A protocol for molecular detection of *Phaeomoniella chlamydospora* in grapevine wood

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PETRI DISEASE IS A SERIOUS DECLINE AND dieback disease of young grapevines. The principal causal organism, *Phaeomoniella chlamydospora,* is distributed mainly by infected propagation material. Pathogen detection and accurate diagnosis are currently based on fungal isolation in artificial growth media. The fungus is extremely slow-growing, however, and cultures are often overgrown by co-isolated fungi before it can be identified. To avoid this problem, we have developed and validated an efficient and cost-effective protocol for the molecular detection of *Pa. chlamydospora* in grapevine wood. This novel molecular technique, using a species-specific PCR, detected as little as 1 pg of genomic *Pa. chlamydospora* DNA. The protocol was validated with grafted grapevines from different nurseries, including grapevines that were first treated with hot water. The basal end of the rootstock was analysed for *Pa. chlamydospora* by means of both isolations in artificial medium and molecular detection. The latter was found to be considerably more sensitive than isolations, and detected *Pa. chlamydospora* in samples that recorded both positive and negative in isolations. The identity of PCR products obtained from a subset of samples that tested positive only for *Pa. chlamydospora,* based on molecular detection, was confirmed to be *Pa. chlamydospora*-specific through restriction digestion with *AatII*. The pathogen was not isolated from samples treated in hot water. However, as expected, *Pa. chlamydospora* DNA was detected in samples exposed to hot water at rates similar to those detected in material not immersed in hot water.

Background

Several trunk diseases can lead to a decline in the productivity and health of grapevines. Decline and premature dieback are responsible for considerable economic losses and cause great concern in most grapevine-growing countries. In South Africa, decline and dieback of young grapevines are most frequently attributed to Petri grapevine decline, or black goo as it was previously known.[1] The organism mainly responsible is the fungus *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams.[2,3] The disease commonly occurs in grapevines of 1–5 years of age.[1] As the plant matures, the fungus predisposes the wood to infection by various wood-rotting fungi, such as *Fomitiporia punctata* (Fr.) Murrill, and can lead to the development of esca disease.[4]

Symptoms are generally not visible in one-year-old wood.[1] Stress conditions induced by severe pruning, drought, poor drainage, nutritional deficiencies and soil compaction are prerequisites for symptom expression.[5,6] Typical symptoms of Petri disease include stunted growth, short internodes, small leaves, small trunks and branches and a general decline of young vines, resulting in plant death.[5,7–11] Internal wood symptoms include vascular streaking and blockage of the xylem vessels. The fungus is present in apparently healthy propagation material in a latent or endophytic form. Infected rootstock propagation material is therefore considered to be a major inoculum source.[8,12] It is suspected that cuttings might be infected from diseased mother plants.[2,4,13] However, Fourie and Halleen[14] investigated isolations from the basal ends of rootstocks and found the incidence of *Pa. chlamydospora* to be extremely low. Isolation of *Pa. chlamydospora* in artificial growth media is problematic, since the fungus is extremely slow growing (it needs up to 4 weeks from isolation to identification) and its cultures are often overgrown by co-isolated fungi before it can be identified and so may lead to false negative results. A more sensitive detection technique is therefore needed.

Molecular detection by means of DNA extraction and species-specific polymerase chain reaction (PCR) offers a fast alternative, which avoids the above problems.[15] Protocols and methods for extraction and amplification of fungal DNA from pure cultures have been developed[16] and adapted for detection of *Pa. chlamydospora* from inoculated tissue culture plants.[17] However, because of the presence of PCR inhibitors, these methods are not suitable for the detection of DNA from lignified wood. Ridgway *et al.*[15] therefore devised an extraction protocol and species-specific PCR method to detect this pathogen in grapevine wood. Their technique requires the use of a CTAB buffer and the DNeasy Plant Mini kit (Qiagen, Germany)[18] for purification. Subsequently, a PCR procedure is performed using the species-specific primers Pch1 and Pch2.[15] This PCR was found to be very sensitive, detecting <1 pg of *Pa. chlamydospora* genomic DNA. Although very time-efficient, the purification kit is nevertheless very expensive for large-scale sampling experiments.

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Hot water treatment at 50°C for 30 min has been shown to be effective in eliminating or reducing pests and pathogens such as nematodes, phylloxera and Pierce's disease.[19] *Phytophthora cinnamomi* was also effectively controlled by subjecting grapevine cuttings to the hot water treatment.[20] Previous studies indicated that exposure to hot water is effective in eliminating certain fungal pathogens and endophytes from grapevine tissue.[21] Varying results were achieved with hot water treatments to eliminate *Pa. chlamydospora*. Whiting *et al.*[22] and Rooney and Gubler[23] reported that hot water was not effective in controlling *Pa. chlamydospora*. By contrast, Fourie and Halleen[24] observed a drastic reduction in the levels of *Pa. chlamydospora* after hot water exposure of naturally infected rootstock cuttings or uprooted nursery grapevines. Moreover, subsequent colonization of treated rootstocks was also inhibited.[24]

The aim of the study reported here was to develop and validate a rapid and cheap DNA extraction protocol and a sensitive species-specific PCR for the detection of *Pa. chlamydospora* in symptomatic or asymptomatic grapevine wood. Molecular detection of the pathogen following hot water treatment of propagation material was also evaluated.

Materials and methods

Grapevine material. Potted grapevines that were naturally infected with the organism mainly responsible for Petri disease (this was proved previously by isolation of the fungus; results not shown) were collected from ARC Infruitec-Nietvoorbij, Stellenbosch. The material was prepared for DNA isolation by removing the rooted basal end and cutting the rootstock into 2 cm pieces, as well as a small part of the scion (up to 6 cm above the graft union). The bark was removed with a potato-peeler and discarded. The woody pieces were surface sterilized by submerging the material in 70% ethanol for 30 s, followed by 60 s in 3.5% sodium hypochlorite and 30 s in 70% ethanol. Surface-sterilized wood pieces were stored at 4°C.

DNA extraction. Previously published extraction protocols[15–17,25] were modified and combined as five different extraction procedures, which were subsequently tested on grapevine wood. DNA extracted by four of these protocols was successfully used to amplify DNA from *Pa. chlamydospora* cultures, but not from wood samples (results not shown). However, a newly developed extraction protocol, based mostly on the methods of Lee and

Taylor[16] and Ridgway *et al.*,[15] showed promising results and was subsequently used. This procedure involved snap-freezing with liquid nitrogen and grinding the frozen sample to a powder by means of a pestle and mortar. Powdered wood sample (0.5 g) was combined with 550 μl CTAB extraction buffer (2% CTAB, 200 mM Tris, pH 7.5; 1.4 M NaCl; 20 mM EDTA, pH 8.0) in a 2-ml Eppendorf tube and incubated at 65°C for 1 h. Thereafter, 400 μl chloroform:isoamylalcohol (24:1) was added, followed by centrifugation at 1300 × g for 15 min. The watery supernatant was transferred to a new Eppendorf tube, 50 μl 7.5 M ammonium acetate solution (pH 7.0) and 600 μl cold isopropanol were added and the samples were incubated at –20°C. After 1 h, the samples were centrifuged at 15 800 × g for 10 min. The supernatant was discarded and 1 ml cold 70% ethanol was added before incubation at –20°C for 30 min. After incubation, the samples were centrifuged at 15 800 × g for 5 min and the supernatant discarded. The DNA pellet was dried on a bench, dissolved in 100 μl sterile double-distilled H_2O and stored at 4°C.

PCR amplification. PCR was performed using the *Pa. chlamydospora*-specific primers Pch1 and Pch2, which were developed by Tegli *et al.*[26] The reactions (total volume 25 μl) were performed using 0.65 units Biotaq polymerase (Bioline, London), 0.2 mM each of dATP, dTTP, dGTP and dCTP, 3 mM $MgCl_2$, 5 pmol of each primer, 1× enzyme buffer, 1 mg/ml bovine serum albumin (BSA) and 5 μl DNA solution. The amplification was performed on a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, California). The cycling conditions were: 5 min at 96°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, 1 min and 30 s at 72°C and a 7-min extension step at 72°C to complete the reaction. DNA was isolated from a pure *Pa. chlamydospora* culture, using the method of Lee and Taylor,[16] and a 1:1000 dilution (yielding a final DNA amount of 1.4 pg) was used as a positive control in PCR reactions. Five microlitres of each PCR product was separated by electrophoresis at 80 V on a 1% agarose gel, stained with 0.5 μg/ml ethidium bromide, in a 0.5 × TAE buffer (0.04 M Tris, 0.02 M glacial acetic acid and 1.27 mM EDTA, pH 7.85) and visualized under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, U.K.). A negative control reaction (no template DNA) was also included during PCR preparation and amplification to ensure absence of

contamination.

To determine the sensitivity of the PCR reaction, under conditions that are closer to those when performing a diagnostic test with woody material, known quantities of *Pa. chlamydospora* DNA were added to a DNA solution, which consisted of extracts from wood that had tested negative with the species-specific primers (Pch1 and Pch2). The different concentrations of the purified DNA were determined with a fluorometer, FL600™ (Bio-Tek, http://www.biotek.com), and added to the DNA wood extract to achieve final concentrations of 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 100 fg/μl, 10 fg/μl and 1 fg/μl. Five microlitres from each of these spiked DNA extracts was used in PCR reactions and analysed with gel electrophoresis as described previously.

Validation: To validate the protocol, one-year-old nursery-grown grapevines from three different rootstock cultivars collected from each of three different nurseries were randomly sampled after uprooting. The different rootstocks used were 101–14 Mgt, Ramsey and Richter 99 (18 grapevines per batch). The occurrence of *Pa. chlamydospora* in the basal end of each rootstock (1–3 cm from basal end) was determined according to the isolation methods used by Fourie and Halleen.[24] The rest of each 2 cm piece was subsequently used for DNA extraction and PCR amplification. Detection percentages (number of positive samples as a percentage of the total number of samples tested) were calculated and the data subjected to analysis of variance using SAS (SAS Institute Inc., Cary, North Carolina).

Hot water treatment. Forty-eight grapevines of Chardonnay/101–14 Mgt were sampled from a nursery after uprooting. The plants were separated into six groups of eight grapevines each. Three of these batches were treated with hot water (drenched in water at 50°C for 30 min) and three untreated bunches were used as the control. The presence of *Pa. chlamydospora* in the hot water treated and untreated plants was determined by means of isolations and molecular detection as described previously.

Identification of putative Pa. chlamydospora *PCR products obtained from wood.* Enzyme restriction digestion was used to determine whether all 360-bp PCR amplicons obtained from the wood samples were indeed *Pa. chlamydospora*. A subsample was randomly selected from the samples that tested positive with molecular detection, but negative with isolations. The PCR amplicons were cut from agarose gels and purified using a QIA-

quick® Gel Extraction kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Purified PCR amplicons were restriction enzyme digested with *AatII* (Roche Diagnostics South Africa, Randburg), since this enzyme differentiates between *Pa. chlamydospora* and other closely related fungi.[27] Restriction digestion was conducted according to the manufacturer's instructions (Roche Diagnostics). The digested PCR products were separated by 1.5% agarose gel electrophoresis as previously described.

Results

DNA extraction and PCR amplification. Several putative *Pa. chlamydospora* PCR products (360 bp) were obtained following amplification with *Pa. chlamydospora* primers (Pch1 and Pch2), using DNA extracted using the protocol modified from Lee and Taylor[16] and Ridgway *et al.*[15] (Fig. 1). The PCR technique was sensitive enough to detect 1 pg of *Pa. chlamydospora* genomic DNA (Fig. 2).

Validation. Analysis of variance of the detection percentages revealed a significant difference between molecular detection and isolation ($P = 0.0003$). On average, the molecular technique detected *Pa. chlamydospora* in 81% of the samples, whereas only 24% of the samples tested positive for *Pa. chlamydospora* by means of isolations. Isolations that were regarded as negative did not have any *Pa. chlamydospora* growth after 2 weeks' incubation or were overgrown by other fungi and bacteria within a few days. Depending on the incidence of *Pa. chlamydospora* in different batches, molecular detection was found to be substantially more sensitive than isolations (Fig. 3). DNA of *Pa. chlamydospora* was detected in 100% of the samples that tested positive with isolation.

Hot water treatment. Analysis of variance of detection percentages revealed a significant interaction ($P < 0.0001$) between treatment (hot water treated and untreated) and detection technique (molecular detection and isolations). This can largely be ascribed to the 100% identification of *Pa. chlamydospora* in hot water-treated samples using molecular detection, whereas none of the samples drenched in hot water tested positive with isolations. In the untreated samples, *Pa. chlamydospora* was detected by means of PCR in 100% of the samples, and 92% by means of isolation (data not shown).

Identification of putative Pa. chlamydospora *PCR products obtained from wood.* Restriction digestion of 360-bp PCR products, amplified with primers Pch1 and Pch2 from wood, with *AatII* yielded
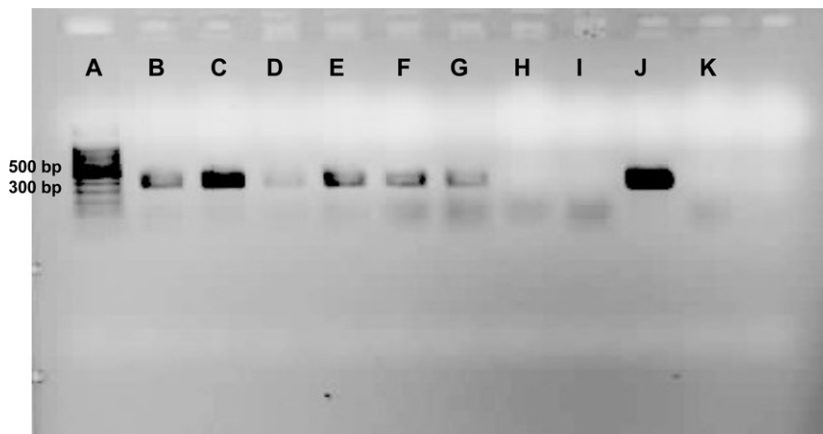

Fig. 1. Detection of *Phaeomoniella chlamydospora* in different parts of the grafting material. A, 100-bp DNA ladder; B, 3–5 cm; C, 5–7 cm; D, 9–11; E, 11–13 cm and G, 1–3 cm from the basal end of the rootstock; F, 2–4 cm; H, 2–4 cm and I, 0–2 cm from the grafting union. J, positive control; K, negative control.
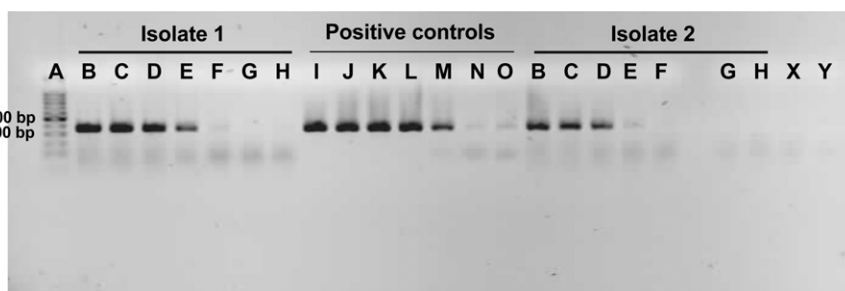

Fig. 2. Determination of the sensitivity of the species-specific PCR with primers Pch1 and Pch2. A, 100-bp DNA ladder; B–H, DNA extracted from grapevine woods that tested negative for *Phaeomoniella chlamydospora* and were spiked with known quantities of *Pa. chlamydospora* genomic DNA (isolates 1 + 2) (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg). I–O, Five positive controls of *Pa. chlamydospora* genomic DNA, suspended in water at different concentrations (1 ng–1 fg). X, Y, Two negative controls: DNA extracted from grapevine wood that tested negative for *Pa. chlamydospora* (X) and water (Y).

products of 127 bp and 233 bp (Fig. 4). These products corresponded in size to those produced by digested genomic DNA from *Pa. chlamydospora*.

Discussion

The detection protocol described above has proved to be robust, fast and highly sensitive. The preparation of the grapevine material may play a significant part in the success of the DNA extraction, especially snap-freezing the samples in liquid nitrogen. Freezing it enabled the grapevine wood to be ground to a powder, which was important for releasing fungal DNA from woody tissue. This also breaks the fungal cell walls, facilitating release of cellular constituents. This molecular technique permits *Pa. chlamydospora* to be detected in a grapevine sample in less than 1 day, whereas diagnosis by means of isolations takes up to 4 weeks. Furthermore, a very low percentage of *Pa. chlamydospora* was detected in some of the rootstocks with isolations; this was due to the cultures being severely overgrown
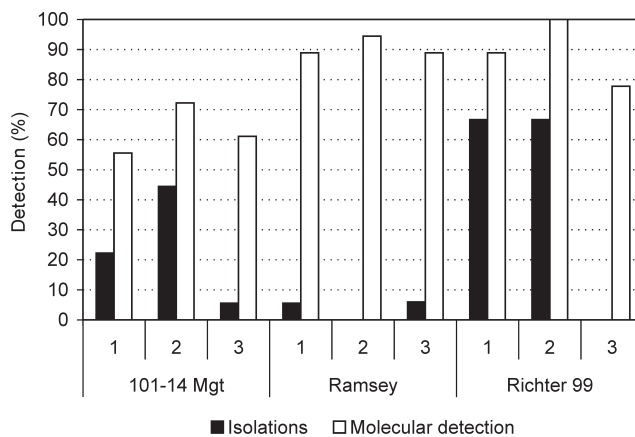

Fig. 3. Detection percentages of *Phaeomoniella chlamydospora* in 101–14 Mgt, Ramsey and Richter 99 rootstocks of grapevines from different nurseries (1, 2, 3) by means of isolations and molecular detection.
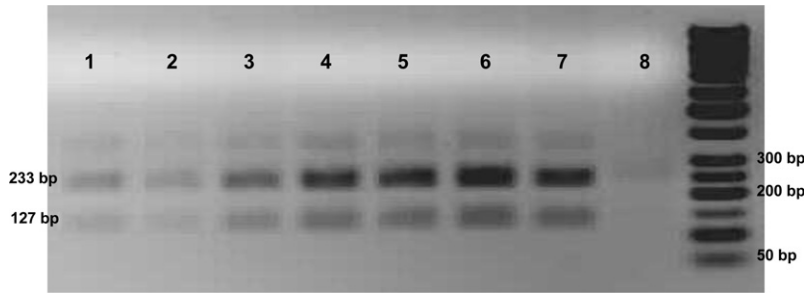
Fig. 4. DNA products resulting from restriction digestion of 360-bp PCR amplicons with *AatII*. The 360-bp PCR amplicons were PCR amplified from wood DNA with *Phaeomoniella chlamydospora* species-specific primers (Pch1 & Pch2). Far right: 50-bp ladder (Fermentas GmbH, St Leon-Rot, Germany); 1–6, wood samples; 7, positive control; 8, negative control.

with bacteria, which led to false negative results.

Previous studies have shown that *Pa. chlamydospora* occurs in rootstock cuttings, from mother plants, at very low incidences.[9,28] By contrast, molecular detection revealed a very high presence of *Pa. chlamydospora* in most of the rootstock combinations, which indicated that the infection percentage increased while the grafted vines were growing in the field. Fourie and Halleen[24] confirmed these results. This might indicate that *Pa. chlamydospora* is introduced into grafted vines during some nursery stages or that infection of young vines occurs in the field, with disease expression being induced only under stress conditions.

The molecular technique can detect as little as 1 pg of *Pa. chlamydospora* genomic DNA. This PCR approach makes the detection protocol faster and more cost-effective than the previously published protocol[15] for DNA extraction and amplification from wood. Initial attempts to use nested-PCR resulted in many false-positive samples, due to cross-contamination of samples with PCR products during the second round of amplification (unpubl. data). Also important, this extraction protocol was found to be 10–15 times cheaper than the use of commercial DNA extraction kits.

Pa. chlamydospora was not detected in the uprooted grapevines that were drenched in hot water using conventional isolation techniques. These results correspond to findings in previous studies,[24] and confirm the importance of hot water treatment for proactive management of Petri disease in grapevine nurseries.[24] However, by contrast, the molecular technique detected the pathogen in all of the experimental samples. These contradictory results are most likely due to the inability of molecular detection to distinguish between dead and viable pathogen tissue. Future molecular studies should be aimed at investigating the effect of exposure to hot water at RNA level,

after varying intervals. In theory, transcripts of RNA will have only a relatively short life span following pathogen death. The detection of pathogen RNA would therefore indicate the presence of viable pathogen propagules only.[29] However, the stability of RNA may depend on the transcript type. The stability of the targeted RNA transcript should therefore first be investigated in cultures grown *in vitro*.

The protocol for DNA extraction and subsequent pathogen detection using a single-step PCR on hot water treated and untreated grapevine samples was found to be robust, rapid, highly sensitive and cost-effective. This approach will be used for the molecular detection of *Pa. chlamydospora* to identify potential inoculum sources and critical infection stages during the various steps in vine propagation. Knowledge of the disease cycle is essential for devising and evaluating strategies for disease management.

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