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A method for direct soil extraction and PCR amplification of endomycorrhizal fungal DNA

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Abstract DNA from endomycorrhizal fungi was extracted directly from a weathered soil (alfisol) mixed with sand. Mycorrhizae were established in a greenhouse culture of *Glomus clarum* with Sudan grass (*Sorghum vulgare* var. *sudanense*) host plants. The extraction procedure included enzymatic digestion of cell walls, sodium dodecyl sulfate lysis of cells, polyvinylpolypyrrolidone absorption of organic compounds, and ethanol precipitation of the DNA. DNA in the extracts was amplified by the polymerase chain reaction using primers from the nuclear 17S rRNA sequence that were general to fungi or were specific to endomycorrhizae.

Key words Endomycorrhizae · DNA · rRNA · PCR · Direct soil extraction

Introduction

Endomycorrhizal fungi are sensitive to physical, chemical and biological soil conditions (Bowen 1987; Wilson and Tommerup 1992). As a result, successful re-establishment of mycorrhizal symbioses during revegetation of barren mined soil or areas deforested by clearcutting or fire requires matching of appropriate or broadly tolerant fungal strains to the soil conditions on the site. Since such sites often exhibit extreme or unique soil environments and plant growth conditions, information about the tolerance of endomycorrhizal fungi would help in selection of useful strains. However, until much more work has been completed on tolerance of the fungi to water availability, metals, organic contents and pH, most screening for appropriate strains will be by simple monitoring of the presence, persistence and identification of adapted fungal strains on existing revegetation projects. A rapid, concise method of identifying endomycorrhizal fungi from field samples would help in this process.

Current culture protocols for identification of endomycorrhizae require several month's growout in trap culture under greenhouse conditions. Because trap culture soil conditions differ from those in the field, fungal strains selected in culture may differ from those in the field. Thus, when the most effective mycorrhizal strain in the field has a low density or tolerance of greenhouse conditions, these strains may be displaced in trap cultures and never be observed. As a result, population distribution in the field may be misinterpreted, and nonadapted strains may be reintroduced to the field.

The use of unique DNA sequence information of the endomycorrhizal symbiont is potentially a more exact and rapid method for identification of desired strains. After identification through spore morphology and response to various stains and treatments (Morton 1988, 1993), DNA sequences unique to all endomycorrhizal fungi (Simon et al. 1992) or unique to distinct species or subspecies taxa (Millner and Meyer 1990) can be used for more routine identification and tracking of the desired fungal strains, as was done with ectomycorrhizae (Gardes et al. 1990) and *Phytophthora* (Érsek, et al. 1994).

Target DNA has been extracted from mixtures of soils and microbes after physical separation of cells from the soil matrix and lysis (Fægri et al. 1977; Torsvik 1980; Holben et al. 1988; Pillai et al. 1991). Ogram et al. (1987) pointed out that density fractionation may not recover microbes tightly sorbed to soil particles, e.g. highly ramified mycorrhizae, and developed a glass bead method which lysed cells directly in the soil matrix. The method produced DNA fragments less than 10 kb in length but the recovery of soil DNA was tenfold higher than cell extraction methods (Steffan et al. 1988). A simplified direct soil extraction method devel-

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oped by Porteous and Armstrong (1991) reduced the sample size to 1 g (from 10–100 g), incorporated polyvinylpolypyrrolidone (PVPP) to adsorb organic matter contaminants, and lysed cells by enzymatic digestion rather than mechanical disruption.

In the present study, the Porteous and Armstrong (1991) procedure was used with slight modification as the first step in extraction of bulk soil DNA, amplification of selected DNA sequences by the polymerase chain reaction (PCR) (Saiki et al. 1988), and the detection of endomycorrhizal fungal DNA. A direct extraction method was selected because endomycorrhizal hyphae are finely intercalated with soil particles and may be difficult to physically separate.

Materials and methods

Soil preparation and greenhouse culture of mycorrhizae

A well-weathered alfisol (fine loamy mixed mesic Ultic Haploxeralf) from the top 10 cm of a mixed oak-conifer woodland located 40 km north of Redding, Calif. was selected as a realistic test medium since it contains moderately high clay (30%) and organic carbon (3.7%), both of which interfere with DNA extraction (Ogram et al. 1988; Steffan et al. 1988). The field soil was sieved to less than 5 mm, mixed, and gamma irradiated. Sterility was confirmed by incubation on nutrient agar at 25 °C for 1 week. To improve drainage of this moderately heavy soil in greenhouse conditions, quartz sand was mixed in a 2:1 ratio. The quartz sand ($\approx$1 mm particle size) was acid washed, rinsed, and autoclaved twice for 1 h at 121 °C. The sand and soil were mixed and inoculated with the filtrate ($\leq$10 μm) from a duplicate nonsterile soil sample so that inoculated and uninoculated treatments had the same background microflora (Ames et al. 1987). The soil mix was then covered and incubated at room temperature for 1 week before being loaded into individual 4 × 20-cm pots.

The mycorrhizal fungus *Glomus clarum* was introduced into some pots as a mixture of spores and infected root fragments in sand obtained from the International Culture Collection of Arbuscular and VA Mycorrhizal Fungi, Morgantown, W.Va; culture # 239). Approximately 10 ml per pot of the inoculum was blended with the sand:soil mixture in a band 2–5 cm below the final soil surface.

Surface-sterilized (5.25% sodium hypochlorite) seeds of Sudan grass (*Sorghum vulgare* var. *sudanense*) were planted in the top 0.5 cm of the sand:soil mixture in inoculated pots and covered with 1.5 cm of sterile, acid-washed sand. Plants were grown in the greenhouse for 3 months with ambient lighting. Distilled water was provided as needed and once every 2 weeks the pots were watered with a 1/4-strength Hoagland's solution (10 ml) without phosphorus. After 6 weeks, when phosphorus deficiency symptoms became severe and no further growth was observed, the pots were watered once with 10 ml of 1/4-strength Hoagland's solution containing 1/10-strength phosphorus. Successful mycorrhizal infection in the inoculated treatments was indicated by a 2- to 4-fold increase in plant heights over the uninoculated treatments.

Hyphae of *Neurospora crassa* were used as positive controls during development of the method. These cultures were grown in 1-l flasks in 500 ml of Vogel's minimal salts solution containing trace elements, sucrose and inositol. Hyphae were sieved onto Mira-Cloth, blotted, cut into 5- to 7-mm squares, weighed and then frozen at –70 °C.

Sampling procedure

Samples were harvested from pots with Sudan grass plus mycorrhizal fungi, uninoculated Sudan grass plants, or soil and background inoculum only (i.e. no plant roots or mycorrhizal fungi). The plant/mycorrhizal and plant only treatments were sampled either as whole soil with coarse roots removed or as a concentrated root sample containing about 0.6 g fresh wt. root tissue. These samples were used to compare the detection of endomycorrhizal infection from concentrated root tissue to direct extraction from soil samples containing ambient soil, hyphae and roots.

The bottom 5 cm of the pots was cut off to remove soil and roots. Roots retained on a 250-μm sieve were cleared and stained with trypan blue (Phillips and Hayman 1970). The percent of root with endomycorrhizal infection was estimated under a dissecting microscope by counting the proportion of 0.3-cm root sections infected with arbuscules, hyphae or spore attachments (Kormanik and McGraw 1982). Mycorrhizal structures on root fragments were periodically confirmed using a compound microscope. Soils for DNA extraction were subsampled, weighed (1 g ± 0.1 g dry wt. equivalent), and placed into 50-ml screw-top centrifuge tubes.

DNA extraction

DNA in the soil samples was extracted by the procedure of Porteous and Armstrong (1991) with minor modifications. All buffers and enzymes were mixed from their component stocks within 5 h of use. Soil samples (1 g) were mixed with 6 ml extraction buffer (0.5 M sorbitol, 15% polyethylene glycol 4000, 2% diethyldithiocarbamate, 100 mM EDTA, 50 mM Tris pH 8, 500 mg PVPP). Lysozyme (100 μl of 50 mg ml^{-1}) and Novozyme™ 234 (Novo Biolabs, Bagsvaerd, Denmark) (120 μl of 50 mg ml^{-1}) were added and the mixture incubated on ice for 2 h. Lysis buffer (3.8 ml of 4% SDS, 100 mM EDTA, 500 μg proteinase K ml^{-1}, 50 mM Tris pH 8) was added, and the slurry was gently mixed by inverting and then incubated on ice for 16 h. The solutions were centrifuged (8 min, 4 °C, 5000 *g*) and the supernatant transferred to duplicate 50-ml sterile tubes. The sediments were washed twice with 3 ml wash buffer (50 mM Tris pH 8, 100 mM EDTA, 4 °C) and recentrifuged. Supernatants from the washes were pooled in the duplicate 50-ml tube. The sedimented samples were centrifuged a final time at 5 min, 4 °C, 15 000 *g* and the combined supernatants were mixed with enough potassium acetate to bring the potassium concentration to 0.5 M at room temperature, and then incubated on ice for 2 h. The precipitated SDS was removed by a 12-min centrifugation (15 000 *g*, 4 °C). Two volumes of 100% ethanol (room temperature) were added and the solution was held at –20 °C for 4 h. The DNA was then pelleted by centrifugation for 12 min (room temperature, 15 000 *g*). The brown-colored pellet containing the DNA was resuspended in 300 μl TE (10 mM Tris pH 8, 1 mM EDTA). *N. crassa* hyphae (30 mg, containing about 6 μg total DNA) was substituted for the soil in two extraction control treatments.

PCR amplification

The endomycorrhizal-specific primer pair used for the mycorrhizal inoculated treatments included the VANS1 and NS4 primer pair (Simon et al. 1992), which amplify a 1.10-kb region specific to rDNA from vesicular-arbuscular or endomycorrhizae. Primers used to amplify general fungal ribosomal DNA in the nonmycorrhizal treatments were the NS1 and NS4 pair (White et al. 1990), which amplify a 1.15 kb-fragment complimentary to the highly conserved 17S small ribosomal RNA subunit. Primers were added at 160 nM with 210 μM dNTP. The reaction volume was 25 μl and contained 10 mM Tris pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl, 200 μg ml^{-1} gelatin and 1 μl of the diluted soil extract (1:150 distilled, deionized water). The extract from the *N. crassa* hyphae extraction control was diluted 1:50 with distilled, deionized water, and a 1-μl aliquot (120 ng DNA) was used for the PCR reaction. A positive amplification control was purified *Neurospora* DNA (1:50 dilution, 2.7 ng DNA). The "hot start" procedure of D'Aquila et al. (1991) was used in which 0.7 U Taq polymerase

was added after the reaction temperature had reached 94 °C. The reaction mixture was then covered with 30 μl of mineral oil. Temperature cycles were 94 °C 1 min, 55 °C 2 min, 72 °C 2 min 30 s, for 50 cycles, and a final 10-min extension at 72 °C.

Electrophoresis

PCR product (10 μl) was mixed with loading buffer and electrophoresed on a 1% agarose gel at 40 V for 2.8 h. The gel was stained with 1 μg ml^{-1} ethidium bromide and visualized with UV light. A 1 kb DNA Ladder (Gibco BAL, Grand Island, NY) AND a HinDIII digest of lambda phage were used as size markers.

Results and discussion

Microscopic evaluation of the cleared and stained root samples indicated that mycorrhizal infection was about 35% in the inoculated treatments and no infection was detected in noninoculated treatments.

Endomycorrhizal-specific PCR products were amplified from direct soil extracts of treatments inoculated with *G. clarum* (Fig. 1, lanes 2 and 4). No endomycorrhizal DNA was detected in the noninoculated, nonmycorrhizal treatments (Fig. 1, lanes 6 and 8). All these reactions used VANS1 and NS4 primer pair, which amplifies only endomycorrhizal fungal DNA, and template DNA from either direct soil extracts or from root tissue. Endomycorrhizal-specific PCR products were not amplified from extracts of treatments without plants or mycorrhizal fungi (Fig. 1, lane 10). The endomycorrhizal primers did not amplify DNA from the nonmycorrhizal fungus *N. crassa* (Fig. 1, lane 12). A water template substituted for soil extracts (Fig. 1, lane 14) showed that the band in the mycorrhizal treatments (Fig. 1, lanes 2 and 4) was not due to amplification of contaminating DNA.

The PCR products from the NS1-NS4 primer pair (nonspecific, general fungal primers) are slightly larger than the endomycorrhizal products (Fig. 1, all odd-numbered lanes and lane 14). DNA was extracted and amplified from soil (Fig. 1, lanes 1, 5, 9), and from concentrated plant roots (Fig. 1, lanes 3, 7). The occurrence of nonspecific fungal PCR products in these lanes shows completion of the various extraction, amplification and visualization steps. Lane 11 (Fig. 1) is a positive control using extracts of *N. crassa* hyphae and lane 13 is a positive control using clean, previously extracted *Neurospora* DNA. These positive controls indicate that the absence of endomycorrhizal-specific products from the noninoculated, nonmycorrhizal treatments is not due to a faulty procedure. Several spurious bands were observed either above the main products (Fig. 1, odd-numbered lanes) or below (Fig. 1, lanes 5 and 9) but these were not identified.

Thus these data demonstrate that this method of direct extraction of DNA from bulk, 1-gm soil samples can be used with PCR to identify the presence of endomycorrhizal fungi. The 50-cycle procedure used gave higher yields than the standard 30 cycles without producing nonspecific amplification. Lack of nonspecific amplification in these reactions may partly be due to the specificity of the primers, some of which allowed amplification at annealing temperatures of 65 °C. Higher Mg concentrations, potentially required because of carryover of EDTA from the soil extraction steps, gave no improvement in amplification. Dilution of the soil extract (upto 100-fold) needed with several soil samples. Variation of the polymerase concentrations also had no influence on amplification product yield. However, because of the variation in amplification from sample to sample, further work is planned to establish sensitivity levels and alternative extraction methods.

Although multinucleate endomycorrhizal spores are a potential high yielding source of DNA from soils (Viera and Glenn 1990), related laboratory trials indicated that these thick-walled, resting-stage propagules were not lysed by this extraction procedure. While in some cases this could be viewed as a deficiency in the method, the results presented here can be said to reflect active hyphae rather than dormant resting propagules. Modification of the enzymatic digestion steps, particularly with chitinase containing Novozymeε, may result in lysis and release of spore DNA.

The evaluation of endomycorrhizal fungi directly from field samples can be expected to be difficult because of low target DNA concentrations and difficulties with cell lysis (Holben 1994). Methods for extracting DNA from other microbial forms has been worked out, e.g. ectomycorrhizal root tips (Rygiewicz and Armstrong 1991) and bacteria (Holben et al. 1988). These protocols yield relatively high DNA amounts compared

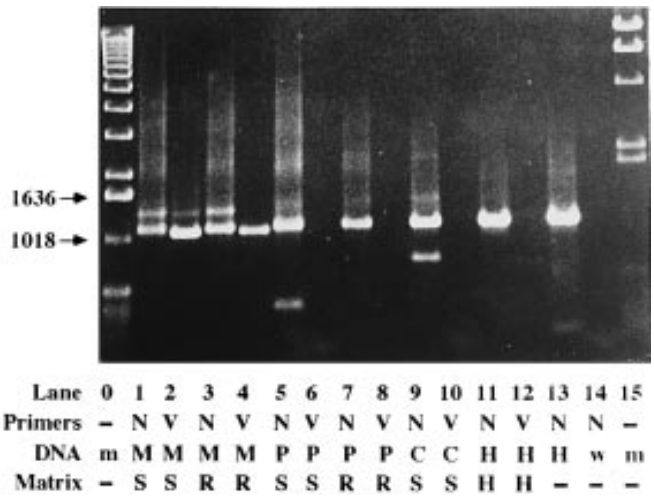

Fig. 1 PCR amplification of DNA extracted directly from soil with or without mycorrhizal inoculation. Lane 0: 1 kb ladder; lane 15: *Hin*dIII digest. Primers: *N* NS1/NS4, nonspecific; *V* VANS1/NS4, specific. DNA source: *C* control (no plants or mycorrhizae); *H* hyphae or DNA from *Neurospora crassa*; *M* mycorrhizal fungus (*Glomus* clarum); *m* size marker; *P* uninfected Sudan grass plants; *w* water. Matrix: *R* root tissue extract; *S* direct soil extract

with soil extracts of endomycorrhizal fungi which grow as fine, single hyphae. Further, since the hyphae are strongly integrated with soil particles and are not easily dislodged, the DNA must be extracted from the entire soil, as opposed to density separation techniques in filtration-extraction protocols which first separate cells from soil particles. For these reasons, development of a direct extraction is a priority and warrants further work, both to optimize detection levels and to monitor DNA degradation and contamination. While extraction efficiencies and exact comparisons with other DNA recovery methods remain to be determined, the protocol described here was successful with soils of moderately high clay and organic content. As morphological taxonomy and systematics improve (Bentivenga and Morton 1994), and as molecular markers for the various taxa are identified, the ability to directly detect endomycorrhizal fungi from soils will increase our ability to monitor and evaluate mycorrhizal function and dynamics.

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