DETECTION OF *PHAEOMONIELLA CHLAMYDOSPORA* IN SOIL USING SPECIES-SPECIFIC PCR

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ABSTRACT

Phaeomoniella chlamydospora is a fungal pathogen of woody grapevine tissue that causes "Petri disease". As one potential source of inoculum is infested soil, a SDS/phenol/chloroform DNA extraction method and PCR assay utilising species-specific primers were evaluated for the ability to detect *P. chlamydospora* in grapevine nursery soil. Using a nested PCR approach, the assay was able to detect 10^2 conidia/ml when a spore suspension was added to sterilised soil samples and 50 fg when *P. chlamydospora* genomic DNA was added directly to the reaction. In this process, preamplification of a 600 bp region of the ribosomal DNA was followed by amplification with primers Pch1 and Pch 2 to produce a 360 bp species-specific product. This highly sensitive diagnostic tool will be used in future studies to determine if pathogen propagules are present in soils collected from New Zealand grapevine nurseries.

Keywords: grapevine, Petri disease, *Phaeomoniella chlamydospora*, soil, species-specific PCR.

INTRODUCTION

Petri disease (previously known as "black goo" and Petri grapevine decline) causes poor growth and decline of grapevines in association with brown/black streaking and the formation of droplets of black liquid in cut woody trunk tissue (Mugnai et al. 1999). A number of fungi have been found in association with this disease, however, *Phaeomoniella chlamydospora* is the most commonly isolated and believed to be the causal agent (Mugnai 1998; Clearwater et al. 2000; Pascoe & Cottral 2000). The presence of *P. chlamydospora* in New Zealand has been confirmed through traditional isolation methods (Clearwater et al. 2000) and molecular identification (Ridgway et al. 2002).

The disease cycle of *P. chlamydospora* is not well understood partially because of practical difficulties associated with working with this fungus. For example, the slow growth habit means its presence is often masked by contaminants (Eskalen et al. 2001). However, two sources of primary inoculum have been conclusively identified. The first is the use of cutting material from infected mothervines. Mothervines have been shown to be infected with *P. chlamydospora* (Ridgway et al. 2002) and the fungus has been isolated from young grafted plants (Mugnai 1998). The second source of primary inoculum is the production of air-borne spores from infected vines in the vineyard (Larignon & Dubos 2000). A third potential source is fungal propagules persisting in soil. Previous researchers have been able to detect *P. chlamydospora* in artificially infested soil at a level of 10^4 conidia/ml using a nested polymerase chain reaction (PCR) (A. Eskalen, pers. comm.). The objective of this work was to determine if this detection level could be improved by using the highly specific primers (Pch1 and Pch2) (Tegli et al. 2000), which have detected less than 1 pg *P. chlamydospora* DNA in infected rootstock mothervines (Ridgway et al. 2002).

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MATERIALS AND METHODS

Extraction of DNA from grapevine nursery soil

Soil samples (approximately 30 g) were collected in sterile 50 ml tubes from recently cultivated land in Auckland being developed as a first time commercial grapevine nursery block. Samples were collected to a depth of 0-10 cm from 10 evenly-spaced points across a 100 m length of field and air-dried at 20°C overnight. DNA was extracted from the soils using a modification of the method of Ridgway & Stewart (2000). Sterile sand (2 g) and soil (2 g) were ground to a fine powder in a chilled mortar and pestle for 60 s and 2 g was transferred to a 15 ml tube. Five millilitres of extraction buffer (0.3% SDS, 0.14 M NaCl, 50 mM NaOAc, pH 5.1) was added, briefly shaken vigorously, incubated at 37°C for 30 min and centrifuged for 5 min at 1000 x g. The resulting supernatant was transferred to a clean tube and extracted four times with 2 ml phenol and 2 ml chloroform, followed by a single extraction with 4 ml chloroform. DNA was precipitated by addition of 60% volume of ice-cold isopropanol and the resulting pellet was washed with 70% ethanol, air dried and resuspended in 400 µl sterile water. The DNA suspension was then processed in a Prep-A-Gene® DNA purification kit (Bio-Rad Laboratories Pty Ltd., New Zealand) as per manufacturers instructions to remove inhibitors.

Non-nested species-specific PCR

Amplification of the DNA extracted from the previously described soil samples was performed according to Ridgway et al. (2002) using the previously described Pch1 (5' CTC CAA CCC TTT GTT TAT C3') and Pch2 (5' TGA AAG TTG ATA TGG ACC C3') primers (Tegli et al. 2000). Each 25 µl amplification reaction contained 20 mM Tris-HCL, pH 8.4, 50 mM KCL, 2.5 mM MgCl₂, 200 µM each of dATP, dTTP, dGTP and dCTP, 5 pmoles of each primer, 1.25 U Taq DNA polymerase (Roche Diagnostics NZ Ltd.) and 1 µl DNA extracted from the soil samples. The reactions were performed in an Eppendorf® Mastercycler® Gradient PCR machine and consisted of 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, with a final extension of 72°C for 7 mins. Two positive controls were prepared: 10 ng of *P. chlamydospora* DNA to ensure the reaction had occurred successfully and 10 ng of *P. chlamydospora* DNA and 1 µl DNA extracted from the soil samples to ensure the samples did not contain inhibitory compounds. The concentration of all DNA was determined by separation on 2% agarose gel electrophoresis and comparison with High Mass ladder under ultra violet light after staining in ethidium bromide. PCR products were separated by 1% agarose gel electrophoresis and visualised under ultra violet light following staining in ethidium bromide.

Nested species-specific PCR

A primary PCR was done on the previously described soil samples using the universal primers ITS-4 (5' TCC TCC GCT TAT TGA TAT GC3') and NS-1 (5' GTA GTC ATA TGC TTG TCT C3') (White et al. 1990). The reaction conditions were as described for the species specific PCR with the exception of the thermal cycle which consisted of 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C, with a final extension of 72°C for 7 mins. The resulting PCR product was diluted 1:200 in sterile water and 1 µl of this dilution was then amplified in a secondary PCR using the species specific PCR protocol outlined above. However, this reaction contained only 1.5 mM MgCl₂.

Confirmation of extraction of DNA from grapevine nursery soil

To ensure that DNA had been successfully extracted from the soil samples and that the PCR reaction was not being inhibited by compounds such as humic acid, the diluted product from the primary ITS-4/NS-1 PCR was also amplified using the internal universal primers ITS-2 (5' GCT GCG TTC TTC ATC GAT GC3') and ITS-5 (5' GGA AGT AAA AGT CGT AAC AAG G3') (White et al. 1990). Reaction conditions were as described for the primary ITS-4/NS1 PCR, but the reaction contained only 1.5 mM MgCl₂.

Determination of detection sensitivity in grapevine nursery soil

The sensitivity of both the non-nested and nested species-specific PCR was determined by adding 50 fg, 500 fg, 5 pg, 50 pg, 500 pg or 5 ng of *P. chlamydospora* DNA to three soil samples testing negative for the presence of *P. chlamydospora*. Samples were then amplified using the non-nested and nested species-specific PCR protocols previously described.

Detection of *P. chlamydospora* DNA in artificially infested soil

Subsamples (10 g) were taken from four of the previously described soil samples, heat sterilised (160°C for 2 h in a glass Petri dish) to denature any *P. chlamydospora* DNA, cooled and spore suspensions of *P. chlamydospora* were added to achieve final concentrations of 10^1 , 10^2 , 10^3 and 10^5 conidia/ml of soil. The culture used was originally isolated from a grapevine onto Malt Extract Agar (Life Technologies/GIBCO BRL) and grown for 21 days at 25°C in the dark. Blocks of this mycelium, which had been stored in water at -5°C, were inoculated into 100 ml of Potato Dextrose Broth (Life Technologies/GIBCO BRL) and incubated at room temperature for 4 days on an orbital shaker at 100 rpm. The resulting spore suspensions were harvested by centrifugation at 3220 x g for 2 min and stored at -80°C in 20% glycerol until use at which time viability was determined to be 100%. DNA extraction and amplification using the non-nested and the nested species-specific PCR protocols was done as previously described.

Identity confirmation with restriction enzyme digestion and sequence analysis

The identity of the products from the nested species-specific PCR for the sensitivity analysis and artificially infested soil was confirmed by digestion with the restriction enzymes *AatII* and *MluNI* (Roche New Zealand Ltd.) which differentiate between *P. chlamydospora* and other closely related fungi (H. Ridgway, pers. comm.). The digested PCR products were separated by 2.5% agarose gel electrophoresis as previously described. One PCR product from each of these groups was sequenced at the Waikato DNA Sequencing Facility (University of Waikato, New Zealand) and analysed via the Blastn function in GenBank (Anon. 2002).

RESULTS

DNA amplification in grapevine nursery soil

Successful DNA extraction from the uninoculated soil samples and the absence of PCR inhibitors was shown by amplification in all ten samples in the nested ITS-5/ITS-2 PCR (Fig. 1). *Phaeomoniella chlamydospora* was not present in any of the samples using both the non-nested and nested species-specific PCR (data not shown).

Determination of detection level of species-specific PCR in grapevine nursery soil

The detection level of the species-specific PCR was determined by adding known quantities of *P. chlamydospora* DNA to the PCR reaction. The detection level in the

FIGURE 1: PCR amplimers generated in a nested PCR using primers ITS-2 and ITS-5 on DNA extracted from grapevine nursery soil. Far left lane: 1 kb DNA ladder, positive control (+) and soil samples 1-10.

non-nested PCR was 500 pg (Fig. 2a) and it was increased 10 fold to 50 fg using a nested PCR protocol (Fig. 2b).

Detection of *P. chlamydospora* DNA in artificially infested soil

DNA of *P. chlamydospora* was amplified in one of the three subsamples with 10^2 conidia/ml added, two of the three subsamples with 10^3 conidia/ml added and all of the subsamples with 10^5 conidia/ml added using the nested PCR approach

FIGURE 2: Demonstration of sensitivity of primers Pch1 and Pch2 in (a) non-nested and (b) nested PCR. Soil samples previously tested negative for the presence of *P. chlamydospora* containing known quantities of *P. chlamydospora* genomic DNA (stated at the top of the gel). Far left lane: 1 kb DNA ladder, positive control (+), soil sample without DNA added (-) and six concentrations.

(Fig. 3). Faint amplimers were observed only in the 10^5 conidia/ml non-nested PCR approach (data not shown).

FIGURE 3: PCR amplimers generated in a nested PCR using primers Pch1 and Pch2 with DNA extracted from soil artificially infested with four concentrations of *P. chlamydospora* conidia (amounts listed at the top of the gel). Far left lane: 1 kb DNA ladder, positive control (+), positive control and soil DNA (++) and four concentrations.

Identification of PCR products

The products resulting from the restriction digests using enzymes *AatII* and *MluNI* corresponded in size to those produced by digested DNA from a known *P. chlamydospora* isolate. The sequences obtained from PCR products were 100% identical to one of the two sequence groups of *P. chlamydospora* (*Phaeoacremonium chlamydosporum*) in GenBank (accession numbers: AF197973, AF197987, AF266652, AF266653, AF266656).

DISCUSSION

The nested species-specific PCR assay described was used to successfully extract and detect *P. chlamydospora* DNA in soil artificially infested with 10^2 conidia/ml in one third of samples. This detection level is an improvement on previous reports of 10^4 conidia/ml and 10^5 conidia/ml (A. Eskalen, pers. comm.). When *P. chlamydospora* DNA was added directly to the PCR reaction a detection level of 50 fg was observed, a 10 fold increase on 5 pg as determined for *P. chlamydospora* DNA in grapevine wood (Ridgway et al. 2002). These two detection levels can be compared by considering that 50 fg is the amount of DNA found in 5 bacterial cells (data for *P. chlamydospora* does not exist) (Torsvik 1996) and the detection level of 10^2 conidia/ml is equivalent to 100 conidia in the 1 g soil sample tested. Hence there is a 20 fold difference between the two detection levels. The real detection level probably lies somewhere between these two values. This lack of certainty about the detection level and the variability in results

between replicates for the artificially infested soil is probably due to the inherent problems with producing spore suspensions, particularly due to inaccuracies in haemocytometer counts and serial dilutions. Also, at the lower concentrations achieving even distribution of the few spores added to the soil is difficult. For these reasons any diagnostic service utilising this assay would probably have to be based on a detection level of 10^3 conidia/ml.

The site from which the soil samples were collected was chosen as it had no history of grapevine production but since it was to be used as a nursery site it was representative of the type of soil in which the assay will be used. While detection of *P. chlamydospora* DNA was observed in artificially inoculated soil, heat sterilisation may have altered the chemical and physical composition of the soil. Hence, it was necessary to successfully extract DNA and determine the detection sensitivity in untreated soil to demonstrate that the assay will be reliable under field conditions.

The results of this work have highlighted the advantages of using a species-specific PCR assay for detecting plant pathogens in soil. Compared to traditional agar plate isolation methods the PCR assay is much faster as results are obtained within 2–3 days compared to 2–3 weeks. Researchers have shown that the primers Pch1 and Pch2 do not amplify DNA from fungi closely related to *P. chlamydospora*, the grapevine (Tegli et al. 2000) or commonly occurring microflora of New Zealand grapevine wood (Ridgway et al. 2002). Traditional methods of isolation are prone to false negatives because contamination by other micro-organisms masks the presence of *P. chlamydospora*. The high specificity of the PCR method avoids these problems. However, a highly specific detection system could potentially have limited applicability if the existing population has a large amount of genetic diversity. This is unlikely to be the case in New Zealand populations of *P. chlamydospora* as a recent study revealed a very low level of diversity among isolates (Pottinger et al. 2001). The main disadvantage of most PCR based assays is that the primers amplify ribosomal DNA from both viable and nonviable fungal propagules. Finally, for Petri disease the correlation between the inoculum levels detected by PCR and the inoculum threshold for infection is unknown.

The nested species-specific PCR assay described in this work represents a highly sensitive, highly specific means of detecting *P. chlamydospora* in soil. It should now be possible to determine if this medium is a potential source of inoculum, potentially valuable information for the development of a disease management strategy.

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