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PCR primers for specific detection of *Phytophthora cinnamomi*

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Abstract. This paper describes the identification of PCR primers for the specific detection of *Phytophthora cinnamomi*. An internal standard DNA fragment amplified by the same PCR primers but giving an amplicon of a different size is added to the PCR reactions to detect false negative reactions caused by inhibition of amplification.

Phytophthora cinnamomi Rands causes root rot in a wide variety of plants and has been implicated as a major causative organism of eucalypt dieback, a disease that is devastating the forests of Western Australia, and placing a large number of native Australian plant species at risk of extinction (Shearer *et al*. 2007). Activities such as mining and road building that involve the movement of large volumes of soil contribute to the spread of the disease through the movement of infested soil into non-infested areas.

Disease control would be significantly enhanced by the availability of a rapid, high throughput test for detection of the pathogen in soil. Current methods for detection of *P. cinnamomi* involve baiting a soil–water slurry with material that is colonised by the pathogen, followed by plating of the bait on a semi-selective medium and microscopic examination (Marks and Kassaby 1974). The technique is low throughput, tedious and prone to false negatives (Huberli *et al*. 2000). An alternative approach is to use PCR for detection of the pathogen. In this technique, DNA is extracted from the material and amplified by PCR using species specific primers. Successful amplification indicates the presence of the pathogen. DNA detection tests have been developed for several *Phytophthora* species (Stammler and Seemuller 1994; Grote *et al*. 2002; Hussain *et al*. 2005; Bilodeau *et al*. 2007). The present paper describes the identification of *P. cinnamomi* specific primers.

Strains of various species of *Phytophthora* were obtained from the Murdoch University Culture Collection and maintained on cornmeal agar plates at 4°C. Mycelium for DNA extraction was prepared as described previously (Dobrowolski and O'Brien 1993) and DNA was then extracted from the mycelium using the method of Graham *et al*. (1994).

Soil DNA was extracted by mixing 0.5 g soil in 1 mL CTAB buffer (Graham *et al*. 1994) and incubating the slurry at 55°C for 10 min. Soil particles were removed by centrifugation at 15 338*g* for 5 min and 200 μL of the supernatant was collected and extracted for DNA as described above for mycelium.

The primers LPC2 (5′GTCCACACCTACCCAGAGAT′3) and RPC3 (5′ CGTGTATGAGGAAGCGTAGG′3) were synthesised by Pacific Oligos. Amplification was carried out in 20-μL reactions containing 1 μL DNA, 1.25 μM primers, 6% DMSO, 100 μM dNTPs, 2U Taq DNA polymerase (Biotech International, Perth, Western Australia), in PCR buffer (Biotech International). Temperature cycling consisted of one cycle of 94°C for 10 s, 25 cycles of 94°C for 5 s, 60°C for 30 s and 72°C for 1 min, followed by a holding temperature of 20°C. Products were analysed by electrophoresis on 5% polyacrylamide in TAE buffer (Sambrook *et al*. 1989) at 100 V until the bromophenol blue dye had migrated to the bottom of the gel ~20 cm from the origin. The gel was stained with ethidium bromide (1 μg/mL) for 10 min and photographed under UV light.

To develop an internal control fragment, sheep DNA was extracted from whole blood using a QIAamp kit (QIAGEN) as described by the manufacturer and used in a random amplified polymorphic DNA (RAPD)-PCR reaction (Duncan *et al*. 1993) with the LPC2/RPC3 primer pair. Products of an appropriate size that were obtained with both primers but not with either primer alone were identified and cloned in pTOPO (Invitrogen). One of these plasmids, pSLR3-LD1, was chosen as the internal standard. For detection of false negatives 5 pg of plasmid DNA was added to the PCR reactions as an internal standard.

We have previously described the isolation of a RAPD-PCR product specific for *P. cinnamomi* in hybridisation tests (Dobrowolski and O'Brien 1993). To convert this to a PCR reaction, the sequence of the RAPD-PCR (GenBank Accession No. EU170014) product was determined as described by (Driessen *et al*. 2004) and pairs of primers were designed to sites at each end of the fragment. The pairs of primers were tested for their ability to specifically identify *P. cinnamomi*. One of several primer pairs tested (LPC2/RPC3) showed specificity for *P. cinnamomi*. Amplification of *P. cinnamomi* DNA resulted in the formation of a 281 bp product that was not observed in the products from the other species of *Phytophthora* tested (Fig. 1). We found that inclusion of 6% DMSO (Frackman *et al*. 1998) enhanced the specificity of the reaction. Tests with *P. cinnamomi* DNA showed that as little as 10 pg of DNA could be successfully detected with the LPC2/RPC3 primers (data not shown). This is comparable to levels of sensitivity reported by other researchers for detection of *Phytophthora* species (Hussain *et al*. 2005; Bilodeau *et al*. 2007; Tomlinson *et al*. 2007). Comparisons of the efficiency of baiting and PCR for the detection of *P. ramorum* in plant tissue samples have shown that PCR detection tests with a sensitivity of detection of 0.5–50 pg DNA successfully detected

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Fig. 1. Specificity of the LPC2/RPC3 primers for *Phytophthora cinnamomi*. DNA was extracted from different species of *Phytophthora* and amplified with the primers. Lane 1, markers; lane 2, *P. cinnamomi*; lane 3, *P. cryptogea*; lane 4, *P. citricola*; lane 5, *P. drechsleri*; lane 6, *P. nicotiana* var. *nicotiana*; lane 7, *P. megasperma* var. *sojae*; lane 8, *P. megasperma* var. *megasperma*; lane 9, *P. parasitica*; lane 10, *P. cambivora*; lane 11, *P. erythroseptica*; lane 12, *P. palmivora*; lane 13, *P. citrophthora*.

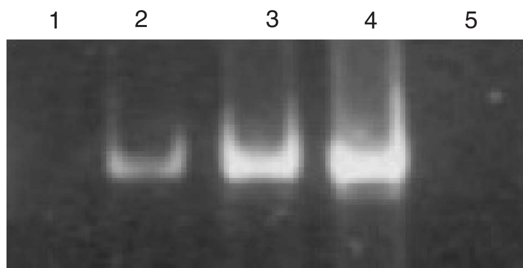

Fig. 2. Inhibition of amplification by soil DNA. Dilutions of soil extracted DNA were added to a standard amplification reaction. Lane 1, undiluted DNA; lane 2, 1/5 dilution; lane 3, 1/10 dilution; lane 4, 1/20 dilution; lane 5, no DNA.

the pathogen in all samples from which the pathogen could be isolated by plating (Lane *et al*. 2005; Hayden *et al*. 2006; Ioos *et al*. 2006). In addition, the tests also detected the pathogen in many samples from which the pathogen could not be isolated.

The addition of soil extracted DNA to the standard PCR reaction containing *P. cinnamomi* DNA resulted in complete inhibition of amplification (Fig. 2). The most effective method to overcome this inhibition was to dilute the extracted DNA in water and add 1 μL of the diluted DNA to the PCR reaction. Although amplification was observed at a 1/5 dilution of the soil extracted DNA, the intensity of the product was greater when a 1/10, or 1/20 dilution was used. For routine use of this detection test a dilution of 1/20 is used. The inhibition observed here is consistent with the observation of several previous studies and is generally ascribed to the presence of humic acids, tannins, pigments and lignin degradation products in the soil (Cho *et al*. 1996; Cullen and Hirsch 1998; Braid *et al*. 2003).

Where a PCR test is being used to screen soil samples for the presence of an organism, failure to observe an amplification product could be due to: (a) the absence of the target organism from the sample, a true negative; or (b) inhibition of amplification by soil components in the DNA extract, a false negative. A strategy to enable detection of false negatives is to use an internal standard DNA fragment that is added to the reaction. This internal standard should be amplified by the same primers used for amplification of the *P. cinnamomi* sequence and it should be derived from heterologous DNA so that it does not interfere with the *P. cinnamomi* amplicon. To develop an internal standard fragment that can be used in screening soil samples for *P. cinnamomi*, sheep DNA was used as a source of heterologous DNA. To identify fragments that are amplified by the LPC2/RPC3 primer pair, sheep DNA was used in a

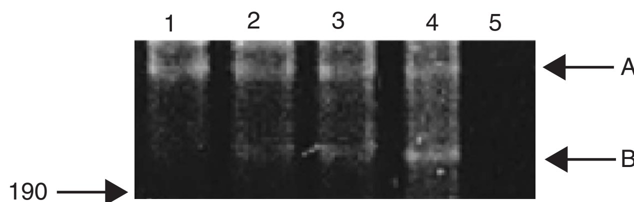

Fig. 3. Test of the internal standard. Varying amounts of *Phytophthora cinnamomi* DNA were amplified in the presence of 10 pg of pSLR3-LD1 DNA. The amounts of *P. cinnamomi* DNA were: lane 1, 5 ng; lane 2, 1 ng; lane 3, 500 pg; lane 4, 100 pg; lane 5, blank reaction. (*A*) *P. cinnamomi* product; (*B*) internal standard. The position of the 190 bp marker is indicated.

RAPD-PCR reaction (Williams *et al*. 1993) with each of the primers alone, or with both primers together. Comparison of the gel separated products of the RAPD-PCR reactions enabled the identification of fragments that were amplified by both primers together. A band of appropriate size was cloned and used as the internal standard. Fig. 3 shows the amplification products in reactions containing both the internal standard fragment and *P. cinnamomi* DNA. Observation of the standard product depends on the amount of *P. cinnamomi* DNA present in the reaction. Although the band from the internal standard is evident at low levels of *P. cinnamomi* DNA, as the level of *P. cinnamomi* DNA increases, amplification of the standard fragment becomes less efficient due to competition for primers and DNA polymerase. Failure to observe an amplification product either from *P. cinnamomi* or from the internal standard indicates inhibition of amplification. This is a false negative.

The use of PCR in the detection of *P. cinnamomi* overcomes the limitations of the traditional baiting method in that it can detect false negatives and can process large numbers of samples in a short time. The test will be of considerable benefit in the management of eucalypt dieback disease.

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