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SHORT COMMUNICATION

Molecular detection of Arbuscular mycorrhizal fungi in calcareous soil and roots of *Zea mays* L.

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Abstract

PCR-based techniques have become mandatory to obtain sufficient quantities of DNA, as these organisms are non-culturable and thus only a small quantity of DNA could be isolated from infected roots. Arbuscular mycorrhizal fungi (AMF) were detected in calcareous soil and roots of maize (*Zea mays* L.) by direct extraction and polymerase chain reaction (PCR) amplification of the small subunit rDNA gene. The maize cultivated roots were used in this study, since they contained a natural inoculum source that could be measured and used to determine the sensitivity of PCR based methods. The sensitivity of detection was improved when an initial PCR amplification using the primers NS1 and NS4 was followed by amplification of the *Glomales*-specific sequences using NS3 and NS4. The availability of a simple method to confirm AM fungal colonization on a broad range of species would be useful in the application and evaluation of endomycorrhizal inoculants in agriculture.

Keywords: Arbuscular mycorrhizal fungi, calcareous soil, *Zea mays*, polymerase chain reaction.

Introduction

Arbuscular mycorrhizal (AM) fungi are symbiotic microorganisms that colonize the roots of most terrestrial plant species (Smith and Read, 1997). AM fungi play an important role in plant nutrient acquisition (especially phosphorus) by the spread of their hyphae into the rhizosphere (Mosse *et al.,* 1981) and hence affect plant community structure and plant diversity (Mukerji and Kaboor, 1990; Kjoller and Rosendahl, 2003). The diversity of AM fungi has been investigated based on the morphological characteristics of their spores in the rhizosphere. On the other hand, study of AM fungal diversity in mycorrhizal roots is lacking because of the difficulty of distinguishing AM fungal species based on hyphal morphology in root. DNA-markers have recently been developed to identify AM fungi (Redekler and Wiemken, 2003; Ram Reddy *et al.,* 2005).

Specifically, a PCR method using AM fungi-specific and its taxon-specific primers has been attempted to detect AM fungal DNA in roots. High quality template DNA must be prepared from AM roots for PCR showed that the boiling method is a useful and easy way to extract DNA from laboratory-grown mycorrhizal roots inoculated with AM fungi (Antoniolli *et al.,* 2000). In contrast to laboratory grown mycorrhizal roots, field-collected mycorrhizal roots contain various amounts of AM fungal tissue and many kinds of PCR inhibitors such as phenolic compounds, polysaccharides and proteins, whose compositions and quantities are always influenced by environmental factors. For field collected mycorrhizal roots, selection of the proper DNA extraction method is therefore necessary. There are few studies on DNA extraction from field collected mycorrhizal roots.

In this study, simple extraction of AM fungal DNA from field collected mycorrhizal roots for PCR is attempted.

Materials and methods

Collection of roots and DNA extraction: Zea mays L. roots were collected from calcareous soils from Ariyalur district, TN, India. The roots were washed in running water and cut into portions approximately 1 cm long. The cleaned root segments were crushed in a pestle and mortar. The DNA was extracted using CTAB method (Ausubel *et al*., 1994). The crushed root was added to 300 µL of 2x CTAB solution (100 mM Tris HCl; pH 8; 1.4 M NaCl; 20 mM EDTA; 2% CTAB and 0.2% 2-Mercaptoethanol). The solution was incubated at 56°C for 90 min. After two extractions with chloroform/isoamyl alcohol (24: 1,v/v), DNA in the aqueous phase was precipitated by the addition of an equal volume of isopropanol and a tenth volume of 3 M sodium acetate was added and incubated at room temperature for 30 min. The DNA pellet was then washed with 70% ethanol and re-suspended in 50 µL sterilized water. DNA solution obtained was diluted 100 to 1,000 fold and used as template for PCR amplification (Simon *et al.,* 1992, 1993).

PCR amplification: Partial small subunit rRNA gene sequences were amplified from the purified total community DNA by PCR using
NS1 (nuclear small) (5'-GTAGTCATATGCTTGTCTC-3'),
NS4 (5'-GTTCCGTCAATTCCTTTAAG-3'),
NS2 (5'-GGCTGCTGGCACCAGACTTGC-3')
and an AMF-specific primer,
NS3 (5'-GCAAGTCTGGTGCCAGCAGCC-3').

DNA was amplified in a 50 µL reaction volume containing either 50 pmol of each primer (NS3 and NS2), 10 pmol (NS1 and NS4), or 20 pmol (NS3 and NS4), 5 mL of 10x PCR reaction buffer (Promega), 5 µg BSA, 2.5 mM $MgCl_2$, 0.2 mM (each) dATP, dGTP, dCTP, dTTP, 2.5 U *Taq* polymerase and approximately 100 ng of DNA. NS3 and NS2 primer pair: An initial 3 min denaturation at 95°C, followed by 40 cycles of 35 s each of denaturation at 94°C, annealing at 53°C, extension at 72°C, and a final 5 min extension at 72°C. Conditions for PCR using NS1 and NS4 primer pairs included: An initial 3 min denaturation at 95°C, followed by 40 cycles of 94°C for 20 s, 55°C for 35 s, and 72°C for 1 min and a final 5 min extension at 72°C. PCR amplification using NS3 and NS4 primer pair followed by latter thermal profile for 25 cycles with 1 µL of PCR product from the NS1 and NS4 amplifications used as the template (Wyss and Bonfante, 1993). PCR product was visualized by electrophoresis on 1% agarose gel followed by staining with ethidium bromide (0.5 µg/mL).

Results

The most probable number (MPN) of mycorrhizae in the calcareous soil was 56 propagules per 100 g soil. Plant DNA will co-extract with AMF DNA when using a direct DNA extraction procedure for plant tissue and this can result in low PCR amplification yields if plant DNA is in great excess (Clapp *et al*., 1995). Even though a direct DNA extraction method was used with *Z. mays* root tissue, PCR amplification was successful using all primer pairs tested (Fig. 1 and 2). The soils were used for comparisons of DNA extraction and amplification efficiency because they shared the same soil texture but differed in band size. Using NS3 and NS2 PCR primers, amplification was detected only in the calcareous soil. This soil has an at least 10-fold higher MPN, than the other soil, suggesting that in a sandy soil, infective numbers may have to exceed to be detected using PCR with these primers. When the product of PCR amplification with NS1 and NS4 primers was used in a second round of amplification with NS3 and NS4, a product was detected in all soils tested, even up to a 1:100 dilution of the original purified template. Sequence analysis of 550-bp cloned PCR band substantiated (Fig. 1 and 2).

Discussion

The intent of this study was to develop PCR-based methodology to determine the presence of AMF in soil and roots. Maize cultivated soils were used because they contained a natural inoculum source that could be measured and used to determine the sensitivity of the PCR based methods. rRNA sequence analysis has proven useful for similar objectives (Clapp *et al*., 1995), in part because multi-copy rRNA genes and multi-nucleated spores (Viera and Glenn, 1990; Giovannetti and Gianinazzi-Pearson, 1994) provide abundant target DNA.

Fig. 1. Ethidium bromide stained agarose gel (1%) showing PCR amplification of 16S rDNA of bacterial isolates obtained from the farming site on various selective media.

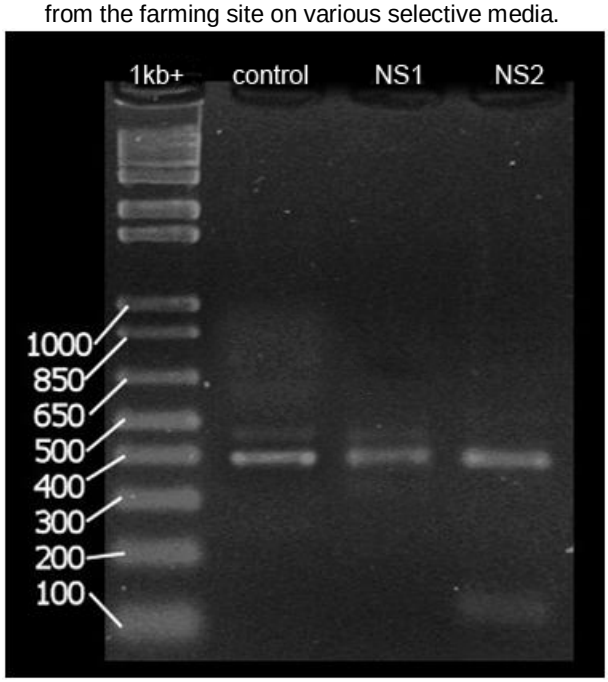
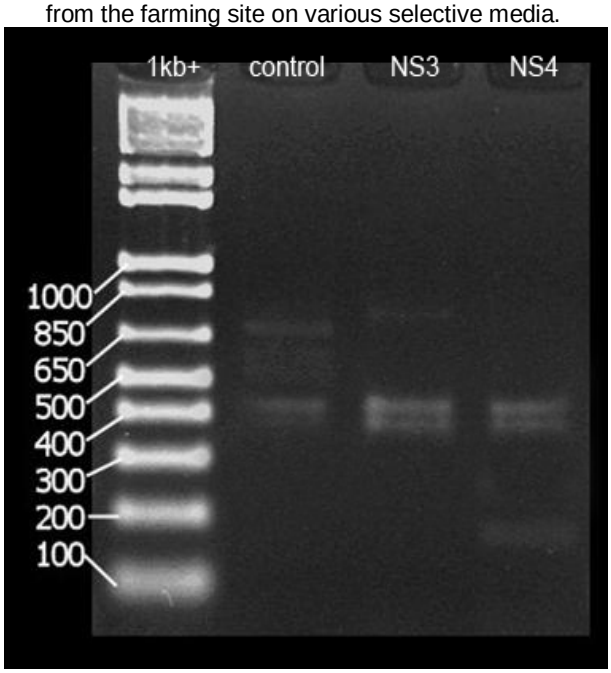


Fig. 2. Ethidium bromide stained agarose gel (1%) showing PCR amplification of 16S rDNA of bacterial isolates obtained from the farming site on various selective media.



The DNA extraction and purification methods were rapid and effectively removed substances are inhibitory to PCR amplification. The sensitivity of detection was improved when an initial PCR amplification using the primers NS1 and NS4 was followed by amplification of the *Glomales*-specific sequences. In this study, a portion of the 18 S rRNA gene was used to identify the presence of AMF in soil.

Analysis of the SSU rDNA (Smaller subunit ribosomal DNA) may be a taxonomic information that may be lacking in examinations of spore morphology. However, this gene evolves relatively slow and may not provide sufficient information to adequately characterize the AMF guild within a community. A more appropriate approach could rely on the sequence variability of the internal transcribed spacer regions (ITS) between the small and large subunits of the rRNA genes (Sanders *et al*., 1995; Redecker *et al*., 1997; Redecker, 2000). As yet, few ITS sequences of a diversity of isolates have been deposited in the Genbank database to allow these comparisons. The above method may be simple, producing high quality DNA in about 1 h. The PCR amplification of AMF sequences in non-inoculated soil emphasizes the potential utility of this method as a tool to identify indigenous AMF. To assess the efficacy of methods for DNA extraction and purification of AMF in soil and roots, some studies have relied on a commercial inoculum and/or material grown under the artificial conditions of a greenhouse (Di Bonito *et al*., 1995; Claassen *et al*., 1996). This confounds interpretations of the sensitivity of the method, because the high concentration of inoculums used with optimized cultural conditions may not reflect the abundance of indigenous populations of AMF. The method described here also eliminates the necessity of isolating spores from soil prior to DNA extraction in order to obtain DNA of sufficient quality for PCR amplification.

Conclusion

The efficiency of extraction is of particular importance in PCR-based medical diagnostic applications where the quantity of fungus in a tissue biopsy may be limited. Isolation and identification of AM fungi from maize root using DNA-based methods. Nevertheless, this experimental approach, in combination with the classic morphological analyses of spores, is highly promising and should provide a workable strategy to better characterize AMF communities within roots. The results of the bioassay should be compared with PCR-based methods which will markedly speed up the assessment of AM fungal infectivity. Employing DNA-based molecular markers have been achieved in areas like phylogeny, taxonomy and functional symbiosis.

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