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Molecular detection of Arbuscular Mycorrhizal fungi in Saline colonization roots of *Trigonella foenum- graecum* L.

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Abstract

Arbuscular mycorrhizal fungi (AMF) were detected in Saline soil colonized roots of plant *Trigonella foenum - graecum* L. by direct DNA extraction. As these organisms are non-culturable and thus only a small quantity of DNA could be isolated from infected roots. The PCR-based techniques have become mandatory to obtain sufficient quantities of DNA by using partial sequence of SSUrRNA of the plant roots by polymerase chain reaction. Since the AMF contained a natural inoculum source that could be measured and to determine the sensitivity of PCR based methods. The sensitivity of molecular detection was done by using the two set of primers (A) NS1; NS4, (B) AML1; AML2 of the *Glomale* - specific primer sequences. And the specific universal primer sequence was concluded in the agarose gel 800bp of NS1;NS4 and 1100bp of AML1;AML2. The availability of a simple method to confirm AM fungal colonization on a broad range of species would be useful in the evaluation of endomycorrhizal inoculants in fenugreek plant cultivation.

Keywords: Arbuscular Mycorrhizal fungi, Saline soil, Polymerase chain reaction.

Introduction

Arbuscular Mycorrhizal (AM) fungi are important for plants because they play a key role in plant nutrient uptake, especially phosphorus uptake (Smith and Read, 1996) and have protective role against various plant pathogenic fungi (Newsham *et al.*, 1995). For molecular techniques have been used to study phylogenetic relationships of AM fungi, and attempts have been made to use ribosomal genes as a tool for the identification of AM fungal species (Redecker *et al.*, 1997 thus, molecular methods have revolutionized our understanding of these organisms and are amongst the most promising tools for their study (Clapp *et al.*, 2003; Dickie and FitzJohn, 2007).

PCR method using AM fungi-specific and its taxon-specific primers has been attempted to detect AM fungal DNA in roots. (Shafiqua and Stephan, 2013).

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). The direct identification of mixed populations of AM fungi in actively colonized root using PCR based detection methods with specific primers (Van denkoornhuy se *et al.*, 2002).

Molecular analysis has become common place in the last two decades in order to get greater insight regarding the structure and function of different organisms. Molecular approaches primarily rely upon exploitation of genetic variations (De Souza *et al.*, 2004). Study of the AMF genome has been modest, considering the fact that these fungi possess large genome compared to other zygomycetes, ranging from

0.13 to more than 1.0 pg DNA per nucleus (Hosny *et al*., 1997). Furthermore, the genomes contain extensively repeated DNA sequences (Zeze, 1999). Molecular methods have been particularly successful for studying rDNA sequence from AMF (Simon, 1996; Pringle *et al*., 2000; Redecker *et al*., 2000). Several investigations have reported that individual spores of AMF which are multinucleate, show a high level of genetic diversity in the internal transcribed spacer (ITS) region of the nuclear rRNA gene (Lloyd-Mac Gilp *et al*., 1996; Antoniolli *et al*., 2000). Ribosomal based DNA sequence analysis has revealed genetic variation both within and between AMF species (Clapp *et al*., 1999, Hosny, *et al*., 1999; Lanfranco, *et al*., 1999).

Plants are frequently used in therapeutics from times immemorial. The documentation of therapeutic utility of plants can be seen from Vedic period. Now a day's therapeutic utility of many plants is identified. In the present study the plant fenugreek is also one of the important plant.(*Trigonella foenum - graecum* L.) belonging to the family Papilionaceae commonly known as Fenugreek is an aromatic, 30-60 cm tall, annual herb, cultivated throughout the country India (Ahmad *et al*., 2005). Seeds of fenugreek are used locally as a yellow dye for cosmetics and medicinal purposes.

Fenugreek seeds and leaves are rich in minerals, proteins and carbohydrates, but low in oil (Gad *et al*., 1982). The seeds are used as spice worldwide, whereas the leaves are used as green leafy vegetables in diets. Fenugreek seeds are bitter to taste and are known for a long time for their medicinal qualities.

Materials and Methods

Collection of roots and DNA extraction

Trigonella foenum- graecum L. roots were collected from saline potted soils. The roots were washed in running water and cut into small 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 56^0C 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).

Agarose gel electrophoresis of DNA

0.8% of agarose gel (w/v) was prepared using 1x TE buffer by melting in a heating mantle and cooling at 60º C. After cooling add one to two drops of ethidium bromide. Each DNA sample was taken and mixed with 6x gel loading dye (0.25% Bromophenol Blue). Then the above mixture was loaded into a separate well with the help of micropipette. The DNA marker was loaded for comparison. The lid of the gel tank was closed. The power supply was connected to run the electrophoresis. After the run was completed gel was scanned under UV visible light.

PCR amplification

Partial small subunit rRNA gene sequences were amplified from the purified total community of DNA by PCR using two set of primers

(A)NS1;NS4,(B)AML1;AML2
NS1 (5'-GTAGTCATATGCTTGTCTC-3'),
NS4 (5'-CTTCCGTCAATTCCTTTAAG-3'),

and

an AMF-specific primer,
AML1 (5'-ATCAACTTTCCTTTAAG-3')
AML2 (5'-GAACCCAAACATTTGGTTTC-3').

DNA was amplified in a 50 µL reaction volume containing either 50 pmol of each primer (NS1 and NS4), 10 pmol (AML1 and AML2), or 20 pmol (NS3 and NS4), and 5 mL of 10x PCR reaction buffer (Promega), 5 µg BSA, 2.5 mM MgCl2, 0.2 mM (each) dATP, dGTP, dCTP, dTTP, 2.5 U *Taq polymerase* and approximately 100 mg of DNA. The conditions for NS1 and NS4 primer pair was set as follows, An initial 3 min denaturation at 95ºC, followed by 40 cycles of 35 sec each of denaturation at 94ºC, annealing at 53ºC, extension at72ºC, and a final 5 min extension at 72ºC. Conditions for PCR using NS1 and NS4 primer pairs areas, an initial 3 min denaturation at 95ºC, followed by 40 cycles of 94ºC for 20 s, 55ºC for 35 s, and 72ºC for 1 min and a final 5 min extension at 72ºC. PCR amplification using AML1 and AML2 primer pair

followed by latter thermal profile for 25 cycles with 1 µL of PCR product from theNS1 and NS4 amplifications were 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

Molecular detection of AMF

In the isolation and purification of DNA, CTAB method was found to be good for successful isolation. This method of isolation of genomic DNA from root sample was used in different plants. The DNA obtained was reasonably of good quantity and quality. Further purification was done by Rnase treatment followed by phenol: chloroform extraction and re-precipitation by ethanol (100%). The DNA extracted will be used in further PCR (polymerase chain reaction) amplification analysis.

PCR amplification was successful for all the primer pairs tested (Figure-1) in the present study. The DNA extraction and amplification efficiency generated different band size. Universal primer (A) AML1; AML2 (B) NS1;NS4 has been used for the identification of AM fungi, AML1 and AML2 generated a single clear PCR product of around 800bp from colonized roots. (Fig-1 lane, 8,9,10,11) and NS1;NS4 generated a clear band of (Fig -1 lane 2,3,4,5). DNA ladder (Fig-1 lane m) was used for comparison likewise positive control lane 2,7, lane 6 negative control The amplicon sequences of lane2,3,4,5 and 8,9,10,11 fell within the order (Glomales, zygomycetes).

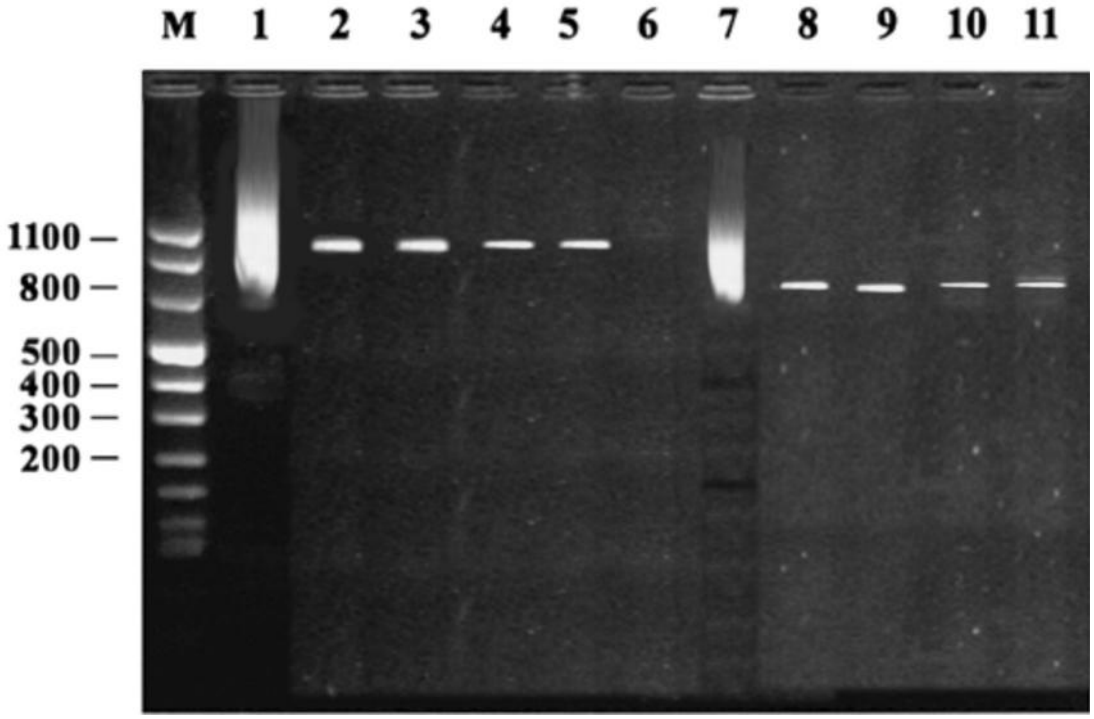

Fig. 1. Ethidium bromide stained agarose gel (0.8%) showing PCR amplification of 16S rRNA sequences obtained from the *Trigonella foenum - graecum* AMF colonized roots.

Lane M - DNA marker
Lane 1 - Positive control (genomic DNA from AMF colonized roots)
Lane 6 - Negative control
Lane 2,3 - PCR amplification with NS1 Universal Primer
Lane 4,5 - PCR amplification with NS4 universal Primer
Lane 8,9 - PCR amplification with AML1 universal Primer
Lane 10,11 -PCR amplification with AML2 universal Primer

Discussion

A similar study was done by Clapp *et al*., 1995 to develop PCR-based methodology to determine the presence of AMF in soil and roots of *Cardiospermum halicacabum* L., and *Ricinus communis* L. and the saline soils were used to determine the sensitivity of the PCR based methods. rRNA sequence analysis has been proven in our studies, likewise multi-copy rRNA genes and multi-nucleated spores (Viera and Glenn, 1990; Giovannetti and Gianinazzi-Pearson, 1994) provide abundant target DNA. The DNA extraction and purification methods were rapid and effectively removed substances which are inhibitory to PCR amplification. The sensitivity of detection was improved when an initial PCR amplification was done by using the primers NS1 and NS4 and it was followed by amplification of the *Glomales* - specific sequences.

Analysis of the SSU rDNA (Smaller subunit ribosomal DNA) may be a taxonomic information that may backing 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). In contrast few ITS sequences of a diversity of isolates have been deposited in the Gene bank 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, studies have relied on a commercial inoculum and/or material grown under the artificial conditions of a greenhouse (Claassen *et al*., 1996). This interpretation of the sensitivity of the method, the high concentration of inoculums used with optimized cultural conditions may not reflect the abundance of indigenous populations of AMF. This 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 importance in PCR-based applications where the quantity of fungus in a tissue biopsy may be limited. Isolation and identification of AM fungi from *Trigonella foenum - graecum* root was done by using 16srRNA-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 present 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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