A Species-Specific Polymerase Chain Reaction Assay for Rapid Detection of *Phytophthora nicotianae* in Irrigation Water

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ABSTRACT

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Phytophthora nicotianae is a common and destructive pathogen of numerous ornamental, agronomic, and horticultural crops such as tobacco, tomato, and citrus. We have developed a species-specific polymerase chain reaction (PCR) assay for rapid and accurate detection of this pathogen in irrigation water, a primary source of inoculum and an efficient means of propagule dissemination. This PCR assay consists of a pair of species-specific primers (PN), customization of a commercial soil DNA extraction kit for purification of DNA from propagules in irrigation water, and efficient PCR protocols for primer tests and sample detection.

The PN primers proved adequately specific for *P. nicotianae* in evaluations with 131 isolates of *P. nicotianae*, 102 isolates from 15 other species of *Phytophthora*, and 64 isolates from a variety of other oomycetes, true fungi, and bacteria. These isolates originated from a wide range of host plants, three substrates (plant tissue, soil, and irrigation water), and numerous geographic locations. The detection sensitivity is between 80 and 800 fg DNA/μl. The assay detected the pathogen in naturally infested water samples from Virginia and South Carolina nurseries more rapidly and accurately than standard isolation methods. Use of this PCR assay can assist growers in making timely disease management decisions with confidence.

Additional keywords: epidemiology, identification, population biology.

The presence of *Phytophthora nicotianae* Breda de Haan (synonym *Phytophthora parasitica* Dastur) in irrigation water is well documented. This species has been isolated in nursery irrigation water in California (25), North Carolina (22), Oklahoma (39), and Virginia (13). It also was recovered from water sources used for irrigating citrus (1,20,38,42), tobacco (5), and vegetable fields (36). Contaminated irrigation water can be a primary source of inoculum for *Phytophthora* diseases in nurseries (15). Plant pathogens such as *P. nicotianae* in nursery irrigation water are of increasing importance to ornamental crop producers recycling irrigation runoff to conserve water and prevent water contaminated with fertilizer and pesticides from leaving the nursery.

Infested irrigation water is not only an important source of inoculum, but also an efficient means of spreading pathogens from a single site of infection to an entire nursery, greenhouse, or farm and from one geographic location to other locations using the same water system (28,31,38). Rapid, accurate, and sensitive detection is important for managing *Phytophthora* diseases and preventing pathogen spread. Standard isolation methods can take 1 to 2 weeks to process a water sample, which is inadequate for timely pest-management decisions. As a result, many nurseries chlorinate irrigation water continuously and apply fungicides routinely (C. X. Hong and S. N. Jeffers, *personal observations*). Enzyme-linked immunosorbent assays (ELISA) and other serological methods offer potential as detection tools for irrigation water, but many questions must be resolved before these methods can be used effectively, and the results can be misinterpreted by growers (2,26, 40). False positive results with ELISA kits for *Phytophthora* spp. due to cross reaction with *Pythium* spp. have been reported (2).

This is a major concern because the population of *Pythium* spp. can be greater than that of *Phytophthora* spp. in irrigation water (2,13,34,40) and many of these species are weak or nonpathogens.

DNA-based techniques have become an effective means for identification of plant pathogens (6,11,23,24). Because of advantages in speed, sensitivity, specificity, and flexibility, polymerase chain reaction (PCR) methods have been used extensively and offer great promise in future plant pathogen detection systems (3,8,18,30,33,35,37). *P. nicotianae*-specific primers have been used for rapid identification of pure cultures (6) or detection of the pathogen in tobacco and tomato plants as well as in zoospore suspensions in the laboratory (21). The primer pair designed by Ersek et al. (6) was based on the sequence of a *P. parasitica*-specific DNA probe that may contain a repetitive sequence unit (11), whereas the other pair was derived from a *P. nicotianae* elicitor gene, *parA1* (19). *parA1* is a good candidate for designing specific PCR primers due to its high copy number (19) and the specificity potential (21). But the sequence of *parA1* coding and noncoding regions recently has been found to share a high degree of similarity with those of elicitor genes of many other oomycetes including *Pythium* spp. (10,32). Cross reaction with other oomycetes is likely if the primers are located within homologous regions or if they were not tested thoroughly.

There has been little work on PCR detection of *P. nicotianae* in irrigation water. Irrigation water contains numerous microbes and residues of fertilizers and pesticides, which may cause cross reactions or reaction inhibition during molecular detection. Neither of the two pairs of primers (6,21) has been tested with irrigation water samples. The detection specificity and sensitivity of these primers for irrigation water is unknown. In this study, we have developed a species-specific PCR assay for detection of *P. nicotianae* in irrigation water. Our specific objectives were to (i) design a pair of *P. nicotianae*-specific primers that provide the specificity and sensitivity required for irrigation water samples, (ii) find

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an effective method for purification of DNA from propagules of *P. nicotianae* in irrigation water, and (iii) develop efficient PCR protocols for evaluating the sensitivity and specificity of primers and sample detection.

MATERIALS AND METHODS

Fungi and bacteria. Isolates of *Phytophthora* spp., *Pythium* spp., other common water mold fungi, waterborne bacteria, and true fungi used in this study are listed in Tables 1, 2, and 3. A total of 131 isolates of *P. nicotianae* (Table 1) and 102 isolates of 15 other species of *Phytophthora* (Table 2) from a wide range of host plants and three substrates (plant tissue, soil, and irrigation water) in numerous geographic locations were tested. Also tested were 21 isolates of *Pythium* spp. and 12 isolates of true fungi from nine host plants in three states (Table 3) as well as 16 isolates of other common water mold fungi and 15 isolates of bacteria from recycled irrigation water in Virginia (Table 3).

Isolates of *Phytophthora* spp., *Pythium* spp., and other water mold fungi were grown on V8 juice agar (V8A) (9) in 6-cm-diameter petri dishes for 1 week at 20°C in the dark and maintained on V8A slants at 15°C in the dark. Isolates of true fungi and bacteria were grown on potato dextrose agar (PDA) in 10-cm-diameter petri dishes at 23°C in the dark for 2 weeks and 3 days, respectively, and maintained on PDA slants at 4°C.

Zoospores used in this study were produced by an isolate (1B11) of *P. nicotianae* recovered from a diseased annual vinca

(*Catharanthus roseus*) plant. Zoospores were produced by incubating finely chopped mycelium plugs bearing numerous sporangia for 2 to 4 h (depending on the temperature) in 25 ml of prechilled sterile soil water extract (SSWE) under fluorescent light. Zoospore suspensions were filtered through two layers of cheesecloth to remove mycelia and agar blocks; zoospores then were encysted by vortexing 1 ml of the original suspension in a microcentrifuge tube for 1 min to facilitate counting. Zoospore concentrations initially were determined by using a hemacytometer on a light microscope at $\times 100$ and by plating zoospore suspensions on PARP-V8 agar (9) in 6-cm-diameter petri dishes. The results from the plating method confirmed those determined by direct enumeration on the microscope. Subsequently, the hemacytometer was used as a primary means for determining zoospore concentration.

Concentrations of initial suspensions were approximately 10^4 zoospores/ml. To obtain higher concentrations of zoospores, 1 to 1.5 ml of the original suspension was centrifuged in a 1.5-ml microcentrifuge tube at $10,000 \times g$ for 1 min, and the pellet was resuspended in a smaller volume of SSWE. To obtain lower concentrations, the original suspension was diluted as needed with sterile nanopure water (SDW), 10 mM Tris-Cl, or irrigation water that had been passed through an Osmonics membrane filter with 5- μ m pores (Osmonics Laboratory Products, Minnetonka, MN).

DNA extraction. DNA of pure cultures was extracted using a modified boiling method. For fungi, 2 cm² of 1- to 2-week-old mycelium were scraped from the surface of a culture on V8 agar and placed in a 1.5-ml microcentrifuge tube containing 500 μ l of

TABLE 1. Polymerase chain reaction amplification of DNA extracted from 131 isolates of *Phytophthora nicotianae* recovered from various substrates and host plants in different geographic locations using *P. nicotianae*-specific primers (PN) designed in this study and the *P. parasitica*-specific primers (PP) reported by Ersek et al. (6)

Isolate/origin		No. isolates tested	Amplification detected		
Host or substrate	Location ^a		PN	PP	Source ^a
<i>Abies</i> sp.	West Virginia	1	1	1	3
<i>Antirrhinum majus</i>	South Carolina (SC)	2	2	2	6
<i>Buxus</i> sp.	SC, Virginia (VA)	5	5	5	5,6
<i>Catharanthus roseus</i>	SC, VA	26	26	26	5,6
<i>Ceratostigma plumbaginioides</i>	SC	1	1	1	6
<i>Chamaerops humilis</i>	SC	1	1	1	6
<i>Chionanthus retusus</i>	SC	1	1	1	6
<i>Citrus limon</i>	California (CA)	1	1	1	3
<i>Citrus</i> spp.	CA, India	7	7	7	1,3
<i>Daphne</i> spp.	SC, VA	21	21	21	5,6
<i>Delosperma nubigenum</i>	SC	1	1	1	6
<i>Fatsia japonica</i>	SC	3	3	2	6
<i>Forsythia</i> sp.	SC	1	1	1	6
<i>Gaura lindheimeri</i>	SC	1	1	1	6
<i>Hedera</i> spp.	SC	12	11	10	6
<i>Hemerocallis</i> sp.	SC	1	1	1	6
<i>Juniperus</i> sp.	SC	1	1	1	6
<i>Kniphofia</i> hybrid	SC	1	1	1	6
<i>Ligustrum</i> sp.	SC	1	1	1	6
<i>Ilex crenata</i>	SC	1	1	1	6
<i>Michelia figo</i>	SC	1	1	1	6
<i>Nicotiana tabacum</i>	North Carolina (NC)	3	3	3	3
<i>Penstemon</i> sp.	SC	1	1	1	6
<i>Petunia</i> sp.	SC	2	2	2	6
<i>Phlox paniculata</i>	SC	1	1	1	6
<i>Pinckneya pubens</i>	SC	1	1	0	6
<i>Rhododendron</i> sp.	VA	1	1	1	4
<i>Salvia</i> sp.	SC	1	1	1	6
<i>Solanum tuberosum</i>	NC	1	1	1	3
<i>Verbena</i> sp.	SC	2	2	2	6
<i>Vinca minor</i>	NC, SC	8	8	7	2,6
<i>Viola xwittrockiana</i>	SC	1	1	1	6
Nursery irrigation water	Oklahoma, SC, VA	12	12	12	5,6,7
Tester	VA	2	2	2	4
... ^b	... ^b	5	5	5	3

^a Isolates were provided by: 1 = J. Adaskaveg, University of California, Riverside; 2 = D. M. Benson, North Carolina State University, Raleigh; 3 = M. E. Gallegly, West Virginia University, Morgantown; 4 = M. A. Hansen, Virginia Tech, Blacksburg; 5 = C. Hong, Virginia Tech, Virginia Beach; 6 = S. N. Jeffers, Clemson University, Clemson, SC; and 7 = S. L. von Broembsen, Oklahoma State University, Stillwater.

^b ... = Not known.

10 mM Tris-HCl (pH 7.5). DNA was released by boiling mycelia in a heat block for 20 min and vortexing for 3 min. Similarly, individual colonies of bacteria on PDA were harvested and boiled in 100 µl of the same buffer. The supernatants (containing raw DNA) were used immediately or stored at -20°C until further use. DNA of *P. nicotianae* isolate 1B11 also was purified using the DNeasy Plant Maxi Kits (Qiagen Inc, Valencia, CA) following the manufacturer's instructions.

DNA of organisms artificially and naturally present in water was extracted using the UltraClean Soil DNA Kit (MO BIO Laboratories, Inc., Solana Beach, CA). Propagules were collected by filtration or centrifugation prior to DNA extraction. For filtration, a predetermined volume of zoospore suspension or naturally infested irrigation water was passed through a 47-mm-diameter nylon membrane filter with 5-µm pores using a vacuum pressure of 5.53 Pa (41.5 cm Hg), which captured propagules on the filter. For centrifugation, zoospore suspensions or irrigation water were centrifuged (Sorvall RC 5D Plus centrifuge; Kendro Laboratory Products, Newtown, CT) at 10,000 × g for 10 min to pellet the propagules. DNA was extracted from propagules on filters or in pellets using the UltraClean Soil DNA Kit with some modifications to the first step. Briefly before starting extraction, the filter was cut into fine pieces while wet, and these pieces were placed

into the bead tube (included in the kit for loading a soil sample). Alternatively, the propagule pellet was resuspended with the solution from the bead tube and was poured back into the tube. The remaining extraction steps followed the manufacturer's instructions. Extracted DNA was eluted with 100 µl of SDW unless otherwise stated.

Primers and DNA amplification. A pair of *Phytophthora nicotianae*-specific primers, referred to as the PN primers, was designed based on specific sequences in the elicitor gene *parA1* (19) (GenBank Accession No. S67432) of the pathogen. The sequences of the forward primer (CCACCACGCAGCAAAGTGGCGC) and the reverse primer (TTGAGTACCAGGCCGCTCGTAG) are located at nucleotides 70 to 91 and 287 to 308 of *parA1*, respectively. The specific sequences for the primers were determined by a BLAST search at the NCBI web site and by comparisons of *parA1* with elicitor genes of other oomycetes. Another primer pair reported previously by Ersek et al. (6) as specific to *P. parasitica* was used in this study for comparison and will be referred to as the PP primers. An additional primer pair, ITS6 and ITS7 (4), was used to assess the quality of DNA preparations from Oomycete isolates. All primers were synthesized at Sigma Genosys (Woodlands, TX).

PCR reactions were performed in a total volume of 25 µl and each contained 2 µl of boiled mycelium extract or DNA extract

TABLE 2. Polymerase chain reaction amplification using *Phytophthora nicotianae*-specific primers designed in this study of DNA extracted from 102 isolates of 15 other species of *Phytophthora* recovered from various substrates and host plants in different geographic locations

Species	Host/substrate	Isolate/origin	No. of isolates		
			Tested	Positive	Source ^a
<i>P. cactorum</i>	<i>Syringa meyeri</i>	Wisconsin (WI)	1	0	4
	<i>Abies fraseri</i>	WI	1	0	4
	<i>Malus domestica</i>	New York, South Carolina (SC), Virginia (VA)	3	0	2,4
	<i>Fragaria xananassa</i>	WI	1 ^b	0	4
<i>P. cambivora</i>	Nursery irrigation water	VA	1	0	3
	Nursery irrigation water	VA	6 ^c	0	3
<i>P. capsici</i>	Nursery irrigation water	VA	3	0	3
	Mating tester	... ^d	2	0	2
<i>P. cinnamomi</i>	<i>Camellia japonica</i>	California (CA)	1	0	4
	<i>Ginkgo biloba</i>	SC	1	0	4
	Nursery irrigation water	Oklahoma (OK), VA	4	0	3,5
	Mating tester	... ^d	2	0	2
<i>P. citricola</i>	<i>Juglans</i> sp.	CA	1	0	3
	<i>Prunus dulcis</i>	CA	1	0	3
	<i>Hedera helix</i>	SC	1	0	4
	Native flora	SC	1	0	4
	<i>Acer saccharum</i>	WI	1	0	4
	<i>Juglans nigra</i>	WI	1	0	4
	<i>Rhododendron</i> sp.	WI	1	0	4
	Nursery irrigation water	VA, OK	2	0	3,5
	<i>Rosa</i> sp.	SC	1	0	4
	<i>Buxus sempervirens</i>	SC	1	0	4
<i>P. citrophthora</i>	<i>Actinidia deliciosa</i>	CA	1	0	4
	<i>Citrus limon</i>	CA	1	0	4
	<i>Theobroma cacao</i>	Brazil	1	0	4
	Nursery irrigation water	CA, OK, VA	12	0	3,5
	<i>Aster</i> sp.	CA	1	0	4
	<i>Verbena</i> sp.	SC	1	0	4
	Native flora	SC	2	0	4
<i>P. drechsleri</i>	Nursery irrigation water	OK	1	0	4
	<i>Beta vulgaris</i>	Idaho	1	0	4
	Nursery irrigation water	VA	11	0	3
<i>P. heveae</i>	Nursery irrigation water	VA	1	0	3
<i>P. infestans</i>	<i>Lycopersicon esculentum</i>	North Carolina, West Virginia, Ireland	4	0	1
<i>P. megasperma</i>	<i>Prunus dulcis</i>	CA	2	0	3
	Nursery irrigation water	VA	10	0	3
<i>P. palmivora</i>	Nursery irrigation water	VA	3	0	3
<i>P. phaseoli</i>	<i>Phaseolus lunatus</i>	Delaware	2	0	1
<i>P. pseudotsugae</i>	... ^d	... ^d	1	0	1
<i>P. tropicalis</i>	Nursery irrigation water	VA	10	0	3

^a Isolates were provided by: 1 = M. E. Gallegly, West Virginia University, Morgantown; 2 = M. A. Hansen, Virginia Tech, Blacksburg; 3 = C. Hong, Virginia Tech, Virginia Beach; 4 = S. N. Jeffers, Clemson University, Clemson, SC; and 5 = S. L. von Broembsen, Oklahoma State University, Stillwater.

^b The isolate produced an amplicon slightly larger than the specific one.

^c One isolate produced an amplicon discernibly higher than the specific one.

^d ... = Not known.

using the UltraClean Soil DNA Kit, unless otherwise stated. Each reaction used 2.5 µl of the 10× PCR buffer, 2.5 µl of each of the 10 µM primer solutions, 2 µl of the 2 mM dNTP solutions, 0.1 µl (5 U/µl) of *Taq* polymerase (TaKaRa, Shuzo Co. Ltd., Kyoto, Japan), and 13.4 µl of SDW. PCR was carried out in a Perkin-Elmer 480 thermal cycler with initial denaturation at 96°C for 2 min, followed by 40 cycles of 94°C for 30 s, 65°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Alternatively, 10 µl of a known number of zoospores in a suspension was added to a PCR reaction (i.e., zoospore PCR), following the above procedure without additional SDW. An extended initial denaturing time of 3 min was added to the PCR program.

PCR with either the PN or PP primer pair always included a positive control with purified DNA from *P. nicotianae* and a negative control with SDW. An aliquot of 5 µl of PCR products from each sample was resolved by electrophoresis in 1.0 to 1.5% agarose or 3% Nusieve gels stained with ethidium bromide. Images were captured and analyzed using a BioImaging Chemi System (UVP, Inc., Upland, CA).

DNA hybridization assays. Detection of *P. nicotianae* was verified by DNA hybridization. Five microliters of the PCR products were used for electrophoresis in a 3% Nusieve gel, and following denaturing and neutralization, the gel was blotted on an ImmobilonNy+ transfer membrane (Millipore Corporation, Bedford, MA). The blot was probed with digoxigenin (DIG)-labeled *parA1* gene of *P. nicotianae* using the PCR DIG Probe Synthesis

Kit, and DNA was detected using the DIG Luminescent Detection Kit following the manufacturer's instructions (Roche Diagnostics GmbH, Indianapolis, IN). Images were captured and analyzed using the BioImaging Chemi System.

Specificity and sensitivity tests. The specificity of the PN primers was compared with that of the PP primers using raw DNA from 131 isolates of *P. nicotianae* (Table 1). The specificity of the PN primers also was evaluated using raw DNA from 166 isolates of other oomycetes, fungi, and bacteria (Tables 2 and 3).

The sensitivities of the PN and PP primers were compared using the zoospore-PCR procedure described above. An original suspension of 8×10^4 zoospores per ml was diluted to 10^4 , 10^3 , and 100 zoospores per ml with SDW, 10 mM Tris-HCl, or irrigation water filtrate. The original spore suspension or one of the dilutions was added directly to a PCR reaction.

The sensitivity of the PN primers was further compared among five isolates of *P. nicotianae*. These isolates originated from different hosts and geographic locations: 1B11 from *Catharanthus roseus* in North Carolina, 1E2 from nursery irrigation water in Oklahoma, 21J1 from *Citrus* sp. in California, 23B5 from *Abies fraseri* in West Virginia, and 23B8 from *Citrus* sp. in India. For each isolate, zoospores were produced as described previously, but DNA was extracted using the UltraClean Soil DNA kit by direct addition of 100 µl of suspension containing 1,000 zoospores to the bead tube; extracted DNA was eluted in 50 µl of SDW. Serial dilutions (up to 10^{-5}) of these DNA extracts were

TABLE 3. Polymerase chain reaction amplification using *Phytophthora nicotianae*-specific primers designed in this study of DNA extracted from various microorganisms commonly encountered in irrigation water and nursery production

Microorganism	Isolate/origin		No. of isolates		
	Host/substrate	State	Tested	Positive	Source ^a
<i>Pythium aphanidermatum</i>	<i>Chrysanthemum</i> sp.	Pennsylvania (PA)	1	0	2
	<i>Euphorbia</i> sp.	PA	1	0	2
<i>Pythium irregulare</i>	<i>Pelargonium</i> sp.	PA	1 ^b	0	2
	<i>Euphorbia</i> sp.	PA	1	0	2
<i>Pythium myriotylum</i>	<i>Pelargonium</i> sp.	PA	2	0	2
<i>Pythium ultimum</i>	Seedling	California (CA)	1	0	1
	<i>Pelargonium</i> sp.	PA	1	0	2
	<i>Euphorbia</i> sp.	PA	1	0	2
<i>Pythium</i> spp. ^c	Nursery irrigation water	Virginia (VA)	12	0	1
<i>Allomyces</i> sp. ^d	Nursery irrigation water	VA	1	0	1
<i>Mortierella</i> spp. ^d	Nursery irrigation water	VA	2	0	1
<i>Saprolegnia mixta</i> ^d	Nursery irrigation water	VA	2	0	1
<i>Saprolegnia</i> spp. ^d	Nursery irrigation water	VA	5	0	1
<i>Thraustotheca</i> sp. ^d	Nursery irrigation water	VA	1	0	1
Chytrids ^d	Nursery irrigation water	VA	2	0	1
Unknown water molds	Nursery irrigation water	VA	3	0	1
Bacterium/grey ^e	Nursery irrigation water	VA	3	0	1
Bacterium/yellow	Nursery irrigation water	VA	3	0	1
Bacterium/milky	Nursery irrigation water	VA	3	0	1
Bacterium/white large	Nursery irrigation water	VA	3	0	1
Bacterium/white small	Nursery irrigation water	VA	3 ^f	0	1
<i>Alternaria alternata</i>	<i>Daucus carota</i>	CA	1	0	1
<i>Alternaria daucia</i>	<i>Daucus carota</i>	CA	1	0	1
<i>Alternaria microspora</i>	<i>Daucus carota</i>	CA	1	0	1
<i>Alternaria porri</i>	<i>Daucus carota</i>	CA	1 ^g	0	1
<i>Alternaria radicina</i>	<i>Daucus carota</i>	CA	1	0	1
<i>Alternaria solani</i>	<i>Daucus carota</i>	CA	1	0	1
<i>Botrytis cinerea</i>	<i>Prunus persica</i>	CA	1	0	1
<i>Monilinia fructicola</i>	<i>Prunus persica</i>	CA	1	0	1
<i>Monilinia laxa</i>	<i>Prunus persica</i>	CA	1	0	1
<i>Rhizoctonia</i> sp.	<i>Rhododendron</i> sp.	VA	1	0	1
<i>Thielaviopsis</i> sp.	<i>Viola tricolor</i>	VA	1	0	1
<i>Ulocladium</i> sp.	<i>Daucus carota</i>	CA	1	0	1

^a Isolates were provided by: 1 = C. Hong, Virginia Tech, Virginia Beach; and 2 = G. W. Moorman, Pennsylvania State University, University Park.

^b The isolate produced a faint and significantly higher molecular weight band than the specific one.

^c Representing the major groups of *Pythium* spp. isolated from irrigation runoff and irrigation water at two nurseries during monthly samplings in 2000-2001 and at four additional nurseries during quarterly samplings in 2000.

^d Tentative identification.

^e Color of bacterium colonies; no identification of bacteria was attempted.

^f One isolate produced a slightly lower molecular weight amplicon than the specific one.

^g The isolate produced a faint and higher molecular weight band than the specific one.

prepared with SDW and used in PCR reactions along with the original extract.

The sensitivity of the PN primers also was compared using two zoospore-collecting methods and six zoospore quantities. The two methods were filtration and centrifugation, and the six quantities were 2×10^5 , 2×10^4 , 2×10^3 , 200, 20, and 2 zoospores in 100 ml of SSWE for filtration method and in 40 ml of SSWE for centrifugation. DNA was extracted from a filter or pellet after zoospores were collected from a suspension as described above, and extracted DNA was eluted with 50 μ l of SDW.

Another experiment was conducted to investigate the effect of DNA quality on template amplification. Two DNA extraction samples, one from irrigation runoff water and the other from irrigation pond water, previously had tested negative with the PN primers. These DNA extraction samples were compared with SDW by adding purified DNA of *P. nicotianae* isolate 1B11 at various concentrations starting at 1 μ g/ μ l. One microliter of the original or diluted purified DNA was mixed with 9 μ l of DNA extracted from irrigation runoff water or irrigation pond water to make six final template concentrations of 8 ng/ μ l, 800, 80, and 8 pg/ μ l, and 800 and 80 fg/ μ l in PCR reaction mixes. Another set was prepared with SDW only. The two extraction samples and SDW with or without addition of purified DNA of *P. nicotianae* were subjected to PCR with the PN primers. All tests above were repeated.

Detection of *P. nicotianae* in irrigation water. The PCR assay developed in this study was compared with the protocol of Ersek et al. (6) as well as standard isolation methods (1,16) using naturally infested irrigation water collected at commercial nurseries in Virginia and South Carolina. In Virginia, three replicate samples of each of irrigation runoff and irrigation water were collected in 3.8-liter plastic bottles at points where runoff entered a retention pond or at irrigation risers in the nursery from May to September 2001. The samples were transported in a cooled ice

chest to the laboratory in Virginia Beach, VA, where they were assayed by filter-based isolation and the PCR method. Samples were assayed by filter-based isolation following the protocol described previously (16,25). Briefly, three aliquots of 50 ml of runoff water or 100 ml of irrigation water were taken from each replicate bottle and passed through a 47-mm-diameter Durapore membrane filter with 5- μ m pores (Millipore Corporation). Propagules captured on each filter were resuspended in 6 ml of 0.09% sterile water agar in a test tube. A 100- μ l aliquot of resultant suspension was spread over PARP-V8 agar in each of two 10-cm-diameter petri dishes. Isolation dishes were incubated at 23°C in the dark for up to 7 days, and colonies were counted daily after 2 days. Twenty percent of representative colonies were subcultured and identified based on morphological characters (7,41).

In South Carolina, duplicate water samples (0.5 or 1 liter) were collected during September 2001 from three irrigation ponds at two commercial nurseries (G and S) and from a stream running through a landscaped area (CU). One set of samples was assayed by filter-based isolation at Clemson University and one set of samples was sent by overnight courier to Virginia Beach to be assayed by the PCR method. For filter-based isolation, five replicate aliquots of water (75 to 200 ml, depending on the sample) were used for each sample; each aliquot was passed through a 47-mm-diameter Nucleopore polycarbonate membrane filter with 3- μ m pores (Whatman, Inc., Clifton, NJ). Each filter was inverted onto a petri dish containing PARPH-V8 agar (9). Isolation dishes were incubated at 20°C in the dark for 3 days. Filters were removed, the agar surface was rinsed under running tap water and colonies were counted. Petri dishes were incubated for four more days at 20°C and checked for additional colonies. Representative colonies from each dish were subcultured and identified.

All PCR detection was performed in the laboratory at Virginia Tech in Virginia Beach, VA, using the PN or PP primers. Water

Fig. 1. Three percent Nusieve gel electrophoresis of polymerase chain reaction (PCR) products of test species that were similar to the specific band of the *Phytophthora nicotianae*-specific primers designed in this study (top); DNA hybridization of the PCR products with the *parA1* probe (bottom). M = 100-bp DNA ladder; 1D2 = *P. cambivora* from irrigation water; W.097 = *P. cactorum* from *Fragaria xananassa*; P11 = *Pythium irregulare* from *Pelargonium*; AP1 = *Alternaria porri* from *Daucus carota*; SW1 = bacterium from irrigation water; - = Negative control with sterile nanopure water; + = positive control with purified DNA from *P. nicotianae*.

Fig. 2. Agarose gel electrophoresis of the polymerase chain reaction (PCR) products obtained from suspensions of *Phytophthora nicotianae* at 8×10^4 zoospores per ml to 10^4 , 10^3 and 10^2 zoospores per ml. The initial zoospore suspension was prepared with soil water extract (SE) and diluted with sterile distilled water (SDW), 10 mM Tris-Cl, or irrigation water filtrate (IWF; i.e., water that had been passed through an Osmonics nylon membrane filter with 5- μ m pores). The number of zoospores added to a PCR reaction is listed above each lane. M = 100-bp DNA ladder; - = negative control with SDW only. Sizes of the specific bands for the *P. nicotianae*-specific primers designed in this study (top) and the *P. parasitica*-specific primers designed by Ersek et al. (6) (bottom) are indicated by arrows.

samples from Virginia were assayed on the day of sampling. Three 500-ml bottles of water from each sample location in South Carolina were assayed upon their arrival. From each replicate bottle from Virginia or South Carolina, 50 ml (runoff) or 100 ml (pond and stream) of water was used. Pathogen propagules were collected using the filtration method, and DNA was extracted using the UltraClean Soil DNA purification kit as described previously. DNA at original concentration was used for Virginia water samples, while DNA at both original and 5× concentration was used for South Carolina water samples. The original DNA extract was concentrated with an ethanol precipitation procedure described by the kit manufacturer, followed by resuspending the DNA in 20 µl of SDW. PCR products from all water samples were electrophoresed on 3% Nusieve gels and the detection results were verified by DNA hybridization with the *parA1* probe to identify false detection.

RESULTS

Primers and DNA amplification. All DNA preparations from oomycetes were amplified with the ITS6 and ITS7 primers, resulting in an amplicon at ca. 300 bp, demonstrating that they were of sufficient quality as PCR templates. The PCR using the PN primers with purified DNA of *P. nicotianae* produced an amplicon at 230 bp, whereas the PP primers produced one at 950 bp.

Specificity. The PN primers allowed greater detection of *P. nicotianae* isolates than the PP primers (Table 1). Of the 131 isolates tested, the PN primers detected 130 isolates and the PP primers detected 126 isolates; the PN primers did not detect one isolate from *Hedera helix*, while the PP primers failed to detect five isolates from *Fatsia japonica*, *Hedera* spp., *Pinckneya pubens* and *Vinca minor*.

The PN primers were specific when they were evaluated with 102 isolates from 15 other species of *Phytophthora* (Table 2). Only one of seven isolates of *P. cactorum* (W.097) and one of six isolates of *P. cambivora* (1D2) gave an amplification. These two amplicons were discernibly higher than the one from the positive control with the amplicon from isolate W.097 closer to the 230-bp target band on a 3% Nusieve gel (Fig. 1). Amplicons of the *P. cambivora* isolate did not hybridize with the *parA1* probe of *P. nicotianae*, but those from the *P. cactorum* isolate did hybridize with the *parA1* probe (Fig. 1).

The PN primers also were specific when tested with 64 isolates of bacteria and fungi from other genera. With two exceptions, DNA from all isolates of *Pythium* spp. and true fungi was not amplified by the PN primers (Table 3). Two faint bands each associated with an isolate of *Pythium irregulare* (P11) or *Alter-*

naria porri (AP1) were larger than the target band (230 bp) and easy to discriminate (Fig. 1). All other water mold fungi tested negative with the PN primers. Only one of the 15 isolates of bacteria tested had a fragment with size slightly smaller than 230 bp, and this was negative in the DNA hybridization assay (Fig. 1 and Table 3).

Sensitivity. The PN primers were more sensitive than the PP primers (Fig. 2). The zoospore PCR with the PN primers detected *P. nicotianae* at levels of one zoospore in SDW or in 10 mM Tris-HCl solution and 10 zoospores in pond water filtrate. The PP primers detected *P. nicotianae* at one zoospore in SDW or in Tris-HCl with a weaker signal than that detected by the PN primers, and they needed at least 100 zoospores in irrigation water filtrate for positive detection.

The PN primers appeared to be equally sensitive to all five isolates of *P. nicotianae* tested (Fig. 3). The PCR produced positive results for all isolates at three concentrations of DNA extract—including the 10⁻¹ dilutions (data not shown). The positive signal was strong when original DNA extract was used but weakened with decreasing concentration of DNA templates. At the 10⁻² dilution of DNA extract, the specific band differed slightly in intensity among the isolates.

Centrifugation was more effective than filtration at collecting zoospores for DNA extraction and detection under the test conditions (Fig. 4). PCR with the PN primers yielded positive results for all six concentrations from 2 × 10⁵ to 2 zoospores per sample when centrifugation was used to collect the spores from water samples. In contrast, the PCR assay needed a minimum of 20 zoospores per sample for a positive signal when filtration was used. The sensitivity of the PCR assay was equivalent to 0.08 zoospores per reaction for the centrifugation method and 0.8 zoospores per reaction for filtration.

Addition of the purified DNA to both extraction samples from irrigation runoff water and irrigation pond water resulted in positive signals at the same levels as that added to SDW (Fig. 5). The intensity of the specific band appeared similar among the extraction samples and SDW and decreased with decreasing amount of the purified DNA. The PCR assay produced a positive signal at 800 fg/µl but not at 80 fg/µl for both extraction samples and SDW.

Detection of *P. nicotianae* in irrigation water. Using the PCR assay, *P. nicotianae* was detected in three of the five irrigation runoff samples (June, August, and September) and in one irrigation water sample (September) collected at the Virginia nurseries (Fig. 6). Hybridization of the PCR products with the *P. nicotianae*-specific probe derived from *parA1* confirmed all detection results. In contrast, the pathogen was detected with the PP primers

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) products using the *Phytophthora nicotianae*-specific primers designed in this study with DNA from five isolates of *P. nicotianae* with different origins: 1B11 from *Catharanthus roseus* in North Carolina; IE2 from nursery irrigation water in Oklahoma; 21J1 from *Citrus* sp. in California; 23B5 from *Abies fraseri* in West Virginia; and 23B8 from *Citrus* sp. in India. For each isolate, DNA was extracted from 1,000 zoospores using the UltraClean Soil DNA extraction kit. Original DNA extract (0) and 10⁻² (2) and 10⁻³ dilutions (3) were used for template amplification. – = Negative control with sterile nanopure water; M = 100-bp DNA ladder.

Fig. 4. Agarose gel electrophoresis of polymerase chain reaction products obtained with *Phytophthora nicotianae*-specific primers designed in this study using filtration or centrifugation to collect zoospores of *P. nicotianae* in sterile soil water extract. A 100-ml aliquot of suspension containing 2 × 10⁵ (A), 2 × 10⁴ (B), 2 × 10³ (C), 200 (D), 20 (E), or 2 zoospores (F) was passed through an Osmonics nylon membrane filter with 5-µm pores or a 40-ml aliquot of suspension containing the same numbers of zoospores was centrifuged at 10,000 × g for 10 min. DNA was extracted from the zoospores collected on the filters or in the pellets using the UltraClean Soil DNA extraction kit and eluted in 50 µl of sterile distilled water. – = negative control with sterile nanopure water; M = 100-bp DNA ladder.

in two samples of runoff water (June and August) but not in any sample of irrigation pond water. Filter-based isolation only detected *P. nicotianae* in one runoff sample (August); zoospore concentrations were estimated to be 450 to 570 CFU/liter.

P. nicotianae was detected in the irrigation water samples from two (G1 and CU) of the four locations in South Carolina when original DNA extracts were used (Fig. 6). The pathogen also was detected in the water samples collected from one other location (G2) when the 5× concentrated DNA extract was used (Fig. 6). Like the Virginia samples, DNA hybridization confirmed all PCR detection results of water samples from South Carolina. In contrast, the PP primers only detected the pathogen from the CU location using the original DNA extract but not using the 5× concentrated one. Filter-based isolation detected *P. nicotianae* in only one (CU) of the four South Carolina locations; zoospore concentration was estimated to be 65 CFU/liter.

DISCUSSION

Rapid and reliable detection of pathogens is important for formulating strategies for disease management in nurseries. This study describes such a detection assay for *P. nicotianae* by using species-specific PCR. *P. nicotianae* is an economically important pathogen on numerous ornamental crops as well as many other crops, e.g., citrus, tomato, and tobacco (7). The PCR assay targeted this pathogen in irrigation water, which can be a primary source of inoculum at ornamental crop nurseries (15).

The PCR assay provided more rapid detection of *P. nicotianae* than standard filter-based isolation methods. The standard isolation methods are tedious and time-consuming and require considerable expertise to differentiate species of *Phytophthora* based on morphology, which can be difficult due to intraspecific variation and the overlap of morphological characters among species (7,41). It takes at least a week to confirm *P. nicotianae* detection using standard isolation methods, which can delay disease-management decisions. In contrast, the PCR assay developed in this study can provide definitive diagnosis within a few hours; it is easy to use and requires minimal training.

The PCR assay has adequate specificity for detection of *P. nicotianae* in irrigation water. The PN primers detected more isolates of *P. nicotianae* than the previously described (6) PP primers. This indicates the ability of our PCR assay to detect the pathogen from more diverse origins with a lower risk of producing false negatives. In addition, the PN primers had only insignificant cross reactions (actually unspecific amplifications) among a large number of diverse microorganisms. These results were not unexpected because the sequences of the PN primers have a low

similarity with those of the elicitors of other oomycetes. The BLAST hits included 11 isolates from five species of *Phytophthora* with the majority having an E-value ≥ 6 (data not shown). High guanine-cytosine (GC) contents (68.18 and 59.09%) and melting temperatures (79.3 and 69.3°C) of the forward and reverse primers also may have facilitated specific binding to complementary target sequences.

Specificity is essential for detecting *P. nicotianae* in irrigation water. Irrigation water is a complex ecosystem whose microbial community is diverse. For example, at least seven species of *Phytophthora* were recovered from nursery effluents in California (25) and five species recovered in Oklahoma (39). Similarly, 10 species of *Phytophthora* were recovered from nursery irrigation water in Virginia over the past 2 years (13) and at least five species have been found in South Carolina (S. N. Jeffers, unpublished data). Many other species of *Phytophthora* and genera of fungi also have been found in irrigation water associated with other crops (20,28,29,36).

Adequate specificity of the PCR assay reported here was evidenced by very few unspecific amplicons among a large number of diverse microorganisms. These microorganisms included major groups of *Pythium*, other water mold fungi, bacteria, and some important fungal pathogens, which were in addition to the 15 other species of *Phytophthora*. The species of *Phytophthora* tested represent the six morphological groups described by Waterhouse (7,41) and all species that have been recovered from nursery irrigation water in the United States (13,22,25,39). Among the species that did not react with the PN primers were three species on the same internal transcribed spacer (ITS) clade as *P. nicotianae*: *P. pseudotsugae*, *P. phaseoli*, and *P. infestans* (4). Nonreactive species also included *P. capsici*, *P. citrophthora*, *P. heveae*, and *P. tropicalis*, which are in the same morphological group (II) as *P. nicotianae* (41).

The amplification of DNA from the isolates of *P. cambivora* (1D2) and *P. cactorum* (W.097) should not present a problem. The population of *P. cambivora* in irrigation water is very limited. This species was isolated only once during monthly assays of irrigation runoff and irrigation water at two commercial nurseries and quarterly assays at four other nurseries in Virginia over the past 2 years (C. X. Hong, unpublished data). It also was recovered from 1 of 31 irrigation sources surveyed for occurrence of *Phytophthora* spp. in British Columbia, Canada (28) and from surface water irrigation sources in California (29). Isolation of this species from runoff or irrigation water has not been documented anywhere else. The potential of this unspecific amplification of DNA from *P. cambivora* with the PN primers is small, and unspecific amplification can be identified by running the PCR products of water samples sided with a positive control on 3% Nusieve gels or DNA hybridization with the species-specific probe. The *P. cactorum* isolate (W.097) was reexamined morphologically and also using a single strand conformational polymorphism (SSCP) analysis (P. Kong, unpublished data). This isolate has the typical morphological characters (7,41) and SSCP profile of *P. cactorum*. The cause of the amplification of DNA from this isolate remains unclear. Natural hybridization between *P. nicotianae* and *P. cactorum* has been reported (27) so isolate W.097 could be another natural hybrid between the two species.

The amplification of DNA from the one isolate of each of *Pythium irregulare* (P11), *Alternaria porri* (AP1), and a bacterium (SW1) also is not a major concern. The amplicons of P11 and AP1 were distinguished easily from the specific band (230 bp) for *P. nicotianae*. Both amplicons also had very weak signals even with 5 µl of PCR products on 3% Nusieve gel so they may not be easily seen on regular gels. The amplicon of DNA from a bacterial isolate was a concern although it occurred only once in specificity tests. This amplicon had a strong signal with a size similar to the specific band; both were hard to differentiate on 1% agarose gels. However, this unspecific amplicon can be separated from the

Fig. 5. Agarose gel electrophoresis of polymerase chain reaction (PCR) products using the *Phytophthora nicotianae*-specific primers designed in this study: + = purified DNA of *P. nicotianae* that was added to and diluted in sterile nanopure water; R = DNA extracted from an irrigation runoff water sample; and P = DNA extracted from an irrigation pond water sample. The runoff and pond water samples previously tested negative with the *Phytophthora nicotianae*-specific primers. The final template concentrations (µl) in PCR reaction mixes are indicated above the lanes. M = 100-bp DNA ladder.

The sensitivity of the PCR assay can be further enhanced by use of concentrated DNA extracts. The assay appeared tolerant to the inhibitors that may have been in the extraction samples from irrigation runoff water and irrigation water. This tolerance allowed use of concentrated DNA extract from water samples for template amplification to increase detection sensitivity. Detection of *P. nicotianae* in the water sample collected from location G2 in South Carolina illustrated how using concentrated DNA extract can improve sensitivity of the PCR assay. This detection result

The PCR assay with the PN primers was more sensitive than the PP primers. This could have been attributed partially to the different sizes of amplicons: 230 bp for the PN primers and 950 bp for the PP primers. In general, the smaller the size of the amplicon, the more efficient the replication will be. It also could have been due in part to the primers' tolerance to inhibitors in the raw DNA extract from irrigation water samples. This was evidenced by detection in the water sample from location CU in South Caro-

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lina. PN and PP primers detected *P. nicotianae* with the original DNA extract, but only the PN primers detected it with the 5x concentrated one.

Development of this PCR assay has significant practical implications. Use of the assay can provide rapid and accurate detection of *P. nicotianae* in water samples at an adequate level of sensitivity, which will allow growers to make timely disease management decisions with confidence. The PCR assay also could be an effective research tool for studying the ecology of *P. nicotianae* and evaluating management strategies for diseases caused by this pathogen.

In spite of the demonstrated detection specificity and sensitivity, the PCR assay developed in this study must be used with care. Filtration is the preferred method for collecting zoospores from water samples because it can reduce the risk of false positive detection from cross reaction with bacteria, and possibly other microorganisms smaller than the 5- μ m pore size of the filter, that may be present in irrigation water. Also, samples testing negative with original DNA extract should be rerun with concentrated extracts to reduce the risk of false negatives. It also is advisable to include an internal control by adding well purified DNA of *P. nicotianae* to DNA extracted from irrigation runoff water and irrigation pond water samples when using the PCR assay for diagnosis. Customization of this assay for detection of the pathogen in plants and soil is underway. In the future, we plan to compare this PCR assay with other similar assays, including two nested PCR protocols reported recently (12,17) using naturally infested water samples, and also to improve the quantitative capability of the assay.

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