Abstr., p. 391
78. Schaad NW, Jones JB, Chun W, eds. 2001. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. St. Paul, MN: APS Press. 3rd. ed.
79. Schaad NW, Ogenorth D, Gaush P. 2002. Real-time PCR for one-hour on-site diagnosis of Pierce's disease of grape in early season asymptomatic vines. *Phytopathology* 92:721–28
80. Schaad NW, Panopoulos NJ, Hatziloukas E. 2002. Direct polymerase chain reaction assay, or BIO-PCR. *U.S. Patent No. 6410223*
81. Schaad NW, Shaw JJ, Vidaver A, Leach J, Erlick BJ. 1999. *Crop biosecurity*. <http://apsnet.org/online/feature/biosecurity/top.htm#Schaad>
82. Schaad NW, Song WY, Hatziloukas E. 2000. PCR primers for detection of plant pathogenic species and subspecies of *Acidovorax*. *U.S. Patent No. 6146834*
83. Schena L, Nigro F, Ippolito A. 2002. Identification and detection of *Rosellinia necatrix* by conventional and real-time Scorpion-PCR. *Eur. J. Plant Pathol.* 108:355–66
84. Schoen CD, Knorr D, Leone G. 1996. Detection of *Potato leafroll virus* in dormant potato tubers by immunocapture and fluorogenic 5' nuclease RT-PCR assay. *Phytopathology* 86:993–99
85. Sen D. 2002. Aptamer rivalry. *Chem. Biol.* 9:851
86. Shao CY, Howe CJ, Porter AJ, Glover LA. 2002. Novel cyanobacterial biosensor for detection of herbicides. *Appl. Environ. Microbiol.* 68:5026–33
87. Sharma V, Carlson SA. 2000. Simultaneous detection of *Salmonella* strains and *Escherichia coli* 0157:7 with fluorogenic PCR and single-enrichment-broth culture. *Appl. Environ. Microbiol.* 66:5472–76
88. Shier WT, Shier AC, Xie W, Mirocha CJ. 2001. Structure-activity relationships for human estrogenic activity in zearalenone, mycotoxins. *Toxicol.* 39:1435–38
89. Stanton J, Hugall A, Moritz C. 1997. Nucleotide polymorphisms and an improved PCR-based mtDNA diagnostic for parthenogenetic root-knot nematodes (*Meloidogyne* spp.). *Fund. Appl. Nematol.* 20:261–68
90. Subbotin SA, Halford PD, Warry A, Perry RN. 2000. Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitizing solanaceous plants. *Nematology* 2:591–604
91. Sui DD, Kluepfel DA, Fortnum BA, Lewis. 2001. Multiplex PCR identification and diagnostic system of *Meloidogyne* species. *Phytopathology* 91:S86 (Abstr.)
92. Szemes M, Klerks, MM, van den Heuvel JF, Schoen CD. 2002. Development of a multiplex AmpliDet RNA assay for simultaneous detection and typing of potato virus Y isolates. *J. Virol. Methods* 100:83–96
93. Taylor EJA, Stevens EA, Bates JA, Morreale G, Lee D, et al. 2001. Rapid cycle PCR detection of *Pyrenophora graminea* from barley seed. *Plant Pathol.* 50:347–55
94. Thaler ER, Kennedy DW, Hanson CW. 2001. Medical applications of electronic nose technology: review of current status. *Am. J. Rhinol.* 15:291–95
95. Vandemark GJ, Barker BM, Gritsenko

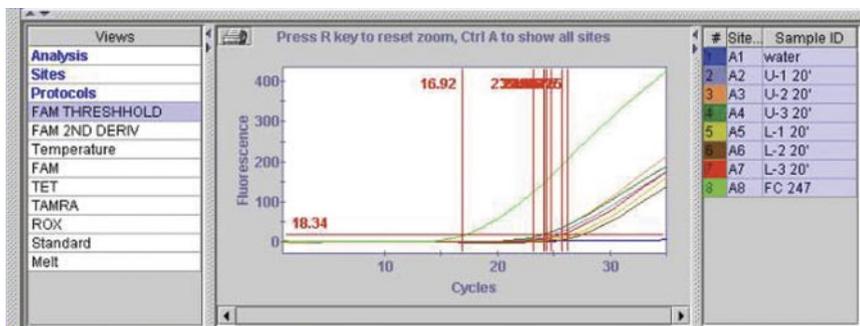
- MA. 2002. Quantifying *Aphanomyces eutiches* in alfalfa with a fluorescent polymerase chain reaction assay. *Phytopathology* 92:265–72
96. Van Vuurde JWL. 1990. Immunofluorescence colony staining. In *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens*, ed. R Hampton, E Ball, S De Boer, pp. 295–305. St. Paul, MN: APS Press
97. Wang ZK, Comstock JC, Hatziloukas E, Schaad NW. 1999. Comparison of PCR, Bio-PCR, DIA, ELISA, and isolation on semiselective medium for detection of *Xanthomonas albilineans*, the causal agent of leaf scald of sugarcane. *Plant Pathol.* 48:245–52
98. Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Appl. Environ. Microbiol.* 66:2853–58
99. Weller SA, Elphinstone JG, Smith N, Stead DE. 2000. Detection of *Ralstonia solanacearum* from potato tissue by post enriched TaqMan<sup>TM</sup> PCR. *OEPP/EPPO Bull.* 30:381–83
100. Weller SA, Stead DE. 2002. Detection of root mat associated *Agrobacterium* strains from plant material and other sample types by post-enrichment TaqMan PCR. *J. Appl. Microbiol.* 92:118–12
101. Wendt KR, Vrain TC, Webster JM. 1993. Separation of three species of *Ditylenchus* and some host races of *D. dipsaci* by restriction fragment length polymorphism. *J. Nematol.* 25:555–63
102. Wheelis M, Casagrande R, Madden LV. 2002. Biological attack on agriculture: low-tech, high-impact bioterrorism. *BioScience* 52:569–76
103. Wilson WJ, Strout CL, DeSantis TZ, Stilwell JL, Carrano AV, et al. 2002. Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. *Mol. Cell. Probes* 16:119–27
104. Whitby SM. 2002. *Biological Warfare Against Crops*. New York: Palgrave. 141 pp.
105. Whitby SM, Rogers P. 1997. Anticrop biological warfare-implications of the Iraq and U.S. programs. *Defense Anal.* 13:303–13
106. Zhang AW, Hartman GL, Curio-Penny B, Pedersen WL, Becker KB. 1999. Molecular detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* from soybean seeds. *Phytopathology* 89:796–804



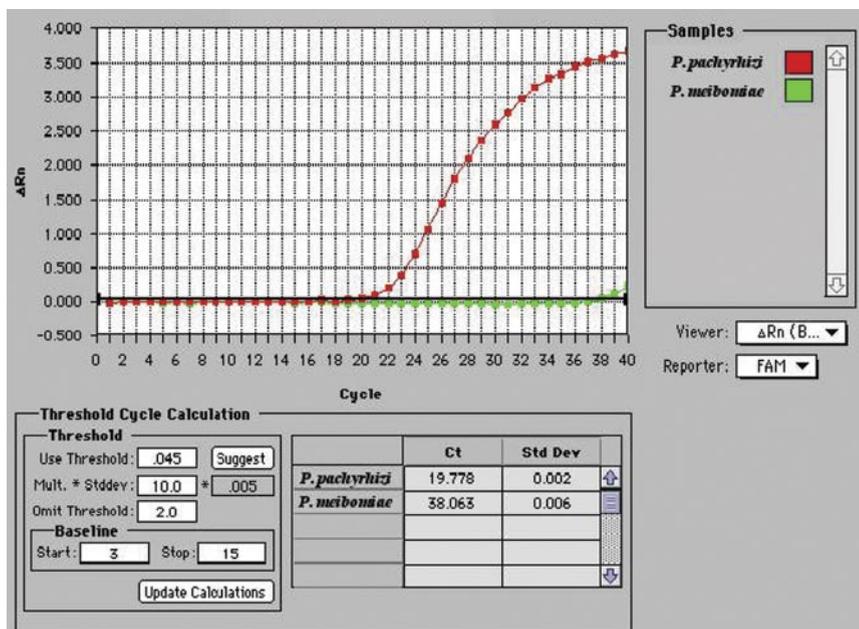
**Figure 1** Bean plants in a windrow to dry in a seed production field near Twin Falls, Idaho. Note the dry pods in the center with black lesions typical of both bacterial blight caused by *Pseudomonas syringae* pv. *phaseolicola* and brown spot caused by *P. syringae* pv. *syringae*. The former is a zero tolerance regulated pathogen whereas the latter is not regulated.



**Figure 2** The principles of real-time polymerase chain reaction (PCR) using TaqMan chemistry. The probe sequence consists of 25 to 30 nucleotides and is labeled at the 5' end with a fluorescent reporter dye such as 6-carboxy-fluorescein (FAM) and at the 3' end with a quencher dye like 6-carboxy-tetramethyl-rhodamine (TAMRA) (Applied Biosystems). During the strand displacement step, the 5' – 3' exonuclease activity of the *Taq* polymerase (AmpliTaq Gold, Applied Biosystems) releases the fluorescent reporter from the probe sequence which is measured by a CCD camera at each cycle of amplification.



**Figure 3** Amplification of *Acidovorax avenae* subsp. *citrulli* (AAC) DNA in stem sections from infected watermelon seedlings by real-time polymerase chain reaction using the Smart Cycler (Cepheid, Sunnyvale, CA) with subsp. *citrulli*-specific ITS primers and probe. The negative water control remains at a cycle threshold (Ct) value of 0 whereas the positive control (AAC strain FC-247) has a Ct value of 16.92. The Ct value is described as the cycle number at which the fluorescence rises above the base line. Plant samples U-1 to L-3 have Ct values from 23 to 27. The left axis is the change in fluorescence that measures probe cleavage and the bottom axis is the cycle number.



**Figure 4** Real-time polymerase chain reaction (PCR) amplification of DNA from the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiaae* by TaqMan PCR using an ABI Prism 7700 Sequence Detection System. *P. pachyrhizi* specific primers Ppm1 and Ppa 2 were used with a 5'-FAM-labeled internal probe (Frederick et al. 2002). The left axis ( $\Delta RQ$ ) is the change in fluorescence that is a measure of probe cleavage efficiency, and the bottom axis is the PCR cycling stage.

## CONTENTS

FRONTISPIECE, <i>Kenneth R. Barker</i>	xii
PERSPECTIVES ON PLANT AND SOIL NEMATOLOGY, <i>Kenneth R. Barker</i>	1
JAMES GORDON HORSFALL: NONCONFORMIST AND FOUNDING FATHER, <i>Paul E. Waggoner</i>	27
CARL FREIHERR VON TUBEUF: PIONEER IN BIOLOGICAL CONTROL OF PLANT DISEASES, <i>Otis C. Maloy and Klaus J. Lang</i>	41
EPIDEMIOLOGY AND MANAGEMENT OF TOMATO SPOTTED WILT IN PEANUT, <i>A.K. Culbreath, J.W. Todd, and S.L. Brown</i>	53
BROME MOSAIC VIRUS RNA REPLICATION: REVEALING THE ROLE OF THE HOST IN RNA VIRUS REPLICATION, <i>Amine O. Noueiry and Paul Ahlquist</i>	77
CULTURAL AND GENETIC APPROACHES TO MANAGING MYCOTOXINS IN MAIZE, <i>Gary P. Munkvold</i>	99
REGULATION OF ANTIBIOTIC PRODUCTION IN ROOT-COLONIZING <i>PSEUDOMONAS</i> SPP. AND RELEVANCE FOR BIOLOGICAL CONTROL OF PLANT DISEASE, <i>Dieter Haas and Christoph Keel</i>	117
THE THREAT OF PLANT PATHOGENS AS WEAPONS AGAINST U.S. CROPS, <i>L.V. Madden and M. Wheelis</i>	155
<i>GIBBERELLA</i> FROM <i>A(VENACEA)</i> TO <i>Z(EAE)</i> , <i>Anne E. Desjardins</i>	177
EVOLUTION OF WHEAT STREAK MOSAIC VIRUS: DYNAMICS OF POPULATION GROWTH WITHIN PLANTS MAY EXPLAIN LIMITED VARIATION, <i>Roy French and Drake C. Stenger</i>	199
MOLECULAR BASIS OF <i>PTO</i> -MEDIATED RESISTANCE TO BACTERIAL SPECK DISEASE IN TOMATO, <i>Kerry F. Pedley and Gregory B. Martin</i>	215
PARASITIC NEMATODE INTERACTIONS WITH MAMMALS AND PLANTS, <i>Douglas P. Jasmer, Aska Goverse, and Geert Smant</i>	245
ECOLOGY OF MYCORRHIZAE: A CONCEPTUAL FRAMEWORK FOR COMPLEX INTERACTIONS AMONG PLANTS AND FUNGI, <i>M.F. Allen, W. Swenson, J.I. Querejeta, L.M. Egerton-Warburton, and K.K. Treseder</i>	271
ADVANCES IN MOLECULAR-BASED DIAGNOSTICS IN MEETING CROP BIOSECURITY AND PHYTOSANITARY ISSUES, <i>Norman W. Schaad, Reid D. Frederick, Joe Shaw, William L. Schneider, Robert Hickson, Michael D. Petrillo, and Douglas G. Luster</i>	305

DEVELOPMENT OF ALTERNATIVE STRATEGIES FOR MANAGEMENT OF SOILBORNE PLANT PATHOGENS CURRENTLY CONTROLLED THROUGH METHYL BROMIDE, <i>Frank N. Martin</i>	325
PATTERNS OF PESTICIDE USE IN CALIFORNIA AND THE IMPLICATIONS FOR STRATEGIES FOR REDUCTION OF PESTICIDES, <i>Lynn Epstein and Susan Bassein</i>	351
INNOVATIONS IN TEACHING PLANT PATHOLOGY, <i>G.L. Schumann</i>	377
OF SMUTS, BLASTS, MILDEWS, AND BLIGHTS: CAMP SIGNALING IN PHYTOPATHOGENIC FUNGI, <i>Nancy Lee, Cletus A. D'Souza, and James W. Kronstad</i>	399
THE ECOLOGICAL SIGNIFICANCE OF BIOFILM FORMATION BY PLANT-ASSOCIATED BACTERIA, <i>Cindy E. Morris and Jean-Michel Monier</i>	429
QUORUM SENSING IN PLANT-PATHOGENIC BACTERIA, <i>Susanne B. von Bodman, W. Dietz Bauer, and David L. Coplin</i>	455
<i>SPIROPLASMA CITRI</i> , A PLANT PATHOGENIC MOLLICUTE: RELATIONSHIPS WITH ITS TWO HOSTS, THE PLANT AND THE LEAFHOPPER VECTOR, <i>Joseph M. Bové, Joël Renaudin, Colette Saillard, Xavier Foissac, and Monique Garnier</i>	483
PATHOGEN SELF-DEFENSE: MECHANISMS TO COUNTERACT MICROBIAL ANTAGONISM, <i>Brion Duffy, Alexander Schouten, and Jos M. Raaijmakers</i>	501
LUTEOVIRUS-APHID INTERACTIONS, <i>Stewart Gray and Frederick E. Gildow</i>	539
ECOLOGY AND EPIDEMIOLOGY OF <i>BENYVIRUSES</i> AND PLASMODIOPHORID VECTORS, <i>Charles M. Rush</i>	567
THE POTENTIAL OF OPTICAL CANOPY MEASUREMENT FOR TARGETED CONTROL OF FIELD CROP DISEASES, <i>Jonathan S. West, Cedric Bravo, Roberto Oberti, Dimitri Lemaire, Dimitrios Moshou, and H. Alastair McCartney</i>	593
ENGINEERING PLANTS FOR NEMATODE RESISTANCE, <i>Howard J. Atkinson, Peter E. Urwin, and Michael J. McPherson</i>	615
ESTABLISHMENT OF BIOTROPHY BY PARASITIC FUNGI AND REPROGRAMMING OF HOST CELLS FOR DISEASE RESISTANCE, <i>Paul Schulze-Lefert and Ralph Panstruga</i>	641
INDEXES	
Subject Index	669
Cumulative Index of Contributing Authors, Volumes 32–41	705
Cumulative Index of Chapter Titles, Volumes 32–41	709

## ERRATA

An online log of corrections to *Annual Review of Phytopathology* chapters (if any, 1997 to the present) may be found at <http://phyto.annualreviews.org/>