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Analysis of the distribution of *Phytophthora cinnamomi* in soil at a disease site in Western Australia using nested PCR

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Summary

The oomycete plant pathogen *Phytophthora cinnamomi* has infected a very large area of native vegetation in the south western corner of Australia. An important aspect of effective disease management depends on being able to accurately map areas of infestation. For this purpose, we have developed a nested polymerase chain reaction (PCR) protocol for the detection of *P. cinnamomi* in soil. The test uses two sets of primers developed from the rRNA ITS sequences of *P. cinnamomi* and can detect as little as 1 pg DNA. The degree of sensitivity was reduced with DNA extracted from soil although this depended on the type of soil. Soils with a high organic content, such as eucalypt forest soil and potting mix were more inhibitory than sandy soils. Inhibition by soil DNA could be reduced by the addition of bovine serum albumin and formamide to the reaction. *Taq* DNA polymerase was very sensitive to inhibitors compared with *Tth*+ or *Taq*F1*. In comparison with baiting (0–10% positive samples), nested PCR proved to be a very much more efficient (90–100% positive samples) method for the detection of *P. cinnamomi* in soil.

1 Introduction

Phytophthora cinnamomi is a pathogen that infects a very wide variety of plant species. It is devastating large areas of native vegetation in the southwest and southeast corners of Australia and placing many species of native plants at risk of extinction (SHEARER et al. 2007). There is evidence that the destruction of vegetation, and therefore, faunal habitat is a significant variable affecting small mammal diversity, density and habitat use (GARKAKLIS et al. 2004). *Phytophthora cinnamomi* is now recognized as a key factor that threatens the viability and biodiversity of many native vegetation ecosystems in Western Australia, Tasmania and Victoria (Dept. of Environment and Heritage, Australia, 2005). *Phytophthora cinnamomi* is a major problem not just in Australia, but also in other parts of the world. Throughout the last century, it has spread worldwide, probably as a result of the increased movement of plants. It has been found in oak (GARBELOTTO et al. 2006; BALCI et al. 2007) and Fraser Fir forests (BENSON et al. 2006) in the United States, and is responsible for diseases of oak in Portugal (MOREIRA and MARTINS 2005), France, (ROBIN et al. 1994) and Mexico (TAINTER et al. 2000).

The pathogen survives mainly within infected organic matter, such as root fragments from infected plants in the soil (SHEARER and TIPPETT 1989). Activities, such as road building, mining, timber logging and eco-tourism, which involve the movement of soil or plant material, spread the disease (SHEARER et al. 2007). One strategy to manage the disease is to prevent the movement of soil from infested to non-infested areas. However, this requires accurate mapping of the infested areas. The most frequent method for mapping infested areas is studying the health of host plant species that are particularly

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susceptible to infection (indicator species). Species of *Protaceae*, *Epacridaceae* and *Xanthorrhoea* are particularly useful indicators of the presence of the pathogen (Davison and Tay 2005).

Problems in determining the disease status of a site arise when there are few or no susceptible indicator species. In this case, the determination relies solely on laboratory analysis of soil samples by baiting. This involves mixing the soil with water to form a slurry, which is baited with susceptible tissue, e.g. rose petals (Marks and Kassaby 1974). After several days' incubation, the baits are plated onto selective agar and after a period of incubation to allow the outgrowth of any infecting pathogens, examined microscopically for the presence of coralloid hyphae that are diagnostic for *P. cinnamomi*.

Detection by baiting is low throughput, and prone to false negatives. Huberli et al. (2000) found that the frequency of isolation of *P. cinnamomi* from soil baits was increased by repeated wetting and drying of the bait. Similar results were reported in a subsequent study by Davison and Tay (2005). McDougall et al. (2002), who studied the spatial distribution of *P. cinnamomi* at seven sites in the eucalypt forest of Western Australia, reported a fivefold higher frequency of positive results with *in situ* baiting with live *Banksia grandis* seedlings than with *ex situ* baiting of soil. These results highlight a key problem with the baiting technique, namely that it does not detect all positive samples and that the distribution of the pathogen across a site may be more widespread that hitherto recognized. This has significant implications for the application of disease management strategies, such as barriers, quarantine or phosphite-spraying regimes.

A recent innovation in plant disease management is the use of DNA tests for detection and identification of pathogens. These are most commonly based on amplification of a species-specific DNA sequence by polymerase chain reaction (PCR). Such tests are rapid, specific and sensitive. Moreover, the equipment and the technology are rapidly becoming standard in many biological laboratories, and hence, these techniques are readily available. DNA detection tests have been developed for a number of species of the genus *Phytophthora* (Bonants et al. 1997; Lacourt and Duncan 1997; Grote et al. 2002; Hussain et al. 2005; Hayden et al. 2006; Bilodeau et al. 2007; Tomlinson et al. 2007). In general, the frequency of detection of *Phytophthora* spp. by such tests is higher than that achieved by baiting or growth of the pathogen on selective media. In view of this, we sought to use PCR to reanalyse the soils analysed in the Davison and Tay (2005) study, as the comparison between the two studies offers a unique opportunity to compare the results of the two methods. We have therefore developed a sensitive nested PCR detection test for *P. cinnamomi* and used this test to re-analyse a number of the sites from the Davison and Tay (2005) study.

2 Materials and methods

2.1 Isolates used in this study

Isolates of a range of species of *Phytophthora* were sourced from the Murdoch University isolate collection. Fresh cultures were regenerated from water storage as required by plating a single agar plug from the water cultures on corn meal agar (CMA) and incubating at 26°C for 3 days in the dark.

2.2 Soils

Soil samples used throughout this study were from a number of locations in Western Australia and were kindly provided by Dr Elaine Davison Curtin University. Soil samples were collected as described by Davison and Tay (2005) and stored in the dark at room

temperature while being transported back to the laboratory. At the laboratory, the soils were stored for no more than 5 days before 1-g samples were lyophilized and stored in sealed tubes at −20°C until extracted for DNA.

Additional soils were sourced from a commercial garden supplier and certified to be *P. cinnamomi* free. These included red sand (Gin-Gin, Western Australia), white sand and a commercial potting mix (a mixture of peat, river sand, crushed bark and sawdust obtained from Soils Ain't Soils, Western Australia). These along with a single sample of Spearwood sand collected from Murdoch University were utilized to assess the level of inhibition of different soil extracts on amplification by PCR.

2.2 Extraction of DNA from mycelium

Several 5-mm diameter plugs from the edge of a 3-day-old CMA agar plate culture were transferred to 9-cm Petri plates containing 20 ml of V8 liquid medium (Miller 1955). After 5-day incubation at 26°C in the dark, the mycelial mats were recovered and lyophilized. DNA was extracted from the lyophilized mycelium by the method of Graham et al. (1994).

2.3 Extraction of DNA from soil

Samples of soil (1 g) were lyophilized and mixed with 0.25-g glass beads (400–600 μm; Sigma-Aldrich, Sydney, Australia) and 1 ml of extraction buffer (Graham et al. 1994) and shaken in a Genogrinder (Glen Mills, Bridgewater, NJ, USA) at 1500 beats per min for 2 min. The slurry was centrifuged at 17 608 *g* for 20 min and the supernatant recovered. DNA was extracted from the supernatant as described previously (Graham et al. 1994). For analysis by PCR, this DNA was diluted 1/50 in PCR-grade water (Fisher Biotech, Perth, Australia) and 1 μl added to the PCR reaction.

2.4 PCR primer design

The ITS sequences from a range of *Phytophthora* species were sourced from Genebank and aligned along with those of several isolates of *P. cinnamomi* from the Murdoch University culture collection (Table 1) using CLUSTAL-W (Thompson et al. 1994). Regions of inter-species variation were targeted as suitable regions for primer design. Six *P. cinnamomi* specific primers were designed using Primer 3 software (Version 0.6; available online from Whitehead Institute for Biomedical Research, Cambridge) and screened against the Genbank database to assess their specificity. Care was taken to situate polymorphisms between closely related species, such as *Phytophthora cambivora* at the 3′ end of each primer (Coelho et al. 1997; Hayden et al. 2004).

2.5 Amplification conditions

Primer combinations were initially tested with DNA extracted from *P. cinnamomi* (MU83), *Phytophthora nicotianae* (MU7), *P. cambivora* (MU136) and *Phytophthora cryptogea* (MU25) to gain a preliminary assessment of the specificity of each primer pair. Unless indicated otherwise, amplification was carried out in 10 μl reactions with 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml gelatin, 2 mM $MgCl_2$, 1 μM primers, 0.1 ng/μl DNA, 0.2 mM dNTP and 0.77 U of Tth Plus DNA Polymerase (Fisher Biotech). PCR cycling consisted of 5-min denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 74°C for 1 min; and a final extension of 74°C for 5 min. The products were analysed by electrophoresis on 2% agarose gels in TBE buffer (Sambrook et al. 1989). For the second round of amplification, the

Table 1. **Isolates of *Phytophthora* and *Pythium* used in this study.**

Species	Isolate number	Source[1]	Location
Phytophthora cinnamomi	MU83	CPSM-MU	
P. cinnamomi	MP 94.03	CPSM-MU	*E. marginata*, Willowdale, WA
P. cinnamomi	MP94.5	CPSM-MU	*E. marginata* Willowdale, WA
P. cinnamomi	MP102	CPSM-MU	
P. cinnamomi	MP97.1	CPSM-MU	Huntley Minesite
P. cinnamomi	MP107	CPSM-MU	
P. cinnamomi	MP32	CPSM-MU	*Banksia* sp. Jarradale, WA
P. cinnamomi	MP94.03	CPSM-MU	*E. marginata*, Willowdale, WA
P. cinnamomi	MU33	CPSM-MU	Soil Cape Arid, WA
P. cinnamomi	MP 97.16	CPSM-MU	*E. marginata*, Jarrahdale, WA
P. cinnamomi	MU35	CPSM-MU	*Banksia* sp., Molly Island, WA
P. cinnamomi	MU84	CPSM-MU	
P. cinnamomi	MP99	CPSM-MU	
P. cinnamomi	MP104	CPSM-MU	
P. cinnamomi	MP105	CPSM-MU	
P. cinnamomi	MP111	CPSM-MU	
P. cinnamomi	MP115	CPSM-MU	
P. cinnamomi	MP119	CPSM-MU	
P. cinnamomi	MP120	CPSM-MU	
P. cinnamomi	MP121	CPSM-MU	
P. cinnamomi	MP125	CPSM-MU	*E. marginata* Huntly, WA
P. cinnamomi	MP103	CPSM-MU	
P. cinnamomi	MP128	CPSM-MU	*Xanthorrhoea preissii* Jarrahdale, WA
P. cinnamomi	MP129	CPSM-MU	
P. cinnamomi	MP130	CPSM-MU	
P. cinnamomi	MP133	CPSM-MU	
P. cinnamomi	MP134	CPSM-MU	
P. cinnamomi	MP80	CPSM-MU	*Corymbia calophylla* Jarradale, WA
P. cinnamomi	MP89	CPSM-MU	*C. calophylla* Jarradale, WA
P. cinnamomi	MP93	CPSM-MU	*C. calophylla Stem*
P. cinnamomi	MP94	CPSM-MU	
P. cinnamomi	MP94.15	CPSM-MU	
P. cinnamomi	EB5	CUT	*Banksia menziesii root collar WA*
P. cinnamomi	MP122	CPSM-MU	
P. cinnamomi	MP97.16	CPSM-MU	
P. cinnamomi	MP127	CPSM-MU	
P. cinnamomi	MP94.11	CPSM-MU	*E. marginata* Willowdale, WA
P. cinnamomi	MP94.48	CPSM-MU	*E. marginata* Willowdale, WA
P. cinnamomi	EB6	CUT	Soil, WA
P. cinnamomi	MP62	CPSM-MU	*E. marginata* Jarradale, WA
P. cinnamomi	MP97.8	CPSM-MU	
P. cinnamomi	MP94.48	CPSM-MU CPSM-MU	*E. marginata* Willowdale, WA
Phytophthora cambivora	MU136	CPSM-MU	
P. cambivora	MU137	CPSM-MU	
Phytophthora citricola	MU1	CPSM-MU	*Pinus radiata* Baudin Plantation
P. citricola	MU2	CPSM-MU	Soil, Nannup, WA
P. citricola	MU3	CPSM-MU	Soil, Walpole, WA
P. citricola	MU131	CPSM-MU	
P. citricola	EB11	CUT	Soil, Iluka minesite, Eneabba, WA
P. citricola	EB13	CUT	
P. citricola	EB3	CUT	Waterbody, Iluka minesite, Eneabba, WA
Phytophthora citrophthora/ Phytophthora meadii	MU 129	CPSM-MU	

Table 1. **Continued.**

Species	Isolate number	Source[1]	Location
Phytophthora cryptogea	MU25	CPSM-MU	*P. radiata* Jarrahwood Plantation
P. cryptogea	MU28	CPSM-MU	Soil, South Coast
Phytophthora drechsleri	MU134	CPSM-MU	*Kuzea baxteri* Murdoch University
P. drechsleri	MU14	CPSM-MU	Soil Bussleton, WA
Phytophthora erythroseptica	MU135	CPSM-MU	
Phytophthora megasojae	MU22	CPSM-MU	*Pinus radiata* Jarrahwood Plantation
P. megasojae	MU23	CPSM-MU	Hopetown WA
Phytophthora megasperma	MU132	CPSM-MU	*Pinus radiata* Sunklands WA
P. megasperma	MU133	CPSM-MU	
P. megasperma	MU17	CPSM-MU	*Soil* Cape Arid, WA
P. megasperma	MU18	CPSM-MU	FRNP
P. megasperma	MP41	CPSM-MU	
Phytophthora nicotianae	MU317	CPSM-MU	*Banksia brownii* Woodland
P. nicotianae	MU7	CPSM-MU	
P. nicotianae	MP5	CPSM-MU	
Phytophthora palmivora	MU128	CPSM-MU	
Pythium species	MU142	CPSM-MU	
Pythium irregulari	WAC7677	AWA	
P. irregulari	WAC7678	AWA	
Pythium spinosum	WAC2013	AWA	
P. irregulari	WAC7406	AWA	
Pythium acanthi	WAC2418	AWA	

[1]CPSM-MU, Centre for Phytophthora Science & Management, Murdoch University; CUT, Curtin University of Technology, Perth; AWA, Agriculture Western Australia.

products of the first round were diluted by 1/100 and 1 μl added to the reaction. The same amplification conditions were used for the second round.

2.6 Procedures to detect cross-contamination between samples

Several measures were taken to prevent cross contamination during sample handling and nested PCR analysis (Hayden et al. 2004). All preliminary handling of soil samples was carried out in a separate laboratory from DNA extraction and PCR analysis. Work areas were surface sterilized for 1 minte using 1% sodium hypochlorite. This was removed using 70% ethanol as excess sodium hypochlorite could in turn degrade target DNA in the samples being analysed. PCR analysis was carried out in a laminar flow work station in which all surfaces and equipment were cleaned as described earlier and irradiated with UV light for 20 min before setting up PCR reactions. The laminar air flow was turned off while setting up the PCR reactions to prevent lateral transfer from one sample to another across the 96-well PCR plates. Fresh packets of micropipette filter tips (Axygen Scientific, Union City, CA, USA) were used for preparation and handling of all stages of the primary and nested-PCR reactions. Primary PCR reactions were spun in an Allegrä X-15R centrifuge (Beckman Coulter, Fullerton, CA, USA) at 2100 *g* for 4 min before removing the sealing film to prevent the samples aspirating from one well to another. A negative control, in which the mycelium or soil was substituted with sterile glass beads, was included between each set of 10 DNA extractions. These negative controls were carried throughout the DNA extraction and were analysed at the end of the associated nested PCR to assess the prevalence of cross-contamination during DNA extraction. As these controls did not contain co-extracted inhibitors, they were likely to be more informative as using non-infested soil samples which could have produced false-negative results owing to varying level of PCR inhibition. Separate PCR negative controls were

incorporated for every 10 PCR samples to assess contamination during PCR analysis. If any of the PCR controls within a 96-well PCR plate produced positive detection results, both the primary and nested PCR reactions for that set were repeated.

2.7 DNA sequencing

To sequence the ITS regions, the region was amplified using the ITS1/ITS4 primer pair (WHITE et al. 1990). The amplification products were cleaned up using a MOBIO Cleanup Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and further concentrated threefold by ethanol precipitation. Both strands of the PCR products were then sequenced with either ITS1 or ITS4 primers using the dye-terminator chemistry (ABI Biosystems, Forest City, CA, USA). The sequence reads were generated on an ABI 3730 XL sequencer (Applied Biosystems).

2.8 Baiting of soils for detection of *Phytophthora cinnamomi*

The baiting procedure is essentially that described by MARKS and KASSABY (1974) using 3 × 5-mm diameter discs of rose petal tissue as bait. The baits were plated on NARPH-elective plates (HUBERLI et al. 2000).

2.9 Population density index (PDI)

The smallest quantity of soil and fine roots from which *P. cinnamomi* could be detected was used as an index of the population density of the pathogen present within each soil. Samples were analysed based on a sample size of 64 g, with fractions of these ranging to 1/64 (1 g) of the original being analysed in triplicate. The reciprocal of the smallest fraction from which *P. cinnamomi* was detected was used as the PDI for each sample (WESTE and RUPPIN 1977).

3 Results

3.1 Primer design

Phytophthora cinnamomi-specific primers have been developed by other researchers (COELHO et al. 1997; KONG et al. 2003); however, in comparative tests using purified *P. cinnamomi* DNA, we find that they are considerably less sensitive than primers based on the ITS regions (Siricord and O'Brien, unpublished). We therefore designed new primers for the ITS regions of *P. cinnamomi*. Because sensitivity is a critical issue, we opted for the design of a nested PCR protocol as several studies have shown that nested PCR is considerably more sensitive than single-round PCR (GROTE et al. 2002; IPPOLITO et al. 2002; HAYDEN et al. 2006).

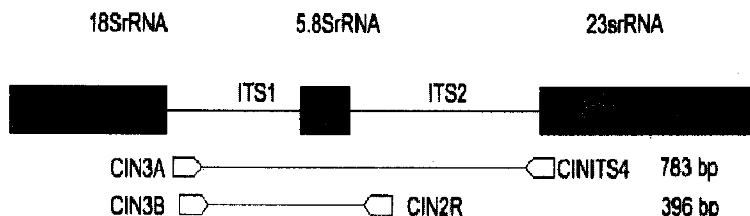

Fig. 1. Location of the primers used for nested PCR detection of *Phytophthora cinnamomi*. The sizes of the primary and secondary amplicons are also indicated.

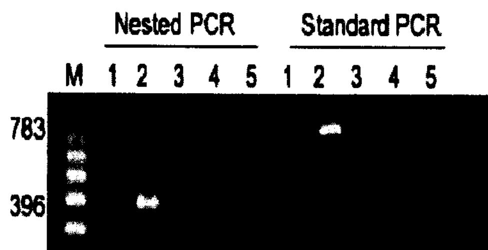

Fig. 2. Specificity of primer pairs. The primers CIN3A/CIN4 were added to PCR reactions containing DNA from various *Phytophthora* spp. for first round amplification. The products were diluted 1/100 and 1 μl added to reactions containing the second-round amplification reactions containing the CIN3B/CIN2R primer pair. The products of both rounds of amplification were analysed by electrophoresis. (1) Negative control; (2) *Phytophthora cinnamomi*; (3) *Phytophthora nicotianae*; (4) *Phytophthora Cambivora*; and (5) *Phytophthora cryptogea* DNA.

The primers CIN3A, CIN3B, CIN4 and CIN2R were designed as *P. cinnamomi*-specific primers from an alignment of ITS sequences (Table 2) of *P. cinnamomi* isolates (Table 1). First-round PCR with the CIN3A/CIN4 amplifies a region of 783 bp (Fig. 1). The products of this amplification were used as template in a second round of PCR with the nested primers CIN3B/CIN2R which amplify a region of 396 bp (Fig. 1). Both pairs of primers produced amplification products only with *P. cinnamomi*. None of the other species tested (Table 1) produced amplification products (Fig. 2).

3.2 Sensitivity of detection

The sensitivity of the primers was tested by the amplification of serial dilutions of *P. cinnamomi* DNA. With the nested PCR protocol, even 1 pg of *P. cinnamomi* DNA could be detected (Fig. 3). In some instances, we could detect down to 0.1 pg, but detection at this level was variable. Addition of 1 μl of the soil extract to the PCR reaction reduced the sensitivity of detection by 10-fold.

The addition of the PCR-enhancing reagents, bovine serum albumin (BSA) (KREADER 1996) or formamide (SARKAR et al. 1990) resulted in a stronger more-consistent signal being detected at 0.1 pg (Fig. 3). These agents also reduced the inhibitory effect of soil extract on amplification although formamide was less effective than BSA. Using a combination of 400 ng/μl BSA and 4% formamide in the PCR reaction, we could detect down to 0.1-pg DNA (Table 3). These were chosen as the standard conditions of amplification as higher concentrations of BSA or formamide proved inhibitory to amplification (data not shown). While not beneficial for overcoming inhibition, formamide was included as a PCR additive to maintain optimal primer specificity. This was deemed to be important as the assay had been designed to detect the pathogen from a broad range of soil samples with diverse microbial communities.

We also investigated whether different types of DNA polymerase might respond differently to the presence of soil extract to the amplification reaction. We tested three types of DNA polymerase for their susceptibility to inhibition by soil extracts. Each enzyme was tested in a series of reactions containing different amounts of soil extract. Amplification products were obtained in reactions containing 0.2% or less soil extract (Fig. 4). No products were obtained with any of the enzymes where the soil extract comprised 0.5% of the reaction. Of the three enzymes tested, *Taq* gave weaker and more variable results compared with *Tth*$^+$ or *TaqF1** DNA polymerases.

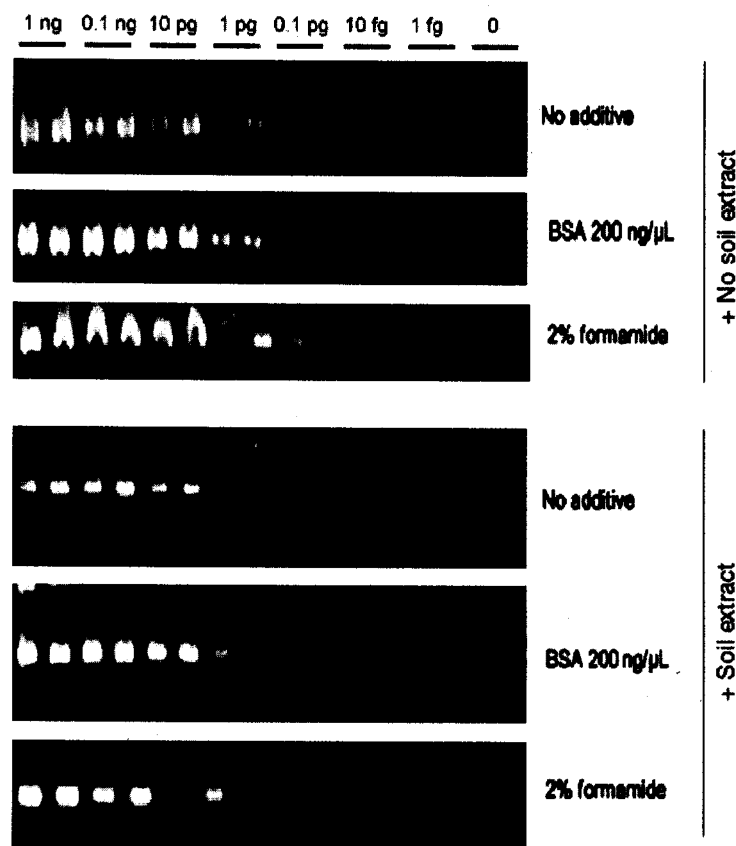

Fig. 3. Sensitivity of detection of *Phytophthora cinnamomi* by nested PCR. Different amounts of *Phytophthora cinnamomi* DNA were added to a series of PCR reactions. The effects of bovine serum albumin and formamide were determined by adding these separately to separate series of reactions. A duplicate group of reactions contained 1 μl of a 1/50 dilution of soil extract. All reactions were carried out in duplicate.

Table 2. ***Phytophthora cinnamomi*-specific primers used for first and nested rounds of PCR.**

Primer	Sequence	Tm (°C)	
CIN3A	CATTAGTTGGGGGCCTGCT	54	1st round
CINITS4	TGCCACCACAAGCACACA	50	1st round
CIN3B	ATTAGTTGGGGGCCTGCT	50	Nested
CIN2R	CACCTCCATCCACCGACTAC	56	Nested

3.3 Inhibitory effect of different soils

The sensitivity of detection of the nested PCR test has been established with DNA extracted from jarrah forest soil (Fig. 3). It is possible that other types of soils may be more inhibitory, and therefore, have a lower detection sensitivity. To address this, we tested five different soils for their effect on PCR amplification. DNA extracts were prepared from the soils and different amounts added to a standard PCR reaction (without BSA or formamide) with added *P. cinnamomi* DNA. The results show that both jarrah forest soil and native

Table 3. **Effect of bovine serum albumin (BSA) and formamide on detection sensitivity in the presence and absence of soil extract.**

Additive			
Soil extract	BSA (400 ng/μl)	4% Formamide	Sensitivity (pg)
–	–	–	0.1
–	+	–	0.1
–	–	+	0.1
–	+	+	0.1
+	–	–	10
+	+	–	0.1
+	–	+	1
+	+	+	0.1

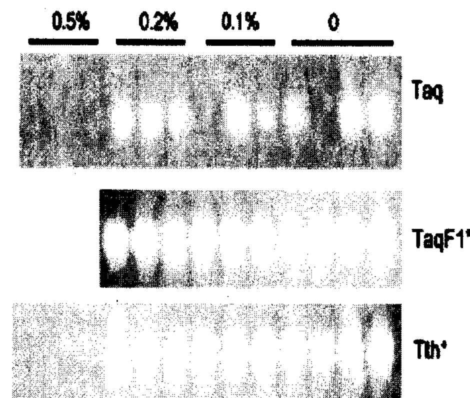

Fig. 4. Sensitivity of Taq, Taq F1* and Tth* DNA polymerases to inhibition by soil extract from *Eucalyptus marginata* forest. PCR amplification was carried out in triplicate with the CIN3A-CIN4 primers, each polymerase and 0, 0.1, 0.2, 0.5, 1.0, 5.0 and 10% jarrah soil extract.

potting mix extracts proved to be the most inhibitory of the five soils (Fig. 5) and the highest levels at which these supported amplification were 0.2 and 0.5%, respectively. The sandy soils were considerably less inhibitory. Amplification was observed in reactions containing up to 2% extract from white sand, and up to 5% with Gin Gin red sand or Spearwood sand. A product was obtained in one of the reactions containing 10% Spearwood sand extract. The results show that although there is variation in the extent of inhibition obtained with DNA extracted from different soils, using the standard PCR conditions with BSA and formamide, we can be confident of detecting the pathogen in a wide variety of soils.

3.4 Nested PCR *vs.* baiting

To compare the efficiency of detection with nested PCR with that of baiting, five soil samples were collected from Mettler reserve, 40 km south east of Wellstead in the Great Southern region of Western Australia (S34°34′29.6″, E118°34′13.8″). The sampling site was located at an intersection in the roadway where the surrounding vegetation displayed distinct symptoms of dieback with a range of soil types present. Preliminary baiting analysis of soil collected from the site confirmed the presence of *P. cinnamomi* throughout

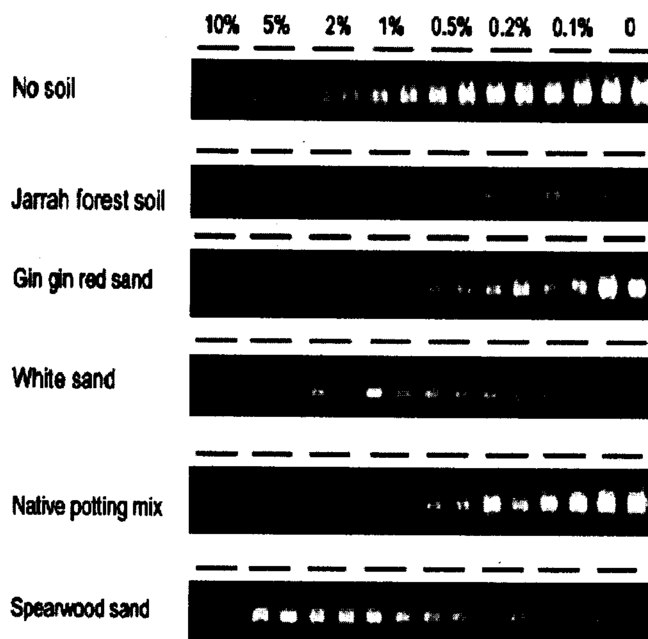

Fig. 5. The inhibitory effect of extracts from different soil types on PCR amplification. PCR amplification was carried out in duplicate with the CIN3A-CIN4 primers, each polymerase and 0, 0.1, 0.2, 0.5, 1.0, 5.0 and 10% soil extract.

(K. Raiter, personal communication). Each soil sample was analysed to determine the smallest quantity of soil from which *P. cinnamomi* could be detected using the PDI described earlier. *Phytophthora cinnamomi* was detected more readily by nested PCR than by baiting with consistent detection achieved by nested PCR from 1-g samples from each of the five soils (Table 4). In contrast, detection by baiting was less sensitive and more sporadic across the range of soil samples. Baiting analysis failed to detect any *P. cinnamomi* in the P2 soil.

3.5 Distribution of *Phytophthora cinnamomi* across a disease front as analysed by nested PCR

In a previous study, Davison and Tay (2005) carried out systematic analysis of the distribution of *P. cinnamomi* at a number of infected sites in Western Australia. In their study, samples were taken in a systematic manner from the centre and the margins of each site according to a grid pattern and analysed by baiting using a modified double baiting procedure. The highest percentage of positive samples from sites at the margins of the disease fronts was 7.2. In the present study, we used both baiting and nested PCR to analyse samples taken from the margin of some of the sites in the Davison and Tay (2005) study. No *P. cinnamomi* was detected from any soil samples by primary PCR alone (data not shown). However, diagnostic DNA fragments were produced from at least 96% of the soil samples taken from each site using nested PCR (Fig. 6). The identity of the amplicon was confirmed by extraction and sequencing of a number of amplicons from the gel. With the exception of two nucleotide polymorphisms at the start of the sequence from site 16, 100% homology was observed between each of the sequences amplified from soil and

Table 4. **Population density index (PDI)[1] of *Phytophthora cinnamomi* in five soils sampled from the Great Southern region of Western Australia determined by baiting and nested PCR analysis of soil samples.**

Sample	S1	S2	P1	P2	W1
Soil characteristics	Orange-brown clay	Orange clay	Grey fine sand from beneath dead *Banksia baxteri*	Grey fine sand from beneath dying *Banksia baxteri.*	Roadside clay, orange/brown
Baiting-pure culture isolations	57	8	6	0	1
Nested PCR detection from soil	192	192	192	192	192

[1]The smallest quantity of soil and fine roots from which *P. cinnamomi* could be detected was used as an index of the population density of the pathogen present within each soil. Samples were analysed based on a sample size of 64 g with fractions of these ranging to 1/64 (1 g) of the original were analysed in triplicate. The reciprocal of the smallest fraction from which *P. cinnamomi* was detected was used as the population density index (PDI) for each sample (WESTE and RUPPIN 1977). Triplicate 1-g samples of each soil were tested by nested PCR for comparison.

numerous ITS1, 5.8S and ITS2 sequences of *P. cinnamomi* in GenBank. The sequence variation observed in the sequence from site 16 was likely to be an artefact of the sequencing reaction, as analysis of the chromatogram in this region was associated with high background signals. As the remainder of the sequence showed 100% homology, with *P. cinnamomi* sequences already present on the Genbank database, it was decided that no further confirmation was required for the purpose of this study.

In contrast, over the five sites, *P. cinnamomi* was detected in only 2.2% of the samples from sites by double baiting (Fig. 6). The highest density detected by baiting was in site 18, where 5/50 samples were positive (Fig. 6). Of these five quadrats, only two were adjacent to each other, whereas the other three were well separated. Two positive samples were observed from adjacent quadrats in site 17, and a single positive sample was obtained from site 16. All of the samples that were positive by baiting were also positive by nested PCR.

4 Discussion

In this paper, we describe the development of a nested-PCR protocol for the detection of *P. cinnamomi* and its application for the detection of the pathogen in a variety of different soil types. Although impurities present in DNA extracted from soil inhibited PCR, it was still a more efficient method for the detection of the pathogen compared with baiting. Analysis of samples from the margins of disease sites in Western Australia by nested PCR reveal quite a different distribution of the pathogen than that revealed by baiting analysis.

We found that soil DNA is inhibitory to amplification although the degree of inhibition varied for different soils. Soil is a very difficult milieu to detect pathogens by PCR as it contains a variety of substances, such as polysaccharides, polyphenols, humic acids tannins and pigments that inhibit amplification of DNA (CULLEN and HIRSCH 1998; SCHENA et al. 2006). In general, the levels of detection with soil extracted DNA are very much lower than those obtained with purified DNA extracted from mycelium. Although there have been many attempts to develop methods to eliminate these inhibitors, none of them have proved effective (CHO et al. 1996; OGRAM 2000; BURGMANN et al. 2001; BRAID et al. 2003; ROBE

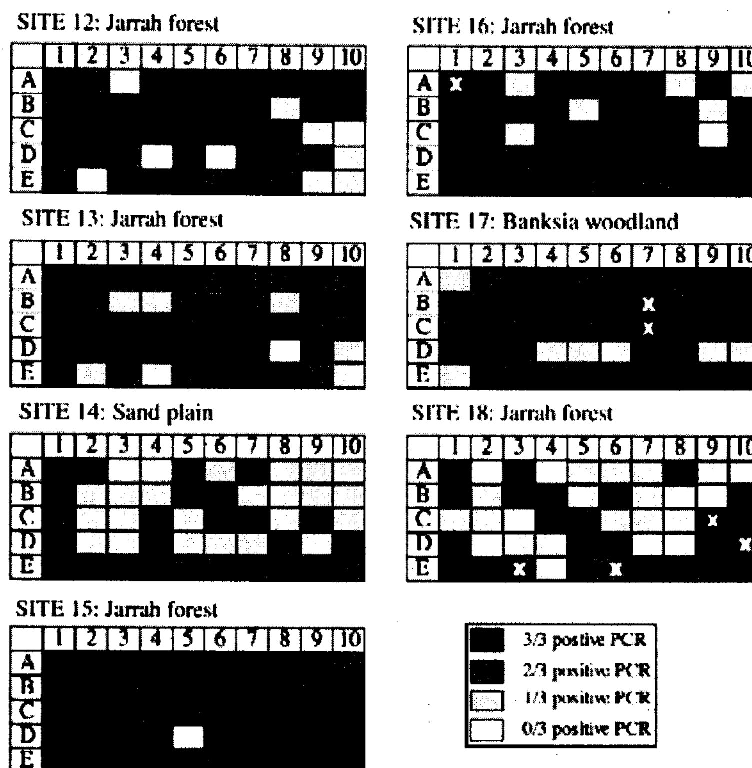

Fig. 6. Mapping the distribution of *Phytophthora cinnamomi* at dieback margin sites by baiting and nested PCR. Each site (12–18) is located along the margin of a dieback site and was divided into 50 quadrats along a 45-m baseline (A to E), and a 20-m baseline (1 to 10) (Davison and Tay 2005). At each site, position A1 and baseline A were in the healthy vegetation and baseline E was in the dieback site. Five hundred grams of top soil was taken from each quadrat for baiting. Before baiting, 3 × 1 g subsamples were removed for analysis by PCR. The baiting results are indicated by X within the quadrat. The PCR results are indicated by the colour of the squares.

et al. 2003). The inhibitory effect of soil DNA can be reduced by increasing the amount of DNA polymerase in the reaction, or by the addition of phage T4 gene 32 protein or BSA (Kreader 1996). Thus, the effect does not appear to be related to a particular activity of the proteins but to an increase in total protein, suggesting that the protein acts by binding inhibitors, thereby preventing them from binding to and inhibiting DNA polymerase.

We have also looked at the susceptibility of different DNA polymerases to inhibition as studies have shown that DNA polymerases differ in their responses to PCR inhibitors. Al-Soud and Radstrom (1998) reported a 5000-fold difference in the sensitivity of different DNA polymerases to PCR inhibitors in blood. They also found that different polymerases responded differently to inhibitors from different milieu (blood, cheese, faeces, meat) and suggested that there is an appropriate polymerase for each one. In this study, we tested three readily available and inexpensive types of DNA polymerases and found that Taq DNA polymerase was more sensitive to PCR inhibitors in soil than either TaqF1* or Tth^{+} polymerases. We used Tth^{+} DNA polymerase in this study.

In comparative tests, nested PCR gave positive results with all samples, whereas only a small number of samples tested positive with baiting. A positive result with baiting depends

on the production of sporangia, release of zoospores by the sporangia and infection of the bait tissue by the zoospores. Bacteria and fungi in the soil can parasitize hyphae and other propagules of *Phytophthora* spp. and can inhibit the production of zoospores by the production of antibiotics and other compounds (MALAJCZUK 1983). Zoospore production is also affected by the chemical composition of the soil. BROADBENT and BAKER (1974) and SHEARER (2003) reported that the use of soils with higher levels of K, P, N and organic matter resulted in higher levels of zoospore production, while MESSENGER et al. (2000) found that high calcium levels were important for zoospore production. The presence of metal ions can also influence the results of baiting. A study by Gerretson-Cornell 1976 (quoted in TSAO 1983) found that the recovery of *P. cinnamomi* was 94, 32 and 0%, respectively, when glass distilled water, deionized water or distilled water from a metal still were used for baiting. Baiting is therefore a complex process sensitive to variations in soil physiochemical and biological characteristics. The results of WILSON et al. (2000), who found that although 30% of samples tested positive for the presence of *P. cinnamomi* using a zoospore-specific assay, the same samples testing negative in a baiting test demonstrates that the production of zoospores is not sufficient to achieve a positive result. Typically, Western Australian soils have a very low frequency of positive detection by baiting compared with other states. Isolation frequencies for West Australian soils range from 0.4 to 10% (PODGER 1978; BLOWES 1980; DAVISON and TAY 2005), whereas the corresponding values for New South Wales and Queensland were 27 and 58%, respectively (BLOWES 1980; PRYCE et al. 2002).

In this paper, we have described the development of a sensitive nested-PCR technique for the detection of *P. cinnamomi* in soil, and shown that it is a more efficient method for the detection of the pathogen compared with baiting. Although PCR detection tests detect the presence of the pathogen's DNA rather than the viable organism, they are invaluable as a means of eliminating samples from further testing. The test will be a valuable tool at cryptic sites where susceptible indicator species are not present, or at sites which have been recently burnt. It will also be valuable to ensure gravel or sand pits are free of the pathogen prior to using the gravel or sand for road building, horticultural purposes or when disease-free material is essential.

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