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Rapid detection of *Phytophthora cinnamomi* using PCR with primers derived from the *Lpv* putative storage protein genes

P. Kong, C. X. Hong*† and P. A. Richardson

Virginia Polytechnic Institute and State University, Department of Plant Pathology, Hampton Roads Agricultural Research and Extension Center, 1444 Diamond Springs Road, Virginia Beach, VA 23455-3363, USA

Phytophthora cinnamomi is an ecologically and economically important pathogen. In this study, PCR assays were developed with primer pair LPV2 or LPV3 for rapid detection and identification of this organism. Both primer pairs were selected from putative storage protein genes. The specificity of these primer pairs was evaluated against 49 isolates of *P. cinnamomi*, 102 isolates from 30 other *Phytophthora* spp., 17 isolates from nine *Pythium* spp. and 43 isolates of other water moulds, bacteria and true fungi. PCR with both primer pairs amplified the DNA from all isolates of *P. cinnamomi* regardless of origin. The LPV3 primers showed adequate specificity among all other species tested. The LPV2 primers cross-reacted with some species of *Pythium* and true fungi, but not with any other *Phytophthora* species. PCR with the LPV3 primers detected the pathogen at levels of a single chlamydospore or 10 zoospores in repeated tests. The PCR assay was at least 10 times more sensitive than the plating method for detection of the pathogen from artificially infested soilless medium, and, to a lesser extent, from naturally infected plants. PCR with LPV3 primers can be a useful tool for detecting *P. cinnamomi* from soilless media and plant tissues at ornamental nurseries, whereas the LPV2 primers can be an effective alternative for identification of this species from pure culture. Applications of these assays for detection of *P. cinnamomi* in other environments were also discussed.

Keywords: detection, identification, molecular genetics, PCR, *Phytophthora sojae*, *Phytophthora root rot*

Introduction

Phytophthora cinnamomi is an ecologically and economically important pathogen that attacks a wide range of host plants (Erwin & Ribeiro, 1996). This species causes jarrah dieback that threatens many native species of forest plants with extinction in Australia. It is also responsible for serious annual losses of avocado and chestnut trees and cranberry vines in the USA. In ornamental crop nurseries, *P. cinnamomi* is a major root rot pathogen of numerous shrubs (e.g. *Rhododendron* spp.) and trees (*Prunus* spp.).

Phytophthora cinnamomi-incited diseases are difficult to manage. This pathogen produces a large number of chlamydospores, which can persist in symptomless plants, debris and soil for a number of years (Zentmyer & Mircetich, 1966). The pathogen also produces sporangia and zoospores, which can be spread through irrigation water, an efficient means for dissemination of infective propagules from a single infection site to an entire farm and from a single farm to all other farms that share the same water sources (MacDonald *et al*., 1994; Pettitt *et al*., 1998; Oudemans, 1999; von Broembsen & Wilson, 2000; Themann *et al*., 2002). The pathogen can also be spread from a nursery to the landscape through contaminated plants. To keep such a widespread and destructive disease under control, rapid and accurate detection is required.

Baiting and culture plating, or a combination of both, are the current standard methods of detection for viable propagules of *Phytophthora* species (Greenhalgh, 1978; Ploetz & Parrado, 1988; MacDonald *et al*., 1994; Hong *et al*., 2002; McDougall *et al*., 2002; Pettitt *et al*., 2002). These classic methods allow detection of multiple species and can be semiquantitative (Themann & Werres, 1997; Werres *et al*., 1997; Ferguson & Jeffers, 1999; Pettitt *et al*., 2002). However, they are labour-intensive and time-consuming. Use of these methods requires expertise in the isolation and morphology of *Phytophthora* species (Tsao, 1990; Dobrowolski & O'Brien, 1993).

Several serological tests have been developed for rapid detection of *Phytophthora* species (Hardham *et al*., 1985; Gabor *et al*., 1993; Cahill & Hardham, 1994a,b; Hahn & Werres, 1997; Wakeham *et al*., 1997). These tests employ either enzyme-linked immunosorbent assay (ELISA), membrane trapping assay or a dipstick format. A number

*To whom correspondence should be addressed.

†E-mail: chhong2@vt.edu

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of these immunoassays are commercially available for on-site use and some kits enable growers to obtain results within 10 min of sample collection. The common problems associated with serological tests are specificity and sensitivity (MacDonald *et al*., 1990; Ali-Shtayeh *et al*., 1991; Benson, 1991; Kratka *et al*., 1996; Latorre & Wilcox, 1996; Themann & Werres, 1997; Haymes *et al*., 2000; Pettitt *et al*., 2002). Of particular concern is cross-reaction of *Phytophthora* diagnostic kits with *Pythium* spp. Some *Pythium* spp. can be more abundant than *Phytophthora* spp. in the environment, and many of these species are weak or nonpathogens. For example, *Pythium* species were 10–100 times more numerous than *Phytophthora* spp. in irrigation water (Hong *et al*., 2001). Application of these kits for detection of *Phytophthora* spp. can result in numerous false positives. An additional concern is that most diagnostic kits cannot differentiate particular species within the genus *Phytophthora*. This is inadequate for pathogen monitoring programmes in ornamental nurseries, where more than a dozen *Phytophthora* spp. may be present, but not all are as important pathogenically as *P. cinnamomi* (Farr *et al*., 1989; Erwin & Ribeiro, 1996).

At least six DNA probes have been developed for specific detection of *P. cinnamomi* (Dobrowolski & O'Brien, 1993; Lee *et al*., 1993; Judelson & Messenger-Routh, 1996; Coelho *et al*., 1997; Lévesque *et al*., 1998; Bailey *et al*., 2002). Five of these DNA probes are effective alternative tools for identification of this pathogen from pure culture and the sixth also has quantitative capability for detecting *P. cinnamomi* in avocado roots (Judelson & Messenger-Routh, 1996). Compared with classic methods, DNA hybridization is a relatively rapid detection and identification technique, but it still takes up to 2 days to perform the procedures. Also, use of DNA probes often requires radioactive labelling to increase detection sensitivity (Lee *et al*., 1993; Judelson & Messenger-Routh, 1996).

Species-specific polymerase chain reaction (PCR) has become increasingly important for pathogen identification and detection because of its speed and sensitivity. Several PCR protocols have been reported for detecting *Phytophthora* spp. The majority of these protocols use primers derived from the internal transcribed spacers (ITS) (Cooke *et al*., 1995a,b; Bonants *et al*., 1997; Tooley *et al*., 1997; Trout *et al*., 1997; Liew *et al*., 1998; Tooley *et al*., 1998; Schubert *et al*., 1999; Bonants *et al*., 2000; Judelson & Tooley, 2000; Winton & Hansen, 2001; Grote *et al*., 2002; Ippolito *et al*., 2002) or elicitin genes (Coelho *et al*., 1997; Lacourt & Duncan, 1997; Kong *et al*., 2003a). DNA sequences of these regions or genes can be easily detected thanks to high copy numbers in the genome. A wealth of data on the level of intraspecific sequence conservation has also facilitated use of these regions or genes for species-specific detection. However, selecting primers from these regions can be a challenge because they are conserved among members of the Pythiaceae family (Gayler *et al*., 1997; Panabières *et al*., 1997; Cooke *et al*., 2000). The rate of evolutionary change in these regions may be low, making it difficult to discriminate some closely related species.

Two species-specific PCR protocols with primers derived from these regions have been reported for *P. cinnamomi*. One was used in combination with colorimetric hybridization (Coelho *et al*., 1997) and the other was accomplished using nested PCR to increase specificity and sensitivity (Cacciola *et al*., 2001). The former appeared highly sensitive but over-specific, only detecting 26 of the 30 isolates of *P. cinnamomi* tested. The latter seemed to have adequate specificity within the genus *Phytophthora*, but remains to be assessed for sensitivity. Both protocols are useful alternatives for identification from pure culture, but they are subject to further tests for specificity with non-*Phytophthora* species and to inhibitors in the DNA from infested plants, soil and irrigation water.

More rapid and reliable protocols are needed for detection of *P. cinnamomi* in formulating and implementing effective strategies for disease management. The aim of this study was to develop an assay to target the pathogen in ornamental nurseries. This assay used PCR with primers derived from a small multigene family, *Lpv*, which encode putative storage proteins in large peripheral vesicles in zoospores of *P. cinnamomi* (Marshall *et al*., 2001). The *Lpv* gene-coding regions contain approximately 12–18 highly conserved 534-bp repeats, flanked by unique sequences. These regions have great potential for developing a specific and sensitive detection protocol. This paper reports on the development of the PCR assay, including: (i) specificity tests of the primers with a wide range of species from *Phytophthora*, *Pythium* and many other genera; (ii) sensitivity tests with zoospores and chlamydospores of *P. cinnamomi*; and (iii) detection tests with artificially infested soil and naturally infected plants, as well as irrigation water collected from two commercial nurseries in eastern Virginia.

Materials and methods

Isolates, culture and propagules

Cultures used in this study included a large number of isolates of *P. cinnamomi* (Table 1), other species of *Phytophthora* (Table 2), *Pythium*, other common water moulds, bacteria and some true fungi (Table 3). Species of *Phytophthora*, *Pythium* and other water moulds were grown on V8 agar in 6 cm Petri plates for 1 week at 20°C in the dark and were maintained at 15°C. Isolates of true fungi and bacteria were grown on potato dextrose agar (PDA) in 10 cm Petri plates at 23°C in the dark for 2 weeks and 3 days, respectively, and were maintained on PDA slants at 4°C.

Zoospores of *P. cinnamomi* were obtained using a standard procedure with some modifications (Hong *et al*., 2002). Mycelium was harvested from one-quarter of the surface area of a 10 cm Petri plate of a 2-week-old culture and then finely chopped. The mycelial plugs were incubated for 6 h under fluorescent light in 25 mL of sterile soil water extract (SSWE) and then overnight in a sporulation solution (Marshall *et al*., 2001). After further incubation in cold SSWE for 4 h, zoospores were harvested by

Table 1 Specificity of the primer pairs LPV1, LPV2 and LPV3 derived from putative storage protein genes of Phytophthora cinnamomi for 49 isolates of the same species from different hosts and geographic locations

Host	Location[a]	No. of isolates tested	PCR-positive			Source[b]
			LPV1	LPV2	LPV3	
Camellia japonica	CA, NC	3	3	3	3	2·5
Ginkgo biloba	SC	2	2	2	2	5
Heliamphora sp.	VA	1	1	1	1	3
Ilex glabra	VA	2	2	2	2	4
Persea americana	CA, Puerto Rico	2	2	2	2	2·5
Rhododendron sp.	NC, VA	34	34	34	34	1·4
Vaccinium macrocarpon	MA	1	1	1	1	5
Vaccinium sp.	VA	3	3	3	3	4
Nursery crops	OK	1	1	1	1	6

[a]Country or abbreviated name of states within the USA: CA, California; MA, Massachusetts; NC, North Carolina; OK, Oklahoma; SC, South Carolina and VA, Virginia.
[b]1, D. Michael Benson; 2, Mannon E. Gallegly; 3, Mary Ann Hansen; 4, Chuanxue Hong; 5, Steven N. Jeffers and 6, Sharon L. von Broembsen.

Table 2 Specificity of the primer pairs LPV1, LPV2 and LPV3 derived from the putative storage protein genes of *P. cinnamomi* for other species within the genus *Phytophthora*

Phytophthora	Host/substrate	Location[a]	No. of isolates tested	PCR positive			Source[b]
				LPV1	LPV2	LPV3	
boehmeriae	NA[c]	NA	1	0	0	0	2
botryosa	*Hevea rubber*	Thailand	1	0	0	0	2
cactorum	*Malus sylvestris*	Rhodesia	1	1	0	0	2
	Rhododendron sp.	OH	1	1	0	0	2
	NA	NA	2	1	0	0	2
cambivora	*Abies* sp.	OR	1	1	0	0	2
	Prunus armeniaca	MD	1	0	0	0	2
	NA	NA	1	1	0	0	2
capsici	*Capsicum annuum*	NC, NM	2	1	0	1	2
	Cucurbita sp.	MD, PA	2	1	0	0	2
	Vanilla planifolia	Tahiti	1	0	0	0	2
	NA	NA	1	0	0	0	2
citricola	*Kalmia latifolia*	WV	1	0	0	0	2
	Rhododendron sp.	WV, NY, OH	3	0	0	0	2
citrophthora	*Citrus limon*	CA	1	0	0	0	5
	Theobroma cacao	Brazil	1	0	0	0	5
	Field soil	CA, SC	3	0	0	0	5
	Irrigation water	CA, OK, VA	4	0	0	0	4·6
cryptogea	*Aster* sp.	CA	1	1	0	0	2
	Godetia sp.	South Africa	1	0	0	0	2
	Lycopersicon esculentum	Ireland	1	0	0	0	5
colocasiae	NA	NA	1	0	0	0	2
drechsleri	*Solanum tuberosum*	SC	1	0	0	0	5
	Irrigation water	VA, OK	7	0	0	0	4·6
	Field soil	SC	2	0	0	0	5
erythroseptica	*Solanum tuberosum*	OH	1	0	0	0	2
	NA	NA	1	1	0	0	2
fragariae	*Fragaria* sp.	MD	1	0	0	0	2
gonapodyides	Irrigation water	England	1	NA	0	0	8
heveae	*Hevea brasiliensis*	Malaysia	1	0	0	0	2
	Field soil	TN	1	1	0	0	2
hibernalis	*Citrus* sp.	Portugal	1	1	0	1[d]	2
ilicis	*Ilex* sp.	Canada	1	0	0	0	2
infestans	*Lycopersicon esculentum*	NC, WV	3	0	0	0	2
	NA	Ireland	1	1	0	0	2
katsurae	*Castanea* sp.	Japan	1	0	0	0	2
lateralis	*Chamaecyparis lawsoniana*	OR	2	1	0	0	2
meadii	*Citrus* sp.	India	2	0	0	0	2

Table 2 *Continued*

Phytophthora	Host/substrate	Location[a]	No. of isolates tested	PCR positive			Source[b]
				LPV1	LPV2	LPV3	
medicaginis	*Medicago sativa*	OH	1	1	0	0	2
megakarya	*Theobroma cacao*	Africa	1	0	0	0	2
megasperma	*Actinidia chinensis*	CA	1	0	0	0	2
	Cornus florida	WV	1	0	0	0	2
	Malus sylvestris	NY	1	0	0	0	2
	Prunus sp.	CA	2	1	0	0	2
	Pseudotsuga menziesii	OR	2	0	0	0	2
	Trifolium sp.	IL	1	1	0	0	2
	NA	OH	1	0	0	0	2
nicotianae	*Abies* sp.	WV	1	0	0	0	2
	Citrus sp.	CA, India	5	0	0	0	2
	Nicotiana tobacum	NC	2	0	0	0	2
	Passiflora edulis	India	1	0	0	0	2
	Solanum tuberosum	NC	1	0	0	0	2
	Irrigation water	VA	1	0	0	0	4
	NA	MD	4	0	0	0	2
palmivora	*Theobroma cacao*	Costa Rica	1	1	0	0	2
	NA	FL	1	1	0	1[d]	2
phaseoli	*Phaseolus lunatus*	DE	1	0	0	0	2
	NA	NA	1	0	0	0	2
pseudotsugae	NA	NA	1	0	0	0	2
ramorum	*Lithocarpus densiflorus*	CA	2	NA	0	0	7
	Quercus sp.	CA	1	NA	0	0	7
	Rhododendron sp.	CA, Germany, Netherlands	5	NA	0	0	7
sojae	*Glycine max*	OH, Canada	2	0	0	2	2
syringae	*Citrus* sp.	CA	1	0	0	0	8
	NA	NA	1	0	0	0	2

[a]Country or abbreviated name of states within the USA: CA, California; DE, Delaware; FL, Florida; IL, Illinois; MD, Maryland; NC, North Carolina; NM, New Mexico; NY, New York; OH, Ohio; OK, Oklahoma; OR, Oregon; PA, Pennsylvania; SC, South Carolina; TN, Tennessee; VA, Virginia; WV, West Virginia.

[b]1, D. Michael Benson; 2, Mannon E. Gallegly; 3, Mary Ann Hansen; 4, Chuanxue Hong; 5, Steven N. Jeffers; 6, Sharon L. von Broembsen; 7, Paul W. Tooley and 8, American Type Culture Collection (ATCC).

[c]Not available.

[d]Showing a weak band of a different size from that of *P. cinnamomi*.

filtering through two layers of cheesecloth. The zoospore suspension was pelleted using a Sorvall® RC 5D Plus centrifuge (Kendro Laboratory Products, Newtown, CT, USA) at 10 000 ***g*** for 2 min. The pellet was then resuspended in SSWE, sporulation solution or sterile distilled water (SDW), and the concentration was adjusted to 50 000 spores mL^{-1}. This initial spore suspension was further diluted with the same media (SSWE, sporulation solution or SDW) to 10 000, 1000 or 100 spores mL^{-1}, before being used for primer sensitivity tests.

Chlamydospores of *P. cinnamomi* were produced by culturing in V8 broth for 2 weeks in the dark at room temperature. Harvested mycelia and chlamydospores were minced in 30 mL of SDW in a Waring blender at high speed for 2 min; then the suspension was filtered through two layers of miracloth to remove larger pieces of mycelium. The filtrate was pelleted in a swinging bucket rotor at 3000 ***g*** for 5 min to separate smaller pieces of mycelium from chlamydospores. The pellet was then resuspended with SDW, and the concentration was adjusted to 2000 spores mL^{-1}. An aliquot of 100 μL of the chlamydospore suspension was spread onto a 10 cm Petri plate containing 1·5% water agar. Single chlamydospores were isolated with a 10 μL capillary pipette under a dissecting microscope then pipetted into a 0·2 mL PCR tube containing 10 μL of SDW.

A vermiculite culture of *P. cinnamomi* was prepared as reported previously for infesting soil (Roiger & Jeffers, 1991). Briefly, 10 mycelial plugs were seeded in 40 g vermiculite mixed with 200 mL V8 broth (200 mL V8 juice, 800 mL H_2O and 2 g $CaCO_3$), then incubated for 2–4 weeks in the dark at room temperature. The four levels of infestation included in this study were 2, 0·2, 0·02 and 0·002% vermiculite culture (v/v). The initial 2% infested soilless medium was prepared by mixing 98 mL of fresh Metro Mix 360 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) with 2 mL of the vermiculite culture in a Waring blender at high speed for 3 min three times. A 10-fold dilution of this initial infested medium was prepared with the same soilless mix.

Table 3 Specificity of the primer pairs LPV2 and LPV3 derived from the putative storage protein genes of *Phytophthora cinnamomi* for other microorganisms

Microorganism	Host	Location[a]	No. of isolates tested	PCR positive		Source[b]
				LPV2	LPV3	
Pythium aphanidermatum	*Chrysanthemum* sp.	PA	1	1	0	3
	Euphorbia sp.	PA	1	0	0	3
Pythium debarynum	NA[c]	NA	1	1	0	5
Pythium intermedium	Field soil	Japan	1	1	0	5
Pythium irregulare	*Euphorbia* sp.	PA	1	1	0	3
	Pelargonium sp.	PA	1	0	0	3
	NA	Korea	1	0	0	2
Pythium mamillatum	NA	NA	1	0	0	5
Pythium myriotylum	*Pelargonium* sp.	PA	2	0	0	3
Pythium rostratum	*Zea mays*	WI	1	0	0	5
Pythium splendens	*Epipremnum aureum*	PA	1	0	0	3
Pythium ultimum	*Digitalis purpurea*	VA	1	0	0	1
	Euphorbia sp.	PA	1	0	0	3
	Fragaria sp.	CA	1	0	0	2
	Gossypium sp.	CA	1	0	0	2
	Pelargonium sp.	PA	1	1	0	3
Allomyces sp.[d]	Irrigation water	VA	1	0	0	1
Mortierella sp.[d]	Irrigation water	VA	2	0	2[e]	1
Saprolegnia spp.[d]	Irrigation water	VA	7	0	0	1
Thraustotheca sp.[d]	Irrigation water	VA	1	0	0	1
Chytrids[d]	Irrigation water	VA	2	0	0	1
Bacterium/grey[f]	Irrigation water	VA	3	0	0	1
Bacterium/yellow	Irrigation water	VA	3	0	0	1
Bacterium/milky	Irrigation water	VA	3	0	0	1
Bacterium/white large	Irrigation water	VA	3	0	0	1
Bacterium/white small	Irrigation water	VA	3	0	0	1
Alternaria alternata	*Daucus carota*	CA	1	1	0	1
Alternaria daucia	*Daucus carota*	CA	1	1	0	1
Alternaria microspora	*Daucus carota*	CA	1	1	0	1
Alternaria porri	*Daucus carota*	CA	1	1	0	1
Alternaria radicina	*Daucus carota*	CA	1	1	0	1
Alternaria solani	*Daucus carota*	CA	1	1	0	1
Botrytis cinerea	*Prunus persica*	CA	1	1	0	1
Cylindrocladium parasitium	*Arachis hypogeae*	VA	1	0	0	4
Monilinia fructicola	*Prunus persica*	CA	1	1	0	1
Monilinia laxa	*Prunus persica*	CA	1	1	0	1
Rhizoctonia sp.	*Rhododendron* sp.	VA	1	0	0	1
Sclerotinia minor	*Arachis hypogeae*	VA	1	0	0	4
Sclerotium rolfsii	*Arachis hypogeae*	VA	1	0	0	4
Thielaviopsis sp.	*Viola tricolor*	VA	1	1	0	1
Ulocladium sp.	*Daucus carota*	CA	1	1	0	1

[a]Country or abbreviated name of states within the USA: CA, California; PA, Pennsylvania; VA, Virginia; WI, Wisconsin; WV, West Virginia.
[b]1, Chuanxue Hong; 2, Frank Martin; 3, Gary W. Moorman; 4, Patrick Phipps and 5, American Type Culture Collection (ATCC).
[c]Not available.
[d]Tentative identification.
[e]Showing a weak band of a different size from that of *P. cinnamomi.*
[f]Colour of bacterial colonies; no identification of bacteria was attempted.

DNA extraction

DNA of pure cultures was extracted using a boiling method. For fungi, mycelium was scraped from a 2 cm^2 surface area of a 1- to 2-week-old culture and placed into a 1·5 mL microcentrifuge tube containing 500 μL of 10 mM Tris-HCl (pH 7·5). DNA was released by boiling mycelia in a heat block for 20 min then vortexing for 3 min. Similarly, individual colonies of bacteria on PDA were harvested and boiled in 100 μL of 10 mM Tris-HCl. The supernatants were used immediately or stored at –20°C until further use. All DNA preparations using this miniprep technique were of sufficient quality for template amplification as demonstrated in a separate study with ITS6 and ITS7 (Kong *et al.*, 2003b). DNA of some cultures was also extracted using Dneasy Plant Mini Kits (Qiagen Inc, Valencia, CA, USA).

Primer pair	Sense	Sequence (5′-3′)	Location	Size
LPV1	Forward	– CTGGCGGCATTGAAGCAAGA-	Pst 2–4 (AF315066) 1–20 nt	412 bp
	Reverse	– CAAGCGCACAGAACGGAGAT-	393–412 nt	
LPV2	Forward	– ACTGGGTCGACAACGACTGCTTG-	Pst 2–4 (AF315066) 230–252 nt	489 bp
	Reverse	– GTCCAAACCGACTCTTGCTCGATG-	695–718 nt	
LPV3	Forward	– GTGCAGACTGTCGATGTG-	Lpv (AF315064) 117–134 nt	450 bp
	Reverse	– GAACCACAACAGGCACGT-	550–567 nt	

Table 4 Sequences of three pairs of primers derived from the putative storage protein genes *Lpv* of *Phytophthora cinnamomi* and evaluated in this study

DNA of soilless mix artificially infested with *P. cinnamomi* was extracted with the UltraClean Soil DNA Kit (MO BIO Laboratories, Inc., Solana Beach, CA, USA). One millilitre of soilless mix was collected arbitrarily from each sample, and DNA was extracted subsequently as instructed by the manufacturer.

DNA of naturally infected nursery plants was purified using Dneasy Plant Mini Kits. About 2 g of fine roots were taken from a container-grown plant and ground in liquid N_2 with a mortar and pestle. One millilitre of the fine powder (~100 mg) was used for the DNA extraction following the manufacturer's instructions.

DNA of microbes in irrigation water samples was extracted using the UltraClean Soil DNA Kit with some modifications. Briefly, irrigation water was passed through a 47 mm nylon membrane filter with 5 μm pores (Osmonics Laboratory Products, Minnetonka, MN, USA) using a vacuum pressure of 41·5 cm Hg. The filter was then cut into fine pieces and placed into the bead tube supplied with the kit. The additional steps in DNA extraction followed the manufacturer's instructions.

Primer design and DNA amplification

In order to obtain specific and sensitive primers, nucleotide sequences of *P. cinnamomi* on databases were searched and compared with others using BLAST 2·2·5 (http://www.ncbi.gov/blast/blast.cgi). Database searches found partial sequences of *Lpv* genes encoding putative storage proteins in large peripheral vesicles in zoospores of *P. cinnamomi*, each *Lpv* containing repeated segments flanked by unique sequences (Marshall *et al*., 2001). BLAST searches with these *Lpv* sequences did not find homology to sequences of any plants or other microbes. Three primer pairs, LPV1, LPV2 and LPV3, were selected from these sequences (Table 4). Primer pairs LPV1 and LPV2 were selected from the sequences of a genomic clone Lpv Pst 2–4 (AF315066) in a nonrepeated region of *Lpv* (Fig. 1). Primer pair LPV3 was selected from the sequence of a cDNA clone Lpv 18 (AF315064), flanking one of the 534-bp repeats in the open reading frame of *Lpv* (Fig. 1). All primers used in this study were synthesized at Sigma Genosys, Woodlands, TX, USA.

Conventional PCR was used to evaluate the three primer pairs with DNA extracted from pure cultures, soil, irrigation water and plant root samples. Each 25 μL PCR reaction contained 2 μL of DNA templates, 2·5 μL of the 10 × PCR buffer, 2·5 μL each of 10 μM forward and reverse primers, 2 μL of 2·5 mM dNTPs, 0·1 μL (5 μ μL^{-1}) of *Taq* polymerase (TaKaRa, Shuzo Co. Ltd, Japan) and 13·4 μL of SDW. The reaction was carried out in a Mastercycler Gradient (Eppendorf Scientific Inc., Westburg, New York, USA) programmed with initial denaturation at 96°C for 2 min, followed by 39 cycles of 94°C for 30 s, 65°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 10 min. The only exception was the use of 60°C instead of 65°C as the annealing temperature for the LPV3 primers because of their low melting temperature.

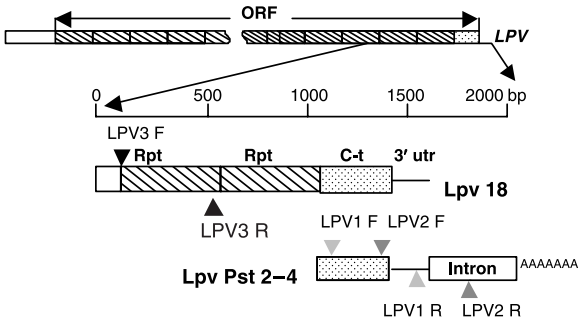

Figure 1 Diagram of LPV1, LPV2 and LPV3 primers in sequences of a cDNA clone Lpv 18 and a genomic clone Lpv Pst 2–4 of *Phytophthora cinnamomi*. ORF, open reading frame; F and R following LPV represent the sense of forward and reverse primers, respectively; Rpt, repeated region; C-t, carboxyl terminus; 3 utr, untranslated region of this gene at the 3′ end.

Spore PCR was performed by direct addition of a zoospore suspension or a single chlamydospore to the reaction mix in primer sensitivity tests. The same reaction mix as described above for conventional PCR was used with adjusted SDW volume after addition of spore preparations. For zoospore PCR, 10 μL of zoospore suspension and 5·4 μL of SDW were used. For chlamydospore PCR, one chlamydospore and 15·4 μL of SDW were used. PCR was performed as above, except that an additional 3 min was added to the initial denaturation process (a total of 5 min).

A 5 μL aliquot of PCR products from each reaction was electrophoresed in 1% agarose gel and then stained with ethidium bromide for visualization under a UV transilluminator. Images were captured and analysed using a BioImaging Chemi System (UVP, Inc., Upland, CA, USA).

Specificity and sensitivity tests

The specificity of the three primer pairs was assessed by PCR with culture DNA from 49 isolates of *P. cinnamomi* originating from different hosts and geographic locations (Table 1). These primers were also tested with DNA from a wide variety of other species of *Phytophthora* (Table 2), *Pythium*, water moulds, bacteria and true fungi (Table 3).

The sensitivity of these primer pairs was evaluated using spore PCR. Zoospore PCR was performed twice at levels of 500, 100, 10 or one spore per reaction, whereas single chlamydospore PCR was conducted 10 times for each primer pair. Additional sensitivity tests were performed with a serial dilution of purified culture DNA, ranging from 400 ag to 40 ag eluted per microlitre reaction mix. All tests were repeated at least once.

The sensitivity of these primer pairs was also assessed using conventional PCR with DNA from soilless mixes artificially infested with *P. cinnamomi* then compared with that of the plating method. DNA of each infested soilless mix sample (1 mL) was extracted as described above and eluted in 50 μL of SDW. Each PCR reaction used 2 μL of purified DNA. An equivalent volume (1 mL) of an infested soilless mix was suspended in 5 mL of 0·1% water agar. A 200 μL aliquot of the suspension was then spread in one of three 10 cm Petri plates containing PARP-V8 agar, a medium selective for pythiaceous species (Ferguson & Jeffers, 1999). These plates were incubated in the dark at 23°C and *P. cinnamomi* colonies were counted daily. Assays with the PCR and plating methods were performed in triplicate and both were repeated. Colonization of plates by *P. cinnamomi* was compared with the presence of specific amplicon by the PCR method.

Detection of *P. cinnamomi in planta*

Sixteen naturally infected azalea (*Rhododendron* spp.) and 2 holly (*Ilex* sp.) plants were collected from two commercial nurseries in eastern Virginia. DNA of fine roots (~100 mg) from each plant was extracted as described previously and eluted in 50 µL of SDW. A 10-fold dilution of purified DNA was prepared with SDW. The original DNA extract and the dilution were subjected to PCR with primer pairs. Roots from the same plants were plated in 10 cm Petri plates containing PARP-V8 agar. Six replicated portions of about 1 g each of roots from a plant were plated in each of two plates. The isolation plates were incubated in the dark at 23°C and examined daily for *P. cinnamomi* colonies. A sample plant was considered positive with the plating method if *P. cinnamomi* was recovered from any of the 12 root portions in the two plates. Detection results were compared between PCR with both primer pairs and the plating method.

Detection of *P. cinnamomi* in irrigation water

Runoff water and pond water samples were collected at a nursery in eastern Virginia, monthly from May to September 2001. DNA was then extracted from a filter following the filtering of 50 mL of runoff water or 100 mL of pond water as described above, and eluted in 50 μL of SDW. A 10-fold dilution of purified DNA was prepared with SDW. The original and diluted DNA samples were subjected to PCR with primer pairs. The same water samples were also assayed with the plating method (Hong *et al*., 2002). The PCR and plating methods were compared for the presence of a specific band and colonization of *P. cinnamomi* in isolation plates.

Results

Primer design and DNA amplification

PCR with primer pairs LPV1 and LPV2 amplified culture DNA preparations of *P. cinnamomi* and produced specific amplicons at 412 and 489 bp, respectively, whereas the LPV3 primers produced one at 450 bp. Spore PCR also amplified DNA released from zoospores or a single chlamydospore directly added to the reaction mix, and resulted in amplicons of the same sizes for the three primer pairs.

Specificity

PCR with the three primer pairs amplified DNA from all isolates of *P. cinnamomi* (Table 1). These isolates were recovered from several important nursery crops, such as species of *Rhododendron*, *Vaccinium* and *Camellia*. They originated in six states across the continental USA and in Puerto Rico.

The LPV2 and LPV3 primer pairs showed higher specificity than LPV1 for *P. cinnamomi* within the genus *Phytophthora* (Table 2). PCR with the LPV2 primers did not amplify DNA from any of the 30 other *Phytophthora* species tested. PCR with the LPV3 primers amplified DNA from five isolates belonging to four species. The *P. hibernalis* isolate originating in Portugal yielded a 300 bp amplicon and an isolate of *P. palmivora* originating in Florida, USA, resulted in a 280 bp amplicon; both were significantly smaller in size than that of *P. cinnamomi*. However, both isolates of *P. sojae* and one out of six isolates of *P. capsici* produced amplicons with sizes similar to that of *P. cinnamomi*. PCR with the LPV1 primers amplified DNA from a number of other species of *Phytophthora*. Consequently, this primer pair was excluded from further evaluation.

The LPV3 primers showed greater specificity than the LPV2 ones when evaluated with non-*Phytophthora* species (Table 3). PCR with the LPV3 primers did not amplify DNA from any species tested except for two isolates of *Mortierella* sp., which had two nonspecific bands (1000 and 600 bp). In contrast, PCR with the LPV2 primers amplified DNA from some species of *Pythium* and true fungi, yielding amplicons with sizes similar to that of *P. cinnamomi* (489 bp).

Sensitivity

Zoospore PCR was subject to the medium used for preparing the spore suspension, with the LPV2 primers appearing

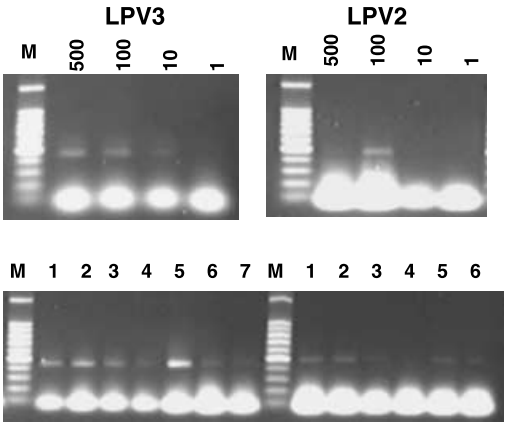

Figure 2 Sensitivity tests of the primer pairs LPV2 and LPV3 specific for *Phytophthora cinnamomi* with the zoospore (top) and chlamydospore PCR (bottom). For zoospore PCR, zoospores were prepared in sterile soil water extract then diluted with sterile distilled water. The number of zoospores per reaction is listed on the top of the lane. For single-chlamydospore PCR, seven positive reactions are presented for the LPV3 primers and six for the LPV2 primers. Track number of a PCR reaction is indicated on the top of each lane. M is a 100 bp DNA ladder.

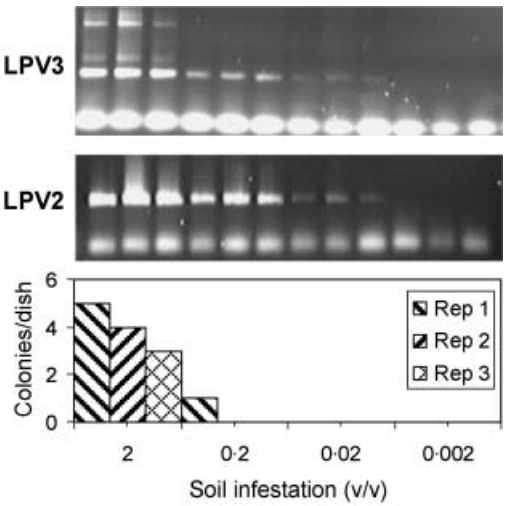

Figure 3 Detection of *Phytophthora cinnamomi* from triplicate (Rep 1, 2, 3) samples of fresh soilless medium (Metro Mix 360) artificially infested with vermiculite culture at four infestation levels of 2, 0·2, 0·02, 0·002% (v/v) using PCR assays with primer pairs LPV3 (top) and LPV2 (middle) developed in this study, and a plating method (bottom).

more sensitive than the LPV3 ones to possible inhibitors. PCR with both LPV2 and LPV3 primers produced positive signals from zoospores suspended in SDW (Fig. 2), but not from those in SSWE or sporulation solution (data not shown). PCR with the LPV3 primers consistently resulted in a positive signal for all numbers of zoospores except for one zoospore per reaction. In contrast, PCR with the LPV2 primers yielded a positive signal only at 100 zoospores per reaction. The detection limits were 4 fg for the LPV3 primers and 40 fg per microlitre reaction mix for the LPV2 primers, respectively, with purified culture DNA (data not shown).

Single chlamydospore PCR with the LPV3 primers appeared to outperform that with the LPV2 primers (Fig. 2). Seven out of 10 single-chlamydospore PCRs produced positive signals for the LPV3 primers, while only six were positive with the LPV2 primers. Similarly, PCR with LPV3 primers seemed to produce more amplicons than that with LPV2.

PCR with both primer pairs LPV2 and LPV3 showed greater sensitivity than the plating method for detecting *P. cinnamomi* from artificially infested soilless mix (Fig. 3). Both primer pairs detected the pathogen in all replicated infested soilless mixes at 0·02% infestation, with the LPV2 primers apparently yielding more PCR products than the LPV3. PCR with the LPV3 primers also yielded two minor bands that were larger than the specific band at 2% infestation. In contrast, the plating method detected the pathogen in only one of the three replicated mixes infested with 0·2% vermiculite culture and failed to detect it in any mixes infested with 0·02% vermiculite culture.

Detection of *P. cinnamomi in planta*

PCR detection of *P. cinnamomi* from plant roots was subject to template DNA composition. All plant samples

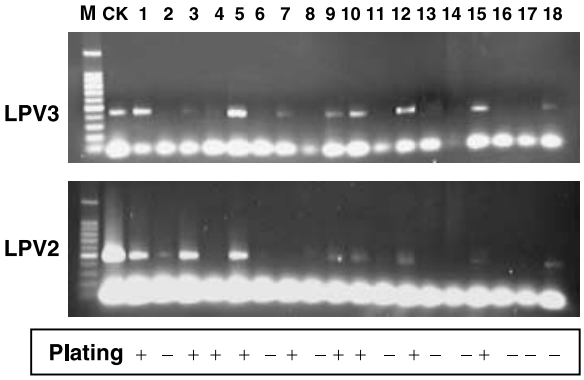

Figure 4 Detection of *Phytophthora cinnamomi* from roots of *Ilex* sp. (lanes 1 and 2) and *Rhododendron* spp. (lanes 3–18) collected from two commercial nurseries in eastern Virginia, using PCR assays with the LPV3 (top) and LPV2 primers (middle), and a plating method (bottom). CK is a positive control, and M is a 100 bp ladder. +, presence; –, absence of the pathogen in isolation Petri plates.

tested negative with both primer pairs when original DNA extracts were used in PCR reactions. However, 10 out of 18 samples tested positive with the LPV3 primers when a 10× dilution of DNA extract was used (Fig. 4). Similarly, nine samples tested positive with the LPV2 primers when a 10× dilution was used.

The PCR detection results with both primer pairs were generally consistent with those of the plating method (Fig. 4). One LPV3-negative sample tested positive with the LPV2 primers (lane 2) and two LPV3-positive samples (lanes 4 and 7) tested negative with the LPV2 primers. A total of nine samples tested positive for the plating method. These nine samples also tested positive with the LPV3 primers, but the LPV3 primers gave an additional positive result (lane 18).

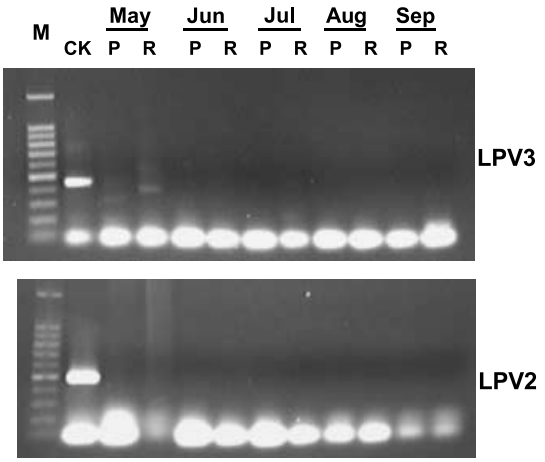

Figure 5 Detection of *Phytophthora cinnamomi* in irrigation water collected from a local nursery in Virginia 2001, using PCR assays with LPV3 (top) and LPV2 (bottom). P and R represent pond water and runoff water, respectively. Month of sampling is indicated above the types of sample.

Detection of *P. cinnamomi* in irrigation water

All irrigation water samples (five runoff water and five pond water) tested negative for both primer pairs with original DNA extract (Fig. 5) and diluted extracts (data not shown). A few unspecific bands were observed in the PCR product for the LPV3 primers (Fig. 5). For example, a 300 bp band was associated with the runoff water sample and 400 and 600 bp bands with the pond water samples collected in May. A 100 bp band was also associated with the pond water sample collected in July (Fig. 5). Similarly, *P. cinnamomi* was not isolated from any water samples using the plating method (data not shown).

Discussion

In response to the need for more rapid and reliable techniques for detection of *P. cinnamomi* from plants and soil, a PCR-based assay with the LPV3 primers was developed in this study.

The PCR assay with LPV3 primers has a time advantage over classic isolation methods and DNA probes, as well as previously reported specific PCR protocols. Use of the PCR assay for detection only takes a few hours to generate disease diagnoses, in contrast to a couple of days with DNA hybridization or longer with traditional methods (Judelson & Messenger-Routh, 1996; Coelho *et al*., 1997; Ferguson & Jeffers, 1999). The PCR assay in this study is also straightforward compared with previously reported ones, which require extra steps such as nested PCR or colorimetric hybridization (Coelho *et al*., 1997; Cacciola *et al*., 2001).

The PCR assay with LPV3 primers appeared to have adequate specificity for detection of *P. cinnamomi* in ornamental nurseries. This was demonstrated by amplifying DNA of all isolates of *P. cinnamomi* regardless of origin (Table 1). It was also indicated by insignificant cross-reaction among a large number of diverse groups and species tested (Tables 2 and 3). Amplification of DNA from one isolate each of *P. hibernalis* and *P. palmivora* (Table 2) and both isolates of *Mortierella* sp. (Table 3) was easily distinguished from *P. cinnamomi* by the size of amplicon.

It is doubtful that amplification of DNA from both isolates of *P. sojae* and one out of six isolates of *P. capsici* would be a major concern. *Phytophthora sojae* is specific for soyabean, while *P. cinnamomi* has a broad and different host range (Farr *et al*., 1989; Erwin & Ribeiro, 1996). *Phytophthora sojae* has not been found in ornamental nurseries. Thus, it is unlikely that use of the PCR assay for detection of *P. cinnamomi* will result in false positives, because of the ability of the LPV3 primers to amplify DNA of *P. sojae*. Instead, this unexpected amplification implies that this PCR assay may have potential for detecting *P. sojae* in soyabean production systems. This possible application deserves further investigation. Similarly, *P. capsici* and *P. cinnamomi* have rather different host ranges. The only common hosts for both these *Phytophthora* species are species of *Lycopersicon* and *Macadamia* (Farr *et al*., 1989), neither of which is an ornamental crop. Therefore, the risk of generating false positives with the PCR assay in this study for detection of *P. cinnamomi* in ornamental nurseries is minimal, especially since only one of six isolates tested showed a cross-reaction. This isolate was re-examined morphologically and using a single-strand conformation polymorphism (SSCP) analysis technique (Kong *et al*., 2003b). It had the typical morphology and SSCP pattern of *P. capsici*. The cause of cross-reaction with this isolate was unclear. Additional analyses are required to determine its identity.

PCR assay with LPV3 primers, in contrast to immunoassays, is capable of specific detection, which is essential for monitoring programmes of plant diseases caused by economically and ecologically important pathogens like *P. cinnamomi*. False positives have been a serious problem for serologically based *Phytophthora* diagnostic kits because of cross-reaction with *Pythium* spp. and inability to differentiate particular species from others within the genus (MacDonald *et al*., 1990; Ali-Shtayeh *et al*., 1991; Benson, 1991; Latorre & Wilcox, 1996). Such problems can be minimized, although not excluded, by use of this PCR assay.

PCR assay with LPV3 primers also demonstrated detection sensitivity for *P. cinnamomi* from soilless mixes and plant roots. The PCR assay was at least 10 times more sensitive than the plating method for detecting *P. cinnamomi* from soilless medium when the same amount of soilless mix was tested (Fig. 3). The PCR assay was also more sensitive than the plating method for detecting the pathogen in plant roots (Fig. 4). Ten plant samples tested positive with the PCR assay, utilizing an equivalent of 4 mg of roots per reaction, while nine tested positive with the plating method, utilizing 12 g of roots in two plates.

PCR assay with LPV3 primers for detection of *P. cinnamomi* is an improvement over other diagnostic methods by virtue of its demonstrated and improved detection

sensitivity. DNA-based assays have been reported for this pathogen (Lee *et al*., 1993; Cacciola *et al*., 2001), but few have been assessed for sensitivity with diseased samples (Judelson & Messenger-Routh, 1996) and none has been compared with traditional isolation methods. These assays may have diagnostic potential, subject to sensitivity tests. Immunoassays were compared with plating and baiting methods using irrigation water (Pettitt *et al*., 2002) and plant root samples (Werres *et al*., 1997). These comparisons indicated that detection sensitivity of immunoassays differs among protocols and kits. Some protocols, such as the zoospore-trapping immunoassay and the dipstick immunoassay, after an enrichment process (16 h incubation), can be as sensitive as classic plating and baiting methods (Pettitt *et al*., 2002). In contrast, the PCR assay in this study was more sensitive than plating methods for detection from both soilless mix and plant samples, with no need for enrichment. This indicates that the PCR assay would result in fewer false negatives than plating methods when detecting the pathogen from these substrates.

Despite the demonstrated detection specificity and sensitivity, this PCR assay must be used with caution and can be improved further for extended applications. *Phytophthora cinnamomi* attacks a broad range of plant species worldwide (Erwin & Ribeiro, 1996). There also appear to be at least three varieties (Old *et al*., 1984; Förster *et al*., 1990; Erwin & Ribeiro, 1996; Ho, 2002). Although 49 isolates were tested in this study, they all belonged to one variety, *P. cinnamomi* var. *cinnamomi*, and originated from limited geographic locations and host plants. Therefore, it is recommended that additional specificity tests be performed prior to use of this PCR assay for detection of isolates of different varieties (*P. cinnamomi* var. *parvispora* and *robiniae*) and of the same variety (*P. cinnamomi* var. *cinnamomi*) on different host plants or at different geographical locations.

Inhibitors in DNA extracted from plants and soil have been a common problem for PCR-based detection (Ersek *et al*., 1994) and were also a major difficulty in this study. Although the PCR assay successfully detected *P. cinnamomi* from the soilless medium (Metro Mix 360), it failed to detect the pathogen from pine bark, a commonly used medium in nursery production. Also, the PCR assay only detected the pathogen from roots using diluted DNA extract. Investigation of DNA extraction methods that can remove or minimize inhibitors is key to extending the application of this PCR assay for detection of the pathogen in other ecosystems such as pine-bark-based media and natural soil.

Application of the PCR assay for detection of *P. cinnamomi* in irrigation water is uncertain. All water samples assayed utilizing both the PCR and plating methods were negative. With the same plating method, this species was not recovered from any other samples taken from two commercial nurseries monthly over a 2-year period and four additional nurseries quarterly for 1 year (Hong *et al*., unpublished data). *Phytophthora cinnamomi* was also among the least isolated species from irrigation water in Oklahoma (von Broembsen & Wilson, 2000). Thus, the lack of detection was probably the result of the absence or limited presence of this pathogen in the water samples. This argument is supported by the fact that neither the original extract of water samples nor 10-fold dilutions of purified DNA were amplified by conventional PCR. Dilution was an effective strategy to improve template amplification for plant samples (Fig. 4), but it did not work for the water samples in this study. The results of zoospore PCR may imply the presence of inhibitors in irrigation water samples, but the purified DNA of water samples with the UltraClean Soil Kit was of sufficient quality for template amplification, as demonstrated in a separate study (Kong *et al*., 2003a). Additional evaluation of this PCR assay with water samples naturally infested with *P. cinnamomi* is warranted.

The LPV2 primers were specific for *P. cinnamomi* among the 31 species of *Phytophthora* tested and amplified DNA from culture, zoospores and chlamydospores. However, LPV2 primers were less specific than LPV3 primers among the non-*Phytophthora* species assessed. As a result, the PCR assay with LPV2 primers is not recommended for use alone to detect *P. cinnamomi* directly from disease samples that may harbour species of *Pythium* and true fungi. Conflicting detection data of plant samples between the PCR assays with the LPV2 and LPV3 primers and the plating method (Fig. 4) support this recommendation.

PCR assay with LPV2 primers, however, can be a useful alternative technique for identifying *P. cinnamomi* from pure culture within the genus *Phytophthora*. When compared with these methods, the PCR assay provides more rapid and reliable identification, while requiring minimal training. *Phytophthora cinnamomi*, characterized by hyphal swelling and large chlamydospores, is considered relatively easy to identify within the genus. However, production of these characteristic structures varies among isolates of different origins and with cultural conditions (Erwin & Ribeiro, 1996). In addition, several other *Phytophthora* spp. and some *Pythium* spp. may produce similar structures (van der Plaats-Niterink, 1981; Erwin & Ribeiro, 1996; Panabières *et al*., 1997). Often, *Phytophthora* isolates cannot be identified to species or are misidentified by classical methods because of the plasticity and overlap among the morphological characters (Yamak *et al*., 2002).

PCR assay with LPV2 primers may have better specificity for identification of *P. cinnamomi* than the methods of Coelho *et al*. (1997) and Cacciola *et al*. (2001). The LPV2 primer pair demonstrated no cross-reaction with 30 other species of *Phytophthora*, while the two previously reported primer pairs tested only eight and 19 species, respectively. The LPV2 primers detected all 49 isolates of *P. cinnamomi* from diverse origins, while the primers of Coelho *et al*. (1997) detected only 26 of the 30 isolates tested, all from cork oak in southern Portugal.

In summary, the PCR assay with LPV3 primers can be a useful tool for detecting *P. cinnamomi* in soilless media and plant tissues, whereas LPV2 primers can be an

effective alternative for identifying the pathogen from pure culture.

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