

A cost-effective protocol for molecular detection of fungal pathogens in soil

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DNA EXTRACTION FROM FUNGI IN SOIL often fails because of humic substances that are co-extracted with the DNA and subsequently inhibit PCR analyses. Moreover, it is difficult to release the fungal DNA because of the diverse fungal structures residing in soil. Since available DNA extraction protocols and commercial kits are expensive or time-consuming, we have devised a superior method by testing different components of these procedures on grapevine nursery soils. The best DNA yield and sensitivity were obtained by a short and easy extraction method with sodium dodecyl sulphate buffer using the FastPrep homogenizer. An easy-to-prepare spin column with polyvinylpyrrolidone was developed to remove PCR inhibitors. In the presence of bovine serum albumin, PCR reactions were possible without further dilutions of the DNA. Our method was more sensitive for detecting *Phaeo-monniella chlamydospora*, the organism responsible for Petri grapevine decline, and *Cylindrocarpon* black-foot pathogens in grapevine nursery soils than the FastDNA SPIN Kit for Soil and enabled us to perform 25 extractions for the price of one with the kit. This is the first report of molecular detection of *Cylindrocarpon macrodidymum* from soil and the first account of *Pa. chlamydospora* from soils in South Africa.

Introduction

There is a growing demand for molecular methods to detect pathogens in plant disease diagnostics, which are supposed to overcome drawbacks of culturing methods. For example, a slow-growing fungus like *Phaeo-monniella chlamydospora*, the organism responsible for Petri grapevine decline, might often not be recorded because it is overgrown by other, co-isolated fungi on artificial medium. Furthermore, morphological identification of this pathogen is possible only after at least 2 weeks' incubation.¹ Molecular analyses are much less time-consuming and results are generally available after 1 or 2 days.

Access to fungal DNA in soil is especially difficult because fungal structures are closely connected with soil particles and plant debris and often occur as thick-walled chlamydospores or sclerotia. Several methods have been attempted to increase DNA yield from soil. Grinding

soil was used by Edgcomb *et al.*² Zhou *et al.*³ preferred 2–3 hours' heating at 65°C, whereas Tsai and Olson⁴ applied a freeze-thaw method. Miller *et al.*⁵ found bead mill homogenization to be more effective than freeze-thawing. The choice of the best extraction buffer is also important for DNA extraction from materials like soil. Sodium dodecyl sulphate (SDS) buffer combined with chloroform or phenol was shown to be more effective than Chelex 100 or guanadinium isothiocyanate.⁵

Hexadecyltrimethyl ammonium bromide (CTAB) buffer proved to be effective for detecting *Pa. chlamydospora* DNA from grapevine wood.⁶ SDS and CTAB were in some cases combined for DNA extraction from soil.^{2,3}

Humic substances, like humic and fulvic acids, are co-extracted with DNA. Humic acids inhibit DNA-transforming enzymes, especially during amplification by *Taq* polymerase.⁷ These substances must therefore be removed prior to analysis using the polymerase chain reaction (PCR). DNA is often separated from humic substances by using expensive Sephadex or Sepharose columns.⁸ During this column purification stage, 100- μ l aliquots are loaded onto columns and the cleaned DNA is sampled in fractions. Alternatively, polyvinylpyrrolidone (PVPP) can be used in the DNA extraction buffer^{3,9} or in a column format.^{10,11} PVPP is an inexpensive substance that can be employed for removing humic and fulvic acids, since it binds phenolic compounds. Other techniques for separating DNA from inhibitors are agarose gel purification^{3,12} and caesium chloride density gradient centrifugation.¹³ Commercial extraction kits designed for soil are available^{14–16} but are generally expensive. The FastDNA SPIN Kit for Soil from Q-BIO-gene (Carlsbad, California) was the most effective in a comparison of different soil extraction methods and commercial extraction kits.¹⁷

The application of special polymerases and the addition of PCR enhancers are other possibilities for reducing the inhibiting effect of humic substances in PCR analyses. For example, the susceptibility of *Taq* polymerase to humic acids decreased

in the presence of T4 gene 32 protein.¹² Bovine serum albumin (BSA) was found to be essential for detecting *Verticillium dahliae* and enhanced detection of *Pythium ultimum* in soil.¹⁸ Another way to reduce the concentration of inhibitors in the PCR mix could be dilution of the template or application of a smaller amount of template; this would, however, lead to a lower sensitivity of detection.

The aim of the project reported here was to develop a simple, cost-effective and sensitive method to extract fungal DNA from soil and to optimize the conditions of the subsequent PCR to detect pathogens in soil samples. The evaluation of this method was based on the various published techniques described above. It focused on the separation of fungal cells and DNA from the soil matrix and the removal of humic substances from DNA because these steps are critical in DNA extraction from soil.

Materials and methods

Soils. Twenty-seven grapevine nursery soils (S1, S2, W1...W14, P1...P11) and one vineyard soil (S0) from different regions of the Western Cape province were sampled during 2003 and were used to test and validate the methods for extracting DNA from soils.

Preparation of PVPP columns. An opening (1 mm diameter) was made in the bottom of a 0.5-ml tube and was covered by glass wool. Thereafter, the lid of the tube was removed and the tube inserted into a 2-ml tube. A mixture of PVPP and high salt TE buffer (0.1 g/ml, buffer: 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8) was transferred to the small tube and spun down for 1 min at 720 \times g. This was repeated until the tube remained full after centrifugation. The column was spun dry before use. The optimum time and centrifugation speed for final column preparation and sample elution was determined by testing all combinations of 2, 5 and 10 min and 320, 720 and 1300 \times g, respectively. The effectiveness of the separation was determined by running the DNA on gel, ITS PCR and subsequent electrophoresis and visual comparison of the colour of columns and final DNA extract.

Development of the DNA extraction method. After several preliminary examinations that included grinding with liquid nitrogen and freeze-thawing (results not shown), four methods were chosen and tested with six of the soils (S0, S1, W4, W8, P1, P6) in three replications.

Methods 1 and 2 (SDS based). Soil (0.5 g)

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was placed in a 2-ml screw-cap tube (or in a normal 2-ml tube for method 2) together with 0.5 g acid-washed sand and 0.5 g glass beads (2 mm diameter). Equal amounts (300 μ l) of phosphate buffer (100 mM NaH_2PO_4 , pH 8.0) and SDS extraction buffer (100 mM NaCl, 500 mM Tris, pH 8.0, 10% SDS) were added and the tube was subsequently inverted. After addition of 400 μ l chloroform:isoamylalcohol (24:1), the tube was shaken in a FastPrep instrument (FP 120, Bio101, Savant, Farmingdale, New York) for 40 s at 4.5 m/s (method 1) or horizontally on a vortex shaker (Vortex-Genie 2, G560E, Scientific Industries, Leicestershire, U.K.) for 10 min at maximum speed (method 2). The soil matrix was spun down for 5 min at 16 000 \times g and the top supernatant phase transferred to a new centrifuge tube. Thereafter, cold ammonium acetate solution (final concentration 2.5 M) was added, the tube inverted and centrifuged for 5 min at 16 000 \times g. Supernatant was transferred to a new centrifuge tube and 600 μ l cold isopropanol added. After 15 min incubation at room temperature, the precipitate was spun down for 5 min at 16 000 \times g and the supernatant discarded. Cold 70% ethanol (1 ml) was added to the pellet, spun down for 5 min at 16 000 \times g and the supernatant discarded again. The DNA pellet was dried and resuspended in 120 μ l double-distilled H_2O . The DNA solution was loaded on a prepared PVPP column, incubated for 5 min and spun through the column.

Methods 3 and 4 (CTAB based). Methods 3 and 4 were essentially the same as methods 1 and 2, except that SDS extraction buffer and phosphate buffer were replaced by 600 μ l CTAB extraction buffer (0.2 M Tris, 1.4 M NaCl, 20 mM EDTA, 0.2 g/l CTAB), respectively. Before adding the chloroform: isoamylalcohol, the tubes were placed in a 65°C water bath for 15 min to reduce the foam produced by shaking the CTAB buffer in the FastPrep instrument or vortex. The ammonium acetate and isopropanol steps were combined.

PCR amplification. Suitability for detecting fungal DNA was tested by performing PCR analyses with the general and fungal ITS primers, ITS1 and ITS4,¹⁹ ITS1F and ITS4,²⁰ as well as primers Pch1 and Pch2, specific for *Pa. chlamydospora*²¹ (yielding a 360-bp band) and Dest1 and Dest4,²² which are specific for the grapevine black-foot pathogens, *C. destructans* and *C. obtusisporum*²³ (yielding a 400-bp band). DNA from *Pa. chlamydospora* (STE-U 5783, culture collection of Department of Plant Pathology, Stellenbosch University) and

Cylindrocarpon destructans (STE-U 3673) from grapevine were used as positive controls in the PCR reactions. The PCR reaction mixture consisted of 0.65 units of *Taq* polymerase (Biotaq, Bioline, London), 1 \times NH_4 reaction buffer (Bioline), 3.0 mM MgCl_2 , 0.2 mM of each dNTP, 0.2 μ M of each primer and 5 μ l DNA solution in a total volume of 25 μ l. T4 gene 32 protein (Roche, Mannheim) (0.6 μ g/reaction),¹² BSA (25 μ g/reaction) and dilutions (1:10, 1:100) of the DNA extracts were tested to improve PCR amplification in a combined experiment with the optimization of the PVPP spin columns. BSA solution for molecular biology (Roche) was compared to a solution prepared from BSA Fraction V (Roche) at the same concentration. PCR was initiated with a denaturation step of 5 min at 96°C, followed by 30 or 40 cycles of 30 s at 94°C, 30 s at annealing temperature [50°C (Pch1 + Pch2), 55°C (ITS1 + ITS4, ITS1F + ITS4) and 60°C (Dest1 + Dest4)] and 1 min 30 s at 72°C, and a final primer extension of 7 min at 72°C. PCR products were analysed by gel electrophoresis for 1 h at 95 V in 1% agarose gel stained with 0.5 μ g/ml ethidium bromide in TAE buffer (40 mM Tris, 1.27 mM EDTA, 20 mM glacial acetic acid) and visualized under UV light using a Gene-System Gel Documentation and Analysis System (Syngene, Cambridge, U.K.).

In order to determine the identity of the putative *Pa. chlamydospora* and *C. destructans/obtusisporum* bands (360 and 400 bp, respectively) obtained with species-specific PCR, a selection of the PCR products was prepared for sequence identification. These products were cut from the gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin). The sequence reaction was carried out (as recommended by the manufacturer) with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and the forward primers (Pch1 and Dest1, respectively) under the following temperature regime: 5 min at 94°C, followed by 25 cycles of 10 s at 96°C, 10 s at 50°C or 60°C (annealing temperatures) and 4 min at 60°C, and a final incubation of 4 min at 60°C. The resulting fragments were analysed on an ABI 3100 Genetic Analyser (AppliedBiosystems, Lincoln, California) and the sequences compared with those of the positive controls of *Pa. chlamydospora* and *C. destructans* as well as other *Cylindrocarpon* and *Cylindrocarpon*-like species from grapevine.²⁴

Validation. The best DNA extraction method was validated with 28 soils and compared to the FastDNA SPIN Kit for Soil from Q-BIOgene. The DNA concen-

tration from all samples extracted with both methods was determined by comparing the fluorescence intensities of DNA bands on ethidium bromide-stained agarose gel with those of standards. DNA yield data were compared with a paired *t*-test and a Pearson's correlation procedure in order to compare robustness and consistency. The efficiency of detecting fungal pathogens was tested by conducting general and specific PCR analyses with all 28 soil samples. The comparison of methods also included calculation of the costs per sample. The costs of the four soil DNA extraction methods tested, the extraction kit, and of the PCR variants were calculated according to current prices in South Africa. Equipment (FastPrep instrument, etc.) and labour costs were not included.

Results and discussion

DNA extractions. All four methods tested allowed the extraction of DNA and detection of fungal DNA from soil (Fig. 1). There were no marked differences in the bands observed when using SDS or CTAB extraction buffer. However, the DNA yield with SDS buffer was often higher and PCR results were more consistent. Furthermore, the method did not require a heating step, which made it more time-efficient than CTAB extraction. The crude DNA extract was also visibly lighter than with CTAB buffer, which indicated a lower humic acid concentration. The best DNA yield and sensitivity were obtained with SDS buffer using the FastPrep instrument (method 1). SDS and simple vortex (method 2), instead of FastPrep, was about 10 times less sensitive in PCR analyses with ITS primers (Fig. 1b, c).

Removal of PCR inhibitors. PCR amplification was not observed with crude DNA extracts that showed a light yellow to dark brown colour (Fig. 2a). In order to remove PCR-inhibiting soil components, the PVPP spin columns were developed (Fig. 2b,d). DNA extracts of most samples were completely colourless (Fig. 2c) after centrifugation through these columns for 5 min at 720 \times g. Subsequent PCR amplification (using the optimized PCR protocol) was generally possible without further dilutions of the DNA extracts (Figs 1b,c, 3c, 4). Longer time or higher speed of centrifugation through the PVPP spin column sometimes resulted in extracts that were yellow in colour and could not be amplified. A lower spin time and speed resulted in a reduced DNA yield (results not shown).

PCR analyses. In spite of the special cleaning step through the spin columns,

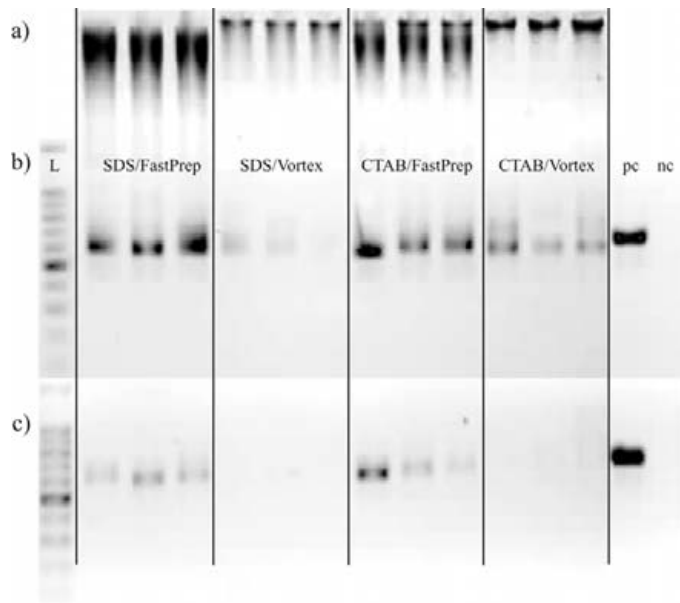


Fig. 1. Gel documentation of (a) DNA yield, (b) PCR products with primers ITS1F + ITS4, template dilution 1:100 and (c) 1:1000 of a grapevine nursery soil (sample W8) with four different extraction methods (three repetitions each). L, 100-bp ladder; pc, positive control; nc, negative control.

PCR results were still inconsistent. PCR amplification of the undiluted soil DNA with T4 gene 32 protein and a 1:100 dilution of the DNA without PCR enhancer were possible but only when centrifugation time or speed for the PVPP spin column was reduced, which generally decreased the DNA yield. However, PCR reactions with undiluted DNA in the presence of BSA showed bands with ITS primers even with higher centrifugation time and speed. Self-prepared solutions from BSA fraction V showed the same efficacy as the ready-to-use BSA solution

for molecular biology (results not shown). The cost of the PCR reaction was reduced by almost one-third when using the solution from BSA fraction V. By using the PVPP spin columns and BSA, humic acids were sufficiently removed and their inhibiting effect suppressed, respectively. Undiluted DNA (5 μ l) added directly to the PCR reaction (40 cycles) can therefore be used, resulting in a robust, sensitive and cheap procedure for the molecular detection of fungal DNA in soil.

Validation of SDS/FastPrep method. Since the best DNA extraction technique was

method 1, consisting of the use of SDS buffer along with the FastPrep instrument (SDS/FastPrep method), this was used for extracting DNA from 28 soils. DNA extracted with the SDS/FastPrep method was visible on agarose gel for all 28 soil samples (Fig. 3a). Average total DNA yield was $3.22 \pm 1.53 \mu\text{g/g}$ soil. PCR reactions with general fungal ITS primers were successful with 5 μ l undiluted DNA for all samples (Fig. 3c). By optimizing PCR reactions with 40 cycles and using specific primers for *Pa. chlamydospora* and *C. destructans/obtusisporum*, DNA was detected in grapevine nursery and vineyard soils (Fig. 4). These results were confirmed by sequencing some of the PCR products. Many of the soils (20 of the 28 samples) yielded 360-bp bands, indicating the presence of *Pa. chlamydospora*. However, not all of the positive results could be confirmed by sequencing, because some bands were often too faint to allow purification. The fungus was also detected in vineyard soil in New Zealand by molecular methods,²⁵ but not in Californian grapevine soils.¹ While *Pa. chlamydospora*, the causal organism of Petri grapevine decline, has been isolated from symptomatic vines,²⁶ roots and rootstocks of nursery grapevines²⁷ and from canes of rootstock mother plants²⁸ in South Africa, this is the first report of its occurrence in soils in South Africa. Almost two-thirds of the 28 soil samples yielded 400-bp bands, indicating the possible presence of *C. destructans* and/or *C. obtusisporum*, causal organisms of black-foot in grapevines. Only a few (3) of these results could be confirmed by sequencing. However, the sequences perfectly matched that of a newly described *Cylindrocarpon* species from grapevine, *C. macrodidymum*,²⁴ providing further evidence that the Dest1 and Dest4 primers are not species-specific²³ as reported by Hamelin.²² Moreover, the sequences of 10 bands could not be determined, possibly due to the presence of more than one species that were detected by this primer pair. Detection of *C. destructans*, *C. obtusisporum* and/or *C. macrodidymum* from the same soil would have resulted in an overlay of sequences and would have made sequence analysis impossible. *C. destructans*, *C. obtusisporum* and *C. macrodidymum* have been reported as pathogens of grapevines^{24,29,30} but their distribution in grapevine nursery and vineyard soils has not been studied. This is the first report of *C. macrodidymum* in soil.

Comparison with an extraction kit. DNA was successfully extracted from all soil samples by using the FastDNA kit

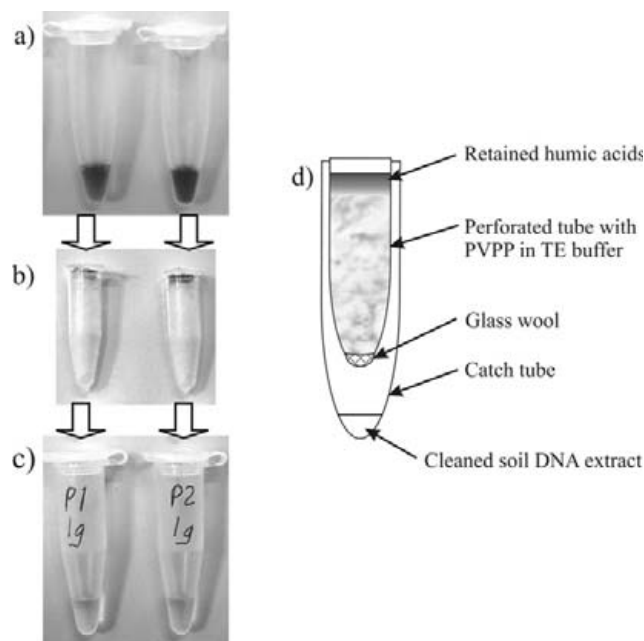


Fig. 2. Removal of PCR inhibitors in the DNA extracts from soil: a, DNA extracts before columns; b, columns after use; c, DNA extracts after centrifugation through PVPP columns; d, schematic presentation of PVPP spin column after use and its components.

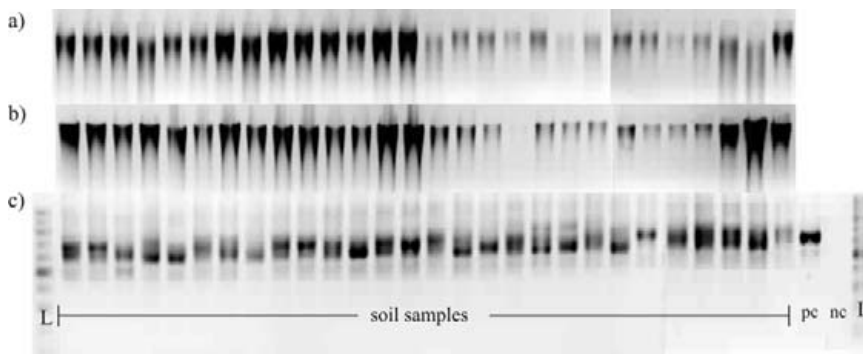


Fig. 3. Validation of the SDS/FastPrep method with 28 soil samples and comparison with the FastDNA SPIN Kit for Soil: DNA yield (a) achieved with SDS/FastPrep method, (b) with the kit, (c) PCR with primers ITS 1F and ITS 4 and DNA extracts from (a). L = 100-bp ladder; pc, positive control; nc, negative control.

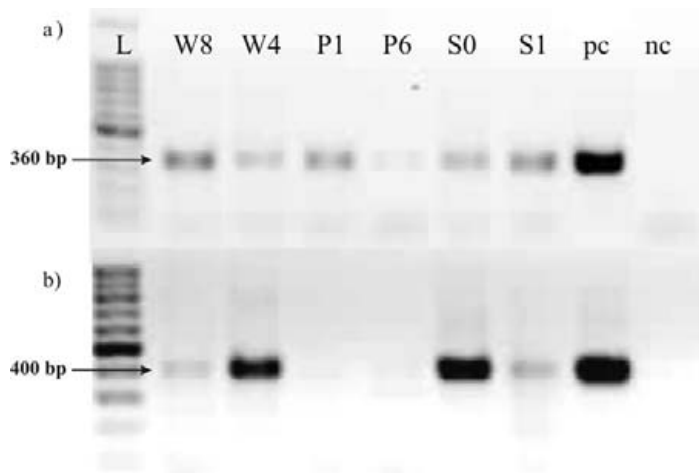


Fig. 4. Detection of (a) *Phaeoconiella chlamydospora* and (b) *Cylindrocarpon* species in different grapevine nursery and vineyard soils (lanes 2–7). L, 100-bp ladder; pc, positive control; nc, negative control.

(Fig. 3b). Total DNA yield was on average $3.97 \pm 2.00 \mu\text{g/g}$ soil, which was significantly ($P = 0.003$) higher than average yields with the SDS/FastPrep method ($3.22 \pm 1.53 \mu\text{g/g}$ soil). However, DNA yields of these methods correlated strongly ($R = 0.795$, $P < 0.0001$), which indicates the robustness of the SDS/FastPrep method.

PCR with ITS 1F and ITS 4 showed positive results for all samples extracted with the FastDNA kit (results not shown). In spite of the higher DNA yield, however, there was no positive reaction with the primers Pch1 and Pch2 with any of the DNA samples extracted with the FastDNA kit. PCR with the primers Dest1 and Dest4 yielded a 400-bp band with 16 soils, compared to 18 with the SDS/FastPrep extraction method. The latter took slightly more time than the FastDNA kit method. However, the SDS/FastPrep method was substantially more cost-effective, since it proved to be almost 25 times cheaper than the FastDNA kit.

Conclusion

We have developed a procedure for the molecular detection of fungal pathogens

in soil, which comprises a method for DNA extraction and an optimized PCR protocol. Among a variety of different extraction methods, we selected the easiest and most successful steps and avoided expensive materials or replaced them by cheaper ones. Owing to its low cost, high sensitivity and simplicity, the SDS/FastPrep DNA extraction protocol is suitable for detecting fungal pathogens in soil with the same or higher efficacy than the commercial extraction kit tested. Notably, the important plant pathogen, *Pa. chlamydospora*, could be detected only by using our extraction method and not with the FastDNA kit. Since the slow-growing *Pa. chlamydospora* seems to be present in very small amounts in soils, it is very difficult to trace it using culturing techniques. Apart from a rapid diagnostic tool, this new protocol will enable us to conduct large-scale investigations in grapevine nursery and vineyard soils to detect different grapevine root and trunk disease agents, as well as other fungal pathogens as a basis for studying their infection cycle and disease management. Since *Pa. chlamydospora* and *C. macrodidymum* were detected in vineyard and

nursery soils, these media should be considered as potential inoculum sources of these pathogens.

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