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Real-time Fluorescent PCR Detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* Using Mitochondrial Gene Regions

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ABSTRACT

Tooley, P. W., Martin, F.N., Carras, M.M., and Frederick, R.D. (2005) Real-time fluorescent PCR Detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. Phytopathology 95: XXX-XXX.

A real-time fluorescent PCR detection method for the sudden oak death pathogen *P. ramorum* was developed based on mitochondrial DNA sequence with an ABI Prism 7700 (TaqMan) Sequence Detection System. Primers and probes were also developed for detecting *P. pseudosyringae*, a newly described species that causes symptoms similar to *P. ramorum* on certain hosts. The species-specific primer-probe systems were combined in a multiplex assay with a plant primer-probe system to allow plant DNA present in extracted samples to serve as a positive control in each reaction. The lower limit of detection of *P. ramorum* DNA was 1 fg genomic DNA, lower than for many other described PCR procedures for detecting *Phytophthora* species. The assay was also used in a 3-way multiplex format to simultaneously detect *P. ramorum*, *P. pseudosyringae* and

plant DNA in a single tube. *P. ramorum* was detected down to a 10^{-5} dilution of extracted tissue of artificially infected Rhododendron 'Cunningham's White' and the amount of pathogen DNA present in the infected tissue was estimated using a standard curve. The multiplex assay was also used to detect *P. ramorum* in infected California field samples from several hosts determined to contain the pathogen by other methods. The real-time PCR assay we describe is highly sensitive and specific, and has several advantages over conventional PCR assays used for *P. ramorum* detection to confirm positive *P. ramorum* finds in nurseries and elsewhere.

Key Words: Sudden Oak Death, *cox* 1, *cox* 2

INTRODUCTION

Phytophthora ramorum (Werres, De Cock & Man in't Veld) sp. *nov* causes sudden oak death, a serious disease of California oak species such as coast live oak (*Quercus agrifolia*) and tanoak (*Lithocarpus densiflorus*) (44). The pathogen also is widespread in Europe primarily as a pathogen of ornamentals (14,28,40,59,60). Because of concern that *P. ramorum* may spread eastward and threaten the vast oak forests of the Eastern U.S., state, federal, and Canadian regulations were drafted in 2001 that restricted movement of *P. ramorum* hosts out of infested areas of California (7,8,42).

In 2003, new *P. ramorum* outbreaks were reported in nursery stock found in nurseries from Oregon, Washington State, Canada, and additional areas of California (22,41, J. Jones, personal communication). Also in 2003, a national *P. ramorum* survey was initiated (12). In 2004 several large west coast production nurseries and some smaller nurseries were confirmed to be infested with *P. ramorum*. These facilities

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shipped over 2 million host plants, of which only a small portion were infected, to 49 states and the District of Columbia (51, J. Jones, personal communication). Efforts were made on the part of several agencies including the U. S. Department of Agriculture Animal and Plant Health Inspection Service (APHIS), U. S. Forest Service, and State Departments of Agriculture to track and test the shipments, monitor for presence of *P. ramorum* in Eastern states, and educate the public about sudden oak death. By the end of 2004, 171 locations (wholesale nurseries and retail outlets) in 20 states were found to contain plants infected with *P. ramorum*. On April 22, 2004 APHIS issued an amended Emergency Order which implemented new restrictions on interstate movement of host nursery stock and associated articles from all commercial nurseries in California that are outside the quarantined area. Nurseries in Oregon and Washington state which ship interstate were added to this regulatory oversight on January 10, 2005. This order also listed 31 confirmed hosts of *P. ramorum* (those for which Koch's postulates had been performed) and a list of 37 additional plant species associated with *P. ramorum* because results of culture or PCR tests had returned results positive for the pathogen. The host range of *P. ramorum* continues to increase as the pathogen is identified on an ever-widening group of plant species (13,24,31,41, J. Jones, personal communication).

In light of the recent movement of *P. ramorum* to the Eastern U. S. through shipment of nursery stock, the availability of rapid, sensitive and specific *P. ramorum* detection methods are needed. Unequivocal identification of *P. ramorum* is the goal of survey workers, as false identification and/or confusion of *P. ramorum* with other *Phytophthora* species could lead to the development of improper quarantine measures and/or rejection of plant shipments by state inspectors. *Phytophthora ramorum* has

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several distinguishing morphological characters that may be used for identification. It is characterized by semi-papillate, deciduous sporangia with short pedicels and high length:width ratios, large chlamydospores, relatively slow growth and low cardinal temperatures for growth (60). To accurately assess morphological features however, requires experience in *Phytophthora* identification as some characteristics often show a continuum among different species. It can also be time consuming, especially when a number of samples have to be processed. Furthermore, it can be difficult to culture the pathogen from infected tissue at certain times of the year (23).

As an adjunct to morphological identification several molecular procedures for identification and detection of *P. ramorum* have been developed and are in use in various laboratories and state and federal agencies. These include classical PCR methods based on ITS regions of ribosomal DNA (13,23,61) and mitochondrial gene regions (39), PCR-SSCP analysis (32), and PCR-RFLP analysis (38). In 2004, a SNP (single nucleotide polymorphism) procedure was also developed to allow differentiation among *P. ramorum* isolates from Europe and North America (33). In 2003, APHIS adopted the ITS-based conventional nested PCR method (13) as an accepted protocol for identification of *P. ramorum* and has stated in an amended order dated April 22, 2004 that positive (conventional) nested PCR tests alone may be used to confirm presence of *P. ramorum* and prohibit movement of affected nursery stock, without requiring confirmatory culturing of the pathogen (54).

Real-time PCR is based on the labeling of primers, probes or amplicon with fluorogenic molecules and allows detection of the target fragment to be monitored while the amplification is in progress (35,46). In 5' fluorogenic real-time PCR (TaqMan), a

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sequence-specific oligonucleotide probe labeled with a fluorescent reporter and a quencher generates fluorescence at a rate directly proportional to the amount of product amplified in the reaction (26). The method is now being applied to a range of organisms in many different research applications (30,34,35,43,50), including detection and quantification of fungal plant pathogens (1,3,18,19,21,46, 47,48,55,56). For *Phytophthora* species, real-time PCR has been used in studies detecting and quantifying levels of various species in host plants and soil (4,29,47,56).

Several real-time PCR assays have been described for detection of *P. ramorum*. Bilodeau et al. (2) described an assay based on the ITS, β-tubulin, and elicitin regions using TaqMan and SYBR Green assays. Hughes et al. (27) have described an ITS-based real-time PCR assay for *P. ramorum* which uses TaqMan chemistry and has been adapted for field use with a SmartCycler (Cepheid, Inc.) instrument. A real-time PCR procedure for detection of *P. ramorum* based on the ITS region using SYBR green has been described by Hayden et al. (23).

Here, we describe the development of a real-time PCR assay for the sudden oak death pathogen *P. ramorum* based upon mitochondrial sequences. In previous work, we characterized the *cox* I and II genes in *Phytophthora* and described a conventional PCR assay for *P. ramorum* (36,37,38,39). In this study, we utilize the same primers as the conventional PCR method previously described (39) except with the addition of TaqMan probes specially designed for *P. ramorum*, *P. pseudosyringae*, and plant DNA. Plant primers were used as a positive control to insure that PCR amplification always occurs with DNA extracted from symptomatic samples. The real-time PCR assay we describe provides a sensitive, specific tool for detection of *P. ramorum*, based on a genomic

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region not used in other *P. ramorum* assays. It offers advantages over conventional PCR procedures as a stand-alone method or confirmatory procedure for workers monitoring for the presence of *P. ramorum* in new geographic regions.

MATERIALS AND METHODS

Cultures and DNA extraction. *Phytophthora* isolates (Table 1) were maintained on Rye A agar (9) at 20 C in darkness and all were used to test primer and probe specificity. Genomic DNA was extracted as per Goodwin et al. (20) from 60 mg of lyophilized mycelium grown on a synthetic medium (63). DNA concentrations were determined using a model ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and by comparison with known DNA standards using agarose gel electrophoresis. Plant genomic DNA was extracted from noninoculated leaves using a Qiagen DNeasy Plant Maxi Kit (Qiagen Inc., Valencia, CA). Leaves of rhododendron 'Cunningham's White' were inoculated with sporangia of *P. ramorum* isolate 0-217 as described by Tooley et al. (53). California bay laurel (*Umbellularia californica*) was artificially inoculated with *P. ramorum*, *P. pseudosyringae*, or both pathogens by placing a 6 mm-diameter agar plug of mycelium on a wound on the leaf and incubating it in a moist chamber for 7 days. Total DNA was extracted by homogenizing two 6-mm diameter leaf disks from lesions on infected leaves in a Fastprep FP120 instrument (Qbiogene, Inc., Carlsbad, CA) and using a Qbiogene FastDNA Kit according to the manufacturer's instructions.

Field samples from California. Samples of total DNA from symptomatic plants collected from the field were processed at the California Department of Food and Agriculture (CDFA) as described previously (39). The presence of *Phytophthora* spp.

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was confirmed by plating tissue on differential medium and DNA was extracted from diseased tissue and tested with the ITS marker system (13) to determine if *P. ramorum* was present. Samples were also assayed using the mitochondrial marker system described in Martin et al. (39). Real-time PCR assays were conducted on 53 samples from 11 hosts in blind fashion; the samples were numbered randomly and the results of culturing and/or conventional PCR were not known until real-time PCR analyses were completed. DNA samples were also diluted 1:10 with sterile water prior to use as undiluted samples some times amplified poorly.

Primers, probes and PCR conditions. The nucleotide sequences of the gene regions from which primer and probe sequences were designed are as described previously (39). Plant primers FMPl-2b and FMPl-3b (Table 2) were constructed from the mitochondrially encoded cytochrome oxidase I gene and generated a target fragment of 143 bp (39). Species-specific primers for *P. ramorum* (FMPr-1a and FMPr-7), and *P. pseudosyringae* (FMPps-1c and FMPps-2c) amplified spacer sequences between the *cox*II and *cox*I genes and produced amplicons of 134 and 158 bp, respectively (39) (Table 2). Primers were synthesized by Qiagen Inc. (Valencia, CA). The TaqMan probes were labeled at the 5' end with either the fluorescent reporter dye 6-carboxylfluoresceine (FAM) or CAL Fluor Orange 560 (CAL Orange) and labeled at the 3' end with the black hole quencher dye (BHQ, Biosearch Technologies, Novato, CA) (Table 2). In multiplex PCR experiments, the plant probe was labeled at the 5' end with TAMRA (N,N,N'-tetramethyl-5-carboxyrhodamine) as a reporter dye instead of CAL Orange.

Real-time PCR was performed using an ABI Prism 7700 Sequence Detection System (Perkin Elmer/Applied Biosystems, Foster City, CA) in a total volume of 25 µl

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containing 100 pg DNA template, 1x TaqMan Universal Master Mix (Perkin Elmer/Applied Biosystems) with an additional 0.5 mM $MgCl_2$. Annealing temperature and magnesium concentration were varied to determine optimum levels for amplification (data not shown). For duplex reactions incorporating both *P. ramorum* and plant primers and probes, an additional 75 uM of dNTPs were added, while for single reactions using *P. pseudosyringae* primers, an additional 1.5 mM $MgCl_2$ were added. Cycling conditions were 50ºC for 2 min, 95ºC for 10 min and 60 cycles of 95ºC for 15 s and 55ºC for 1 min. The FMPr-1a/FMPr-7 and FMPps-1c/FMPps-2c primer combinations were used at a final primer concentration of 1000 nM and probe concentration of 400 nM, whereas the FMPl2b/FMPl3b (plant) primers were used at a final primer concentration of 100 nM and probe concentration of 80 nM. For multiplex reactions, we used conditions identical to those for duplex reactions except that 50 µl reaction volumes were used and the plant probe was at a concentration of 400 nM. A water blank was included as a negative control in each experiment.

Dilution series experiments. Three repeated experiments with two replications each were performed using spectrophotometrically quantified DNA of *P. ramorum* isolate 288 or *P. pseudosyringae* isolate 471 diluted in sterile distilled water. To determine whether the presence of plant DNA affected the DNA dilution series for *P. ramorum*, experiments were performed using a *P. ramorum* DNA dilution series 'spiked' with DNA extracted from uninfected azalea cv. 'Gloria'. Two 6-mm diameter leaf disks were extracted with the Qbiogene FastDNA Kit in a final volume of 100 microliters and diluted 1:10. Two microliters of extract were added to a dilution series of *P. ramorum* DNA from isolate 288 ranging from 10 ng down to 100 ag, and real-time PCR was performed using only

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the *P. ramorum* primers and probe as well as a two way multiplex reaction with the *P. ramorum* primers and probe plus the plant primers and probe (3 replications each). In addition, dilution series were made from total DNA extracted from infected rhododendron 'Cunningham's White' inoculated as described above. Individual dilution series were constructed from three separate extractions and two experiments were conducted each using dilution series from all three extractions.

Data analysis. Data acquisition and analysis were performed using the TaqMan data worksheet and software according to the manufacturer's instructions (Applied Biosystems). The cycle threshold (Ct) values for each reaction were calculated automatically by the ABI Prism sequence detection software (ver. 1.6.3) by determining the PCR cycle number at which the reporter fluorescence exceeded background.

RESULTS

***P. ramorum*-specific primers and probe.** A high level of *P. ramorum* specificity was observed using the primers FMPr-1a and FMPr-7 and the Pr-FAM probe (Table 2) when tested against 45 other species of *Phytophthora* (multiple isolates tested for some species) at a concentration of 100 pg DNA with an annealing temperature of 55º C (Table 1). Only *P. ramorum* showed a Ct value of less than 30 cycles with other species exhibiting no detection after 60 cycles (Fig. 1A, Table 3). Twenty-five diverse isolates of *P. ramorum* were amplified at a concentration of 100 pg DNA using primers FMPR-1a and FMPr-7 and the Pr-FAM probe, with Ct values ranging from 22.56 to 28.91 (Table 3). Primers FMPr-1a and FMPr-7 and the FAM probe worked successfully at 55º C, but at 57º C amplification became inconsistent (data not shown).

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Results from real-time PCR based on a DNA dilution series showed that amplification with the *P. ramorum* primers and probe occurred down to 1 fg of template DNA, which had a Ct value of 42 (Fig. 2A). A standard curve was calculated based on three replicate serial dilutions of DNA extracted from *P. ramorum* isolate 288 and demonstrated the linearity in response of the assay to DNA concentrations (Fig. 2B). Data for the 100 ag quantity was omitted from the standard curve analysis since detection was variable at that low level. Addition of plant DNA in amounts similar to those that would likely be added when assaying field samples slightly reduced the amplification efficiency of *P. ramorum* template amplification (slope of –4.14 compared to –3.68); the regression equation for the spiked DNA standard curve was y = -4.14 Log(x) + 21.96 with a r^2 value of 0.984.

***P. pseudosyringae*-specific primers and probe.** Primers FMPps1c and FMPps2c and the PpsCALOrange probe (Table 2) specifically detected all six isolates of *P. pseudosyringae* when tested at an annealing temperature of 55 ºC and did not amplify any of the other 45 *Phytophthora* species (including the closely related *P. nemorosa*) when tested at a concentration of 100 pg DNA, including 25 isolates of *P. ramorum* (Table 3). Results of a DNA dilution series showed that amplification with the *P. pseudosyringae* primers and probe occurred down to 10 fg template DNA, which had an average Ct value (based on six replications) of 39.94 (data not shown). A standard curve was calculated based on three replicate serial dilutions of *P. pseudosyringae* isolate 471 each containing two replications, and the regression demonstrated the linearity in response of the assay to

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DNA concentrations (Fig. 2C). Data for the 1 fg quantity was omitted from the standard curve analysis since detection was variable at that low level.

Testing primers and probes with plant DNA. Both the *P. ramorum* and *P. pseudosyringae* primers and probes were also tested with DNA of the following plant species using an annealing temperature of 55ºC and no amplification was observed: Rhododendron sp. (cv. 'Cunningham's White'), *Glycine max* cv. 'Williams', *Solanum demissum*, *Solanum cardiophyllum*, *Solanum tuberosum* cv. 'Russet Burbank', *Lycopersicon esculentum*, coast live oak (*Quercus agrifolia*), laurel oak (*Quercus laurifolia*), *Kalmia latifolia* cv. 'Olympic Wedding', California bay laurel (*Umbellularia californica*), *Pieris japonica*, Highbush blueberry (*Vacinnium corymbosum)*, Tan oak (*Lithocarpus densiflorus*), *Citrus* sp., *Zauschneria californica*, *Fragaria x ananassa*, and *Juniperus* sp.

Sensitivity of detection of real time PCR assay with infected tissue. We performed a dilution series from rhododendron leaf disks artificially inoculated with *P. ramorum* to determine the approximate limits of pathogen detection in infected tissue (Table 4). Even at dilutions of 10^{-6} pathogen detection was observed, albeit with a C_t of 55.34. The amount of DNA at each serial dilution of the infected plant extract was estimated using the standard dilution series curve (Fig. 2) with the 10^{-5} dilution extrapolated to have 1.7 fg *P. ramorum* DNA.

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Use of two-way multiplex real-time PCR assay with field samples from California. Samples from naturally infected plant hosts in California received from the California Department of Food and Agriculture were evaluated using the *P. ramorum*, *P pseudosyringae*, and plant primers (Table 5). We performed a two-way multiplex real-time PCR using *P. ramorum* and plant primers and probes. For samples negative for *P. ramorum*, we then performed a second real-time PCR reaction using the *P. pseudosyringae* primers and probe. Results for all 53 samples showed good agreement between the real-time PCR and the results of prior analysis (Table 5). All 14 samples previously determined to be infected with *P. ramorum* were correctly identified with the real-time assay, as were all 6 of the samples infected with *P. pseudosyringae*. Cross reactivity between these two species or with several other *Phytophthora* spp. colonizing the tissue was not observed. Importantly, no examples of false positives were obtained. Use of plant primers and probe allowed confirmation that amplifiable DNA was present in all samples, and was of high quality and did not contain PCR inhibitors that would prevent amplification and result in false negatives.

Three-way multiplex real-time PCR assay. Experiments were conducted using California bay laurel (*U. californica*) artificially infected with *P. ramorum*, *P. pseudosyringae*, or both pathogens using their respective primers and probes and plant primers and probes in 3-way multiplex reactions**.** Initial studies were performed to determine optimum concentrations of dNTPs, magnesium, and primers/probes and optimum probe-fluorochrome combinations to prevent competitive interference between the three components in the multiplex reactions (data not shown). Two multiplex experiments were performed at an annealing temperature of 55º C, with two replications

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each. Cycle threshold values (Table 6) revealed specificity for each pathogen or for plants with each respective primer/probe combination. For the *P. ramorum* primer/probe combination, amplification from samples containing DNA of both pathogens had the same C_t (Table 6) and amplification curve (Fig. 3) to that obtained with *P. ramorum* alone. For the *P. pseudosyringae* primer/probe combination, amplification from samples containing both pathogens not only had a reduced C_t (Table 6) but the amplification curve was substantially reduced compared with that containing *P. pseudosyringae* alone (Fig. 3). Use of the plant primer/probe combination in multiplex PCR resulted in similar levels of amplification with individual pathogen samples as well as the combined sample (Fig. 3).

DISCUSSION

We have described a real-time PCR protocol based on mitochondrial gene regions which offers advantages over conventional PCR procedures and will provide a useful and rapid tool in nationwide efforts to detect the sudden oak death pathogen, *P. ramorum.* The need for such a test, which combines ease of use along with the specificity of conventional PCR and DNA hybridization (due to the inclusion of a specific TaqMan probe sequence) is especially pressing in light of the recent spread of the pathogen to the Eastern U.S. via shipments of nursery stock (51). The PCR method we describe can differentiate *P. ramorum* from other *Phytophthora* spp., some of which can cause similar looking lesions on the same hosts as *P. ramorum*. Using a multiplex format, additional *Phytophthora* species could be added to the assay as well. The described method uses mitochondrial gene regions rather than nuclear regions for detection, and thus offers the

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advantage of targeting a different region of the pathogen genome than in other tests. Several other real-time PCR assays for *P. ramorum* have targeted nuclear genes such as the ITS regions (2,13,23,27,61) and β-tubulin and elicitin genes (2). When used in combination, assays based on different genomic regions are more powerful and reliable than either test used alone, particularly in cases where one test may result in faint positive reactions and the pathogen cannot be cultured on selective agar medium. The fact that mitochondrial sequences are high copy also aids with the sensitivity of the assay.

However, the high AT/CG ratio and abundance of A and T in mitochondrial DNA offers a challenge to development of molecular detection methods. Methods such as increasing the ratio of dATP and dTTP vs. dGTP and dCTP in PCR reactions and/or reducing extension temperatures can enhance amplification of mitochondrial A + T-rich DNAs (45,52). A possible explanation for the reduced sensitivity we observed in multiplex PCR may be the A + T-rich nature of primers and probes we designed for use with our mitochondrial target region. Our primers and probes have a G/C base composition which is far below the 50% composition considered optimum (see Table 2). However, it is known that low G/C content can be compensated for by an increase in primer length (10). In spite of such potential difficulties, mitochondrial gene regions have proven useful in identification and detection studies with a number of different fungi (11,16,38, 64).

The specificity of our assay was determined by evaluating 45 different *Phytophthora* species (for some species multiple isolates were examined). In contrast the specificity of the PCR assay based on the ITS region has been tested with 20 species, some of which (*P. lateralis* and *P. cambivora)* cross-reacted at certain DNA

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concentrations (13, 23). The real-time PCR assay described here also detected a variety of *P. ramorum* isolates, including those from Europe. U.S. and European populations have been shown to be different for several characters including mating type (5,59) and our assay is able to detect *P. ramorum* from either population. The assay also exhibited a linear response between DNA concentration and detection limit and was sensitive enough to detect *P. ramorum* when present at a concentration of 1 fg of culture extracted DNA. The presence of plant extracts in the amplification mix in the amount equal to what would be used in assays of field samples did not alter the sensitivity of the assay. In fact, DNA extractions from infected leaves from a *Rhododendron* sp. could be diluted to 10^{-5} and the pathogen could still be detected. This marker system was initially developed for conventional nested PCR with the first round amplification done using a genus-specific primer pair followed by nested amplification with the species-specific primer pair (39). While it has not been experimentally verified, conducting conventional PCR with the genus-specific primers followed with the described nested real-time PCR procedure would be expected to enhance the sensitivity of pathogen detection.

Hayden et al. (23) reported detection of *P. ramorum* down to 12 fg DNA in an ITS-based PCR assay using SYBR green detection but several other *Phytophthora* species cross-reacted in the assay at DNA template concentrations above 0.7 ng. SYBR green binds indiscriminately to double-stranded DNA, so false positives caused by detection of primer-dimers and nonspecific amplification are possible (49). Vandemark and Barker (56) reported a detection limit of 1 pg DNA for *P. medicaginis* using a fluorescent real-time PCR primer-probe set based on a sequence characterized DNA marker (SCAR). Boehm et al. (4) reported a linear standard curve for detection of *P.*

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infestans using real-time PCR that ranged from 10^{-6} µg to 1 µg of template DNA per ml. This would place the lower limit of detection in the femtogram range similar to the results obtained with our real-time PCR assay.

Multiplex PCR allows for increased sample throughput and lower operating costs since multiple pathogens can be detected within the same plant extract by using different primer/probe combinations in the same reaction. Multiplex real-time PCR assays have been used previously for detecting both host and pathogen in the same reaction (25,62), and conventional (non real-time) multiplex PCR was used to detect *Phytophthora lateralis* in Port-Orford-cedar (61) and multiple fungal pathogens of wheat (17). We evaluated a real-time duplex assay with markers for *P. ramorum* and the plant using infected plant samples from the greenhouse and field samples from California and found a high correlation between the results of the real-time PCR assay and those of culturing and other detection methods. Perhaps due to the presence of PCR inhibitors in the samples with the extraction procedure that was used a 10-fold dilution of field sample DNA was necessary to obtain consistent amplification. Multiplexing amplification had a limited effect on the sensitivity of detection by the *P. ramorum* markers.

In an effort to simultaneously detect two pathogens causing similar foliar symptoms on some hosts, a three-way multiplex amplification was evaluated using markers for *P. ramorum*, *P. pseudosyringae*, and the plant to serve as a positive control. While multiplexing had no effect on the sensitivity of the *P. ramorum* and plant markers, there was a reduction in the detection sensitivity for the *P. pseudosyringae* markers (Fig. 3). However, the Ct values obtained were sufficient to determine whether the target pathogen was present or not in the assay. It is known that PCR efficiencies may be

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decreased when multiple primer sets are present in a single tube. Also, there exist many variables within PCR reactions that can affect the efficiency of multiplexing including the sequence of the oligonucleotides, concentrations of primers and probes, and concentrations of other PCR reaction components (10,15). One or more of these variables may have been responsible for the observed results.

In the future, we plan to extend the utility of this assay by developing primer/probe combinations for *P. nemorosa*, a pathogen present in California which is often isolated from material also infected with *P. ramorum*. We also plan to adapt the assay for use in other PCR machines such as the portable SmartCycler (Cepheid, Inc.) platform for more broad use by other laboratories, federal and state regulatory agencies.

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TABLE 1. Isolates of *Phytophthora* spp. used in this study.

Species	Group[a]	Isolate #[b]	Host	Origin
Phytophthora arecae	II	441[PT], IMI348342	*Theobroma cacoa*	Indonesia
Phytophthora boehmeriae	II	325[PT], P1257[MC]	*Boehmeriae nivia*	Papua New Guinea
Phytophthora cactorum	I	384[PT], NY577	*Fragaria* x *ananassa*	New York
		385[PT], NY568	*Malus sylvestris*	New York
Phytophthora cambivora	VI	443[PT], 33-4-8	*Prunus dulcis*	California
Phytophthora capsici	II	306[PT], Pc-m1	*Capsicum annuum*	New Jersey
Phytophthora cinnamomi	VI	Cn-2[DJM] (A-2 mating type)	*Vaccinium* spp.	Florida
		446[PT], 3210[GB]	*Castenea*	California
		447[PT], 3267[GB]	*Jugulands californica*	California
Phytophthora citricola	III	422[PT], CR4	*Cornus*	UNK
Phytophthora citrophthora	II	461[PT]	*Rhododendron sp.*	Oregon
Phytophthora clandestine	I	IMI287317[DC]	*Trifolium subterranean*	Australia
Phytophthora colocasiae	IV	345[PT], 1696[MC]	*Colocasia esculenta*	China
Phytophthora cryptogea	VI	310[PT], 620[PH]	*Pinus lambertiana*	Oregon
		389[PT], NY508[WW]	*Prunus avium*	California
Phytophthora drechsleri	VI	401[PT], ATCC64494	*Solanum tuberosum*	Egypt
Phytophthora erythroseptica	VI	374[PT]	*Solanum tuberosum*	Maine
Phytophthora fragariae fragariae	V	398[PT], 94-96[JIM]	*Fragaria* x *ananassa*	Oregon
Phytophthora gonapodyides	VI	392[PT], NY414[WW]	*Prunus persica*	New York

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Phytophthora heveae	II	462[PT], 97-251[PC]	*Rhododendron sp.*	Oregon
Phytophthora hibernalis	IV	338[PT], ATCC56353, 3822[MC]	Citrus	Australia
Phytophthora humicola	V	IMI302303[DC]	soil from citrus	Taiwan
Phytophthora idaei	I	IDA3[DC] (Type)	*Rubus idaeus*	Scotland
Phytophthora ilicis	IV	344[PT], P3939[MC], ATCC56615	*Ilex aquifolium*	Canada
Phytophthora inflata	III	IMI342898[DC]	*Syringa* sp.	
Phytophthora infestans	IV	561[PT], P30[JG]	*Solanum cardiophyllum*	Mexico
Phytophthora iranica	I	IMI158964[DC]	*Solanum melongera*	Iran
Phytophthora katsurae	II	IMI360596[DC]	*Cocos nucifera*	Ivory Coast
Phytophthora lateralis	V	451[PT], 91/11/1-5[MG]	*Chamaecyparis lawsoniana*	Oregon
Phytophthora medii	II	IMI129185[DC]	*Hevea brasiliensis*	India
Phytophthora megasperma	V	309[PT], 336[PH]	*Pseudotsuga menziesii*	Washington
		437[PT], IMI133317	*Malus sylvestris*	Australia
Phytophthora megakarya	II	327[PT], P132[CB]	*Theobroma cacao*	Nigeria
		328[PT], P184[CB]	*Theobroma cacao*	Cameroon
Phytophthora melonis	VI	IMI325917[DC]	*Cucumis* sp.	China
Phytophthora mirabilis	IV	340[PT], ATCC 64070, P3007[MC]	*Mirabilis jalapa*	Mexico
Phytophthora nemorosa	IV	482[PT], P-13[EH] Type	*Lithocarpus densiflorus*	California
Phytophthora nicotianae	II	360[PT]	*Solanum tuberosum*	Delaware

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Phytophthora parasitica	II	332[PT], P1751[MC]	*Nicotiana tabacum*	Auatralia
		334 [PT], P3118 [MC]	*Lycopersicon esculentum*	Australia
Phytophthora palmivora	II	329[PT], P131[CB]	*Theobroma cacao*	Nigeria
Phytophthora phaseoli	IV	352[PT], ATCC 60171, CBS 556.88	*Phaseolus lunatus*	unknown
		373[PT]	*Phaseolus lunatus*	Delaware
Phytophthora porri	III	CBS782.97[DC]	*Brassica chinensis*	The Netherlands
Phytophthora primulae	III	CBS620.97[DC]	*Primula acaulis*	Germany
Phytophthora pseudosyringae	IV	470[PT], P193907A[CDFA]	*Manzanita* sp.	Royal Oaks, CA
		471[PT]	*Umbellularia californica*	Napa, CA
		472[PT]	*Umbellularia californica*	Calistoga, CA
		473[PT]	*Umbellularia californica*	Yountville, CA
		484[PT], PSEU16[TJ], NFV-BU97-15	*Fagus sylvatica*	Germany
		485[PT], P96[EH]	*Umbellularia californica*	Contra Costa Co., CA
Phytophthora pseudotsugae	I	308[PT], H270[PH]	*Pseudotsugae menziesii*	Oregon
Phytophthora quercina	V	IMI340618[DC]	*Quercus robur*	Germany
Phytophthora ramorum	IV	Prn-1[PT], PD93/844[sw]	*Rhododendron* sp.	Netherlands
		Prn-2 [PT], PD94/844[sw]	*Rhododendron* sp.	Netherlands
		Prn-3 [PT], PD98/8/6743[sw]	*Rhododendron* sp.	Netherlands
		Prn-4 [PT], PD98/8/6285[sw]	*Rhododendron* sp.	Netherlands
		Prn-5 [PT], PD98/8/2627[sw]	*Rhododendron sp.*	Netherlands
		Prn-6 [PT], PD98/8/5233[sw]	*Viburnum* sp.	Netherlands
		Prg-1 [PT], BBA 69082[sw]	*Rhododendron* sp.	Germany
		Prg-2 [PT], BBA 9/95[sw], CBS101553 (Type)	*Rhododendron catawbiense*	Germany
		Prg-3 [PT], BBA 14/98-a[sw]	*Rhododendron catawbiense*	Germany
		Prg-4 [PT], BBA 12/98[sw]	*Rhododendron catawbiense*	Germany

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		Prg-5 [PT], BBA 13/99-1[sw]	*Rhododendron catawbiense*	Germany
		Prg-6 [PT], BBA 16/99[sw]	*Viburnum* bodnantense	Germany
		Prg-7 [PT], BBA 9/3[sw]	water	Germany
		Prg-8 [PT], BBA 104[sw]	water	Germany
		288[MG]	*Rhododendron* sp.	California
		73101[CDFA]	*Lithocarpus densiflorus*	California
		044519[CDFA]	*Umbellularia californica*	California
		044522[CDFA]	*Lithocarpus densiflorus*	California
		P072648 [CDFA]	*Quercus agrifolia*	California
		201C[DR]	*Rhododendron* sp.	California
		0-217, Pr-52[DR]	*Rhododendron* sp.	California
		Coen[MG]	*Rhododendron* sp.	California
		0-13, Pr-5[DR]	*Lithocarpus densiflorus*	California
		0-16, Pr-6[DR]	*Quercus agrifolia*	California
		C[MG]	*Umbellularia californica*	California
Phytophthora richardiae	VI	ATCC46538[DC]	*Zantedeschia* sp. root	The Netherlands
Phytophthora sojae	V	312[PT], ATCC 48068	*Glycine max*	Wisconsin
Phytophthora syringae	III	442[PT], P1023[CB], IMI 296829	*Rubus idaeus*	Scotland
		469[PT]	*Kalmia latifolia*	Oregon
Phytophthora tentaculata	I	CBS552.96[DC]	*Chrysanthemum leucanth.*	Germany
Phytophthora sp. "O" group[c]		P246[DC], IMI389751	*Salix* roots	U.K.
P. taxon Raspberry[c]		P896[DC], IMI389744	soil	Tasmania

[a] Waterhouse morphological group (57)

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[b] CB = Clive Brasier, DC= DNA supplied by David Cooke, MC=Michael Coffey, KD = Ken Deahl, PH = Phil Hamm (E. Hansen), DJM=Dave Mitchell, DS=Dave Shaw, PT=Paul Tooley, UCR = University of California at Riverside, SW= Sabine Werres, WW=Wayne Wilcox, DR= Dave Rizzo, CDFA=Cheryl Blomquist, California Dept. of Food and Agriculture, PC=Plant Clinic identification by Paul Reeser, JG= J. Galindo

[c] Species groupings of Brasier et al. (6)

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TABLE 2. Polymerase chain reaction primer and fluorescent probe sequences used to develop species-specific assays for *Phytophthora ramorum* and *Phytophthora pseudosyringae*.

Target	Primer/probe	Sequence (5' to 3')	Length	Tm[a]	%GC[b]
P. ramorum	FMPr-1a	GTATTTAAAATCATAGGTGTAATTTG	26	50.0	23.1
P. ramorum	FMPr-7	TGGTTTTTTTAATTTATATTATCAATG	27	51.9	14.8
P. ramorum	PrFAM probe	6-FAM d(CAGATATTAAACAAATTATATATAAAATCAAACAA) BHQ-1[c]	35	56.2	14.3
Plant	FMPl-2b	GCGTGGACCTGGAATGACTA	20	57.2	55
Plant	FMPl-3b	AGGTTGTATTAAAGTTTCGATCG	23	53.5	34.8
Plant	Plant CALOrange probe	CAL Orange d(CTTTTATTATCACTTCCGGTACTGGCAGG) BHQ-1	29	64.5	44.8
P. pseudosyringae	FMPps1c	AGTTTCATTAGAAGATTATTTAC	23	52.1	21.7
P. pseudosyringae	FMPps2c	AAAATTGTTTGATTTTATTAAGTATC	26	52.0	15.4
P. pseudosyringae	PpsCALOrange probe	CAL Orange d(TTAATAAAAAAATTATGATATTTAAACTAATTGGT) BHQ-1	35	56.3	11.4

[a] Melting temperature; Tm was calculated at 50 nM primer and 50 nM salt using the program Primer Express (Applied Biosystems).
[b] Percentage of guanulic and cytidylic acid.
[c] TaqMan probes were labeled at the 5' end with either the fluorescent reporter dye 6-carboxy-fluorescin (FAM) or CAL Fluor Orange (CAL Orange) and labeled at the 3' end with the black hole quencher dye (BHQ, Biosearch Technologies, Novato, CA)

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TABLE 3. Cycle threshold (Ct) values for 25 isolates of *Phytophthora ramorum, Phytophthora pseudosyringae* and other *Phytophthora* species subjected to real-time PCR analysis.

	Ct value[a]	
P. ramorum	*P. ramorum* primers and probe	*P. pseudosyringae* primers and probe
Coen	28.91 ± 0.44	>60 ± 0[b]
201C	26.40 ± 0.13	>60 ± 0
0-13	24.84 ± 0.46	>60 ± 0
0-16	26.83 ± 0.56	>60 ± 0
0-217	25.23 ± 0.11	>60 ± 0
288	28.81 ± 0.05	>60 ± 0
C	27.40 ± 0.75	>60 ± 0
73101	25.41± 0.55	>60 ± 0
044519	25.26 ± 0.13	>60 ± 0
044522	25.28± 0.44	>60 ± 0
Prn-1	25.66 ± 0.15	>60 ± 0
Prn-2	28.45 ± 0.69	>60 ± 0
Prn-3	28.87 ± 0.14	>60 ± 0
Prn-4	27.25 ± 0.01	>60 ± 0
Prn-5	26.73 ± 1.12	>60 ± 0
Prn-6	26.68 ± 0.18	>60 ± 0
Prg-1	26.88 ± 0.21	>60 ± 0
Prg-2	22.56 ± 0.11	>60 ± 0
Prg-3	24.86 ± 0.14	>60 ± 0
Prg-4	27.07 ± 0.18	>60 ± 0
Prg-5	27.49 ± 0.27	>60 ± 0
Prg-6	25.02 ± 0.15	>60 ± 0
Prg-7	28.53 ± 0.42	>60 ± 0
Prg-8	24.37 ± 0.52	>60 ± 0
P72648	25.66 ± 0.76	>60 ± 0
P. pseudosyringae		
470	>60 ± 0	25.41± 0.03
471	>60 ± 0	25.01± 0.40
472	>60 ± 0	24.52 ± 0.64
473	>60 ± 0	24.11 ± 0.06
484	>60 ± 0	27.74 ± 0.33
485	>60 ± 0	24.93 ± 0.25
Other *Phytophthora* species[c]	>60 ± 0	>60 ± 0
negative control	>60 ± 0	>60 ± 0

[a] Data are mean values of two replicated experiments ± standard error.
[b] No fluorescence was detected at 60 cycles of PCR amplification when tested at a concentration of 100 pg DNA.
[c] Other species listed in Table 1.

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TABLE 4. Amount of DNA estimated to be present in dilutions of DNA extracted from *Rhododendron* sp. (cv. 'Cunningham's White') leaf disks infected with *Phytophthora ramorum*.

Dilution from Bio101 kit[a]	Ct avg[b] ± SE	Amt. DNA calculated from standard curve
1:10	27.75 ± 0.32	20.9 pg
1:100	32.06 ± 0.53	1.4 pg
1:1000	35.37 ± 0.62	177 fg
1:10,000	39.57 ± 0.33	13 fg
1:100,000	42.81 ± 0.58	1.7 fg
1:1,000,000	55.34 ± 2.95	ND[c]

[a] DNA was extracted from two 6-mm diameter leaf disks using a Qbiogene Fast DNA extraction kit according to manufacturer's instructions.
[b] Ct values are means of six observations, plus or minus the standard error. Three separate extractions were performed (each using two 6-mm diameter leaf disks), and two replicate real-time PCR experiments were conducted, each containing sample from all three extractions diluted as indicated (n = 6).
[c] ND = not determined due to out of range of the standard curve

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TABLE 5. Real-time PCR results for symptomatic plant samples collected from the field in California and processed by the California Department of Food and Agriculture to determine which *Phytophthora* spp. were present.

Host species	Pathogen identification[a]	Real-time PCR result[b] (Ct value)	
		P. ramorum	*P. pseudosyringae*
Acer macrophyllum (6 samples)	none detected	>60[c]	>60
Aesculus californica (3 samples)	none detected	>60	>60
Arbutus menziesii (2 samples)	none detected	>60	>60
Heteromeles arbutifolia (2 samples)	none detected	>60	>60
Pseudotsuga menziesii	none detected	>60	>60
Rhamnus californica	*Phytophthora* sp.	>60	>60
Rhododendron sp.	*P. ramorum*	34	>60
Rhododendron sp.	*P. pseudosyringae*	>60	30
Rhododendron sp.	*Phytophthora sp.*	>60	>60
Rhododendron sp.	*Phytophthora sp.*	>60	>60
Rhododendron sp. (2 samples)	*P. syringae*	>60	>60
Rhododendron sp. (2 samples)	none detected	>60	>60
Salal sp.	none detected	>60	>60
Sambucus sp.	none detected	>60	>60
Sequoia sempervirens (2 samples)	none detected	>60	>60
Umbellularia californica (8 samples)	*P. nemorosa*	>60	>60
Umbellularia californica	*P. pseudosyringae*	>60	30
Umbellularia californica	*P. pseudosyringae*	>60	34
Umbellularia californica	*P. pseudosyringae*	>60	37
Umbellularia californica	*P. pseudosyringae*	>60	32
Umbellularia californica	*P. pseudosyringae*	>60	39
Umbellularia californica	*P. ramorum*	38	>60
Umbellularia californica	*P. ramorum*	35	>60
Umbellularia californica	*P. ramorum*	41	>60
Umbellularia californica	*P. ramorum*	41	>60
Umbellularia californica	*P. ramorum*	40	>60
Umbellularia californica	*P. ramorum*	44	>60
Umbellularia californica	*P. ramorum*	39	>60
Umbellularia californica	*P. ramorum*	32	>60
Umbellularia californica	*P. ramorum*	35	>60
Umbellularia californica	*P. ramorum*	38	>60
Umbellularia californica	*P. ramorum*	40	>60
Umbellularia californica	*P. ramorum*	37	>60
Umbellularia californica	*P. ramorum*	33	>60
Umbellularia californica (4 samples)	none detected	>60	>60

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[a] Plant samples from the field were the same as discussed previously (39). They were processed at the California Department of Food and Agriculture by plating on selective medium and confirming species identification based on morphological criteria and/or amplification of DNA extracted from infected tissue with the *P. ramorum* specific ITS primers. These were the same samples that were evaluated in a prior publication with the *Phytophthora* genus-specific, *P. ramorum*, *P. nemorosa*, and *P. pseudosyringae* species-specific primer pairs (39).
[b] Real-time PCR was performed following 1:10 dilution of DNA extract for multiplex amplifications using plant and th eindicated species-specific primers and probe. Results using plant primers and probe were positive for all samples, with Ct values ranging from 23 to 34.
[c] No fluorescence was detected at 60 cycles of PCR amplification.

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TABLE 6. Cycle threshold (Ct) values for multiplex experiments with California bay laurel (*Umbellularia californica*) artificially infected with *Phytophthora ramorum*, *Phytophthora pseudosyringae*, or both pathogens using primers and probes specific for *Phytophthora ramorum*, *Phytophthora pseudosyringae*, and plant DNA.

Sample	*P. ramorum* primers and probe	*P. pseudosyringae* primers and probe	Plant primers and probe
P. ramorum 0-217	28.6	>60[b]	30.6
P. pseudosyringae 470	>60	27.5	32.3
0-217 plus 470	28.5	34.2	29.1
negative control	>60	>60	>60
MSD[c]	0.8	5.0	1.2

[a] Data are means of four observations (two experiments with two replications each).
[b] No fluorescence was detected at 60 cycles of PCR amplification.
[c]Minimum significant difference, K-ratio = 100 for Waller-Duncan K-ratio t test for Ct value.

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FIGURE CAPTIONS

Fig. 1. Real-time amplification profiles for *Phytophthora ramorum* (A), and *Phytophthora pseudosyringae* (B) using primers and probes described in Table 2.

Fig. 2 . (A), Real-time PCR amplification profile for representative dilution series of DNA extracted from *Phytophthora ramorum* isolate 288. (B), Standard curve of Ct values calculated from serial dilutions of DNA from *P. ramorum* isolate 288 with standard error bars indicated. (C), Standard curve of Ct values calculated from serial dilutions of DNA from *P. pseudosyringae* isolate 471 with standard error bars indicated.

Fig. 3. Amplification profiles from multiplex real-time PCR analysis of leaf samples of California bay laurel (*Umbellularia californica*) artificially infected with *Phytophthora ramorum*, *Phytophthora pseudosyringae*, or both pathogens. Multiple experiments were performed; these amplification profiles represent results of a single run. The dye used for the *P. ramorum* probe (A) was FAM, that for the *P. pseudosyringae* probe (B) was CAL Orange, and that for the plant probe (C) was TAMRA. See Table 6 for Ct values associated with multiplex analysis.

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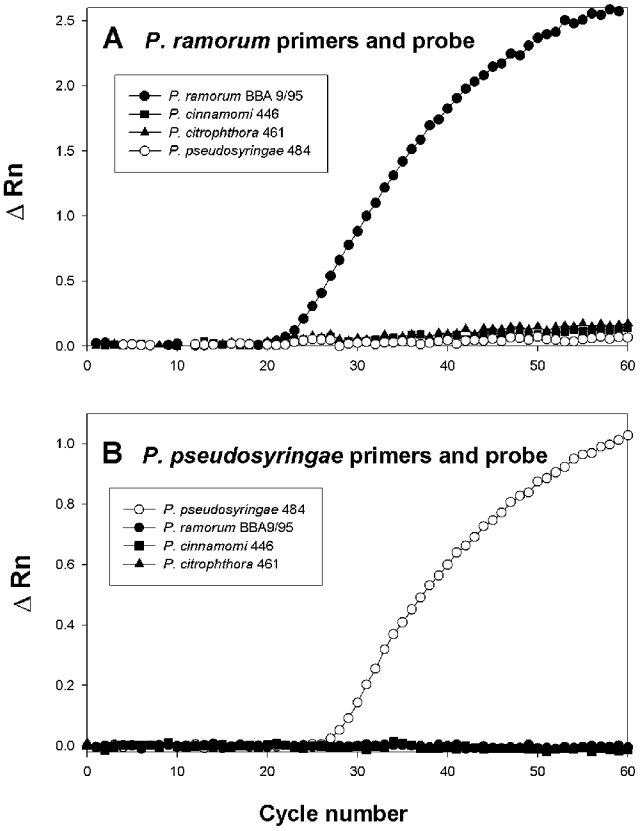

Fig. 1 Tooley et al.
Phytopathology

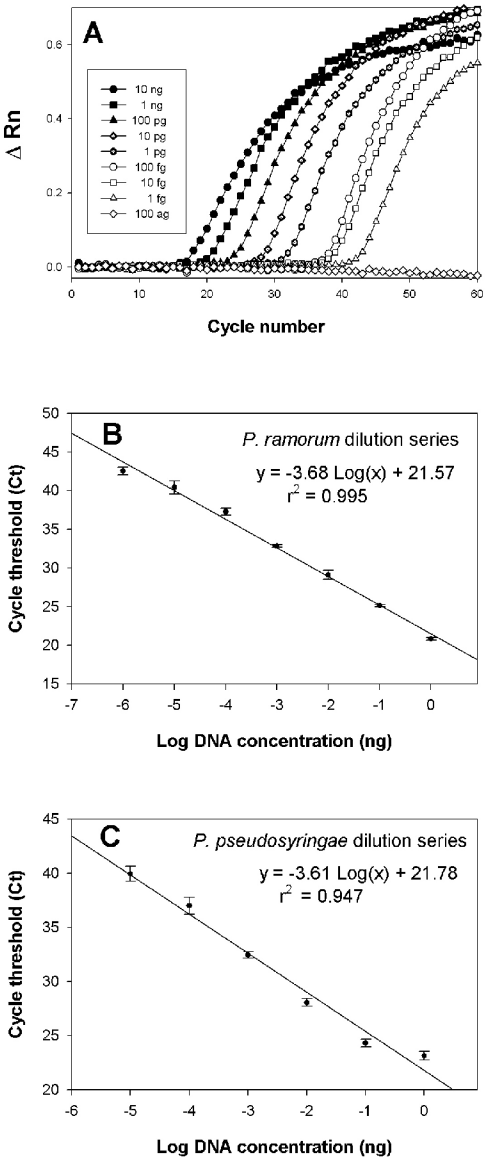

Figure 2. Tooley et al.
Phytopathology

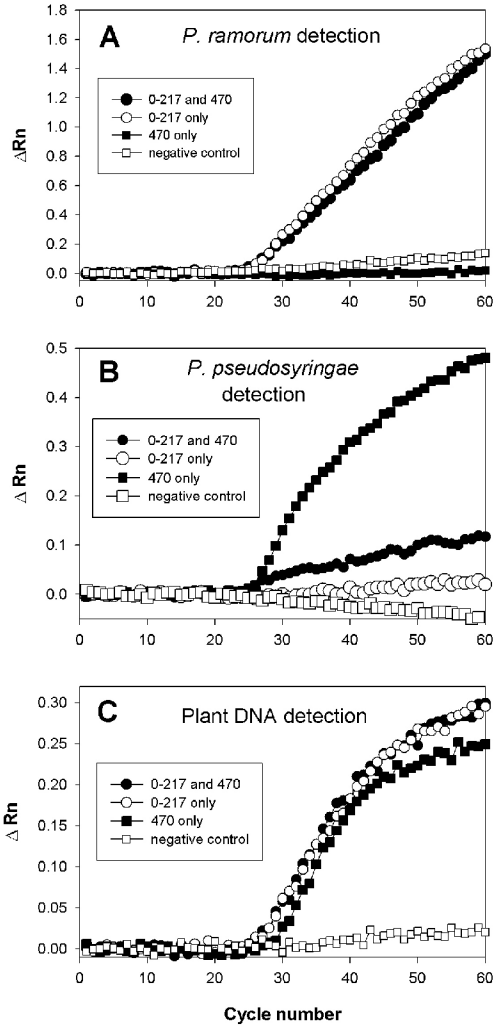

Fig. 3. Tooley et al.
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