A new approach for enhanced multiplication of arbuscular mycorrhizal fungi and isolation of ITS regions from *Glomus deserticola* and *Laccaria fraterna*

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Rootlets induced from the petiole base of *L. purpureus*, using IAA and kinetin was used for enhanced multiplication of arbuscular mycorrhizal (AM) fungus, *G. deserticola*. Using conserved short arbitrary oligonucleotides, as specific primers, we amplified the ITS-region, a molecular marker for fungal identification, from the genomic DNA extracted from cultured spores of *G. deserticola*, and genomic DNA extracted from the mycelium of *L. fraterna*. The capacity of fungal colonization and subsequent spore formation of *G. deserticola*, compared with the natural root system was evaluated. This technology would provide a simple way to multiply AM fungi and to produce spores without microbial contamination useful for further molecular characterization.

Keywords: *Glomus deserticola*, Internal transcribed spacer (ITS), *Laccaria fraterna*, Mycorrhiza.

Arbuscular mycorrhizal (AM) fungi form symbiotic association with the roots of most terrestrial plants including agriculturally important crop species. This fungi enters the cortex of root to obtain carbon from their plant hosts, while assisting the plants with the uptake of phosphate and other mineral nutrients from the soil. This association is beneficial to the plants because, phosphate is a major essential element for growth and development. Besides this, mycorrhizal fungi help in the uptake and accumulation of mineral ions from soil and translocate them to the shoot system of their hosts.

Molecular understanding of AM fungi is limited, due to unavailable *in vitro* multiplication system to get spores without microbial contamination. Molecular techniques are useful to identify mycorrhizal fungi in the absence of morphological features. The development of molecular techniques, based on polymerase chain reaction (PCR) has provided a valuable and alternative approach to morphology, which represents the primary criterion to define the taxonomic position of fungi. Spores of AM fungi contain thousands of nuclei. In fungi, (i) the entire internal transcribed spacer (ITS) region is often between 600 and 800 bp, and can be readily amplified with universal primers, complementary to sequences within the rRNA genes, (ii) the multicopy nature of rDNA repeat makes the ITS region easy to amplify from spore samples, and (iii) the ITS region is often highly variable among morphologically distinct fungal species.

However, simple method to propagate endomycorrhizal fungi has not been achieved yet. The objective of this study is to develop a simple method to multiply AM fungal spores for preparation of DNA, under laboratory conditions to study molecular biology of the fungi. We have developed for the first time a methodology to multiply AM fungi (*Glomus deserticola*) in laboratory by root induction method, using *Lablab purpureus* petiole induced roots and we have identified ITS region from isolated spores using PCR.

Materials and Methods

*Cultures and media*—Spores of *G. deserticola* and culture of *L. fraterna* were obtained from the Culture Collection Center, Centre for Advanced Studies in Botany, University of Madras, Chennai, India. *L. fraterna* was grown on Potato Dextrose Agar medium (PDA) slants at 25°C in dark. Sub-culture was made at every week. Seeds of *L. purpureus* were purchased from Soundara Pandi Nursery, Chennai, India. *Kalanchoe pinnata* and *Allium cepa* were the two selected experimental host plants for the experimental studies, those plants are routinely used in our laboratory.
Multiplication of *G. deserticola* on rootlets of *L. purpureus* petioles—The seeds of *L. purpureus* were surface sterilized in mercuric chloride (0.1%) for 2 min, washed in sterile distilled H₂O for 3 to 5 times, sown in earthen pots containing sterilized soil and kept at 24°C. After 15 days, fully expanded young leaves of *L. purpureus* with petioles were collected and treated with 200 mg/l of indole acetic acid (IAA) for 24 hr. To retain the green colour of leaves, they were sprayed with kinetin solution (100 mg/l). After 24 hr, the leaves were transferred to test tubes in such a way that the petioles were completely immersed in sterile distilled water, and water in the test tube was changed everyday. After 6 days, the petioles initiated rootlets.

The rooted leaflets were transferred to plastic cups containing sterilized vermiculite and spores of *G. deserticola*. The spores were isolated from *G. deserticola* by wet-sieving and decanting method. The residues filtered through 75 and 45 μm sieves were examined for small and detached spores. The spores trapped in the sieves were visualized under light microscope and counted for number of spores.

Assessment of mycorrhizal colonization was carried out by following Phillips and Hayman method. Isolation of AM spores was made by wet-sieving and decanting method. The residues filtered through 75 and 45 μm sieves were examined for small and detached spores. The spores trapped in the sieves were visualized under light microscope and counted for number of spores.

Extraction of DNA from *G. deserticola* spores—Genomic DNA of *G. deserticola* was extracted using Instagene (5% Chelex, Bio-Rad Laboratories, CA, USA). Fifty spores were crushed in 10 μl of Instagene using a micropestle and incubated at 56°C for 30 min. The sample was vortexed for 10 sec, followed by incubation at 100°C for 10 min, vortexed again for 10 sec and centrifuged at 10,000 x g for 3 min. The supernatant containing genomic DNA was removed, transferred to a fresh tube, and stored at -20°C.

Isolation of DNA from *L. fraterna*—DNA of *L. fraterna* was extracted from 7 day old mycelium, grown as stationary culture in 250 ml Erlenmeyer flasks in 50 ml MMN medium, using CTAB extraction procedure. The lyophilized fungal mycelium was ground to a fine paste in liquid nitrogen using a pre-chilled mortar and pestle and incubated in the preheated extraction buffer (1% CTAB, 50 mM Tris, pH 8.0, 10 mM EDTA, 1% mercaptoethanol) for 60 min at 65°C. After cooling to room temperature, an equal volume of chloroform:isoamyl alcohol (24:1) was added and the aqueous phase was separated by centrifugation at 10,000 x g for 10 min. To the aqueous phase, an equal volume of ice-cold isopropanol was added and kept at -20°C for 2 hr. The DNA was pelleted by centrifugation at 10,000 x g for 30 min at 4°C. The pellet was washed with 70% ethanol and air-dried briefly. It was dissolved in a measured volume of TE. RNase-A treatment was carried out in a water bath at 37°C for 1 hr. This was followed by phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and chloroform:isoamyl alcohol (24:1 v/v) extractions. The aqueous phase separated by centrifugation at 12,000 x g for 10 min was precipitated with ethanol. The pellet was washed with 70% ethanol, air-dried briefly and dissolved in TE buffer. The quantity and quality of the DNA preparation was determined by measuring the optical density at 260 nm and 280 nm, and separation in 0.8% agarose gel electrophoresis.

PCR amplification of ITS region from *G. deserticola* and *L. fraterna*—Primer sequences: forward primer ITS1 5'-TCCGTAAGTGAA-CCTCGG-3', reverse primer ITS4 5'-TCTCCG-GCTTATGGATATGC-3' were obtained from Gennei, Bangalore, India. PCR amplification was carried out in a thermocycler (Erecord, Deltacycler). Amplification reaction mixture (50 μl) contained 10X PCR buffer, 50 mM MgCl₂, 10 mM dNTPs; 75 pM of ITS1/ ITS4 primers, 2 units of Taq DNA polymerase (Gennei, Bangalore, India), and 10 μl of genomic DNA isolated from *G. deserticola* as template. The cycling parameters were initial pre-denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. The product was resolved by electrophoresis on 1.2% agarose gel. For *L. fraterna*, 100 ng of genomic DNA was used as template DNA.

Results and Discussion

Induction of rootlets and multiplication of *G. deserticola* in *L. purpureus*—Multiplication of AM fungus *G. deserticola* in the rootlets of *L. purpureus* leaf petiole base was attempted in this study, for the aim of producing spores without microbial contamination. The leaflets were induced by IAA, a rooting hormone and after 6 days, enormous young...
roots proliferated from the petioles of the leaves were put in pot containing vermiculite and *G. deserticola* inoculum, for a period of 90 days. For a comparative analysis, two more host plants, *K. pinnata* and *A. cepa*, with natural roots, were also prepared for multiplication of *G. deserticola* spores. The roots were evaluated for AM fungal colonization at 30, 40, 50, and 60 days post inoculation (Table 1; Fig 1A). The percentage of AM fungal colonization was higher (80%) in *G. deserticola* inoculated induced rootlets of *L. purpureus* on 60 days of post inoculation, compared with the hosts *K. pinnata*, *A. cepa* (50, 56% respectively), with natural roots as the source for multiplication (Table 1). On 90th day of post inoculation, young spores were isolated from the vermiculite base. The number of spore was low in both the two host plant species with natural roots, compared with the induced rootlets of *L. purpureus* (Table 1). The number of *G. deserticola* spores counted was 29/g vermiculite base in *L. purpureus* (Fig 1B; Table 1), whereas the number of spores in *K. pinnata* and *A. cepa* were 12, 15/g vermiculite base, respectively (Table 1). Our results revealed that the spore formation capacity of *G. deserticola* in *K. pinnata* and *A. cepa* was low, and statistically not significant between these two plants. However, induced rootlets of *L. purpureus* were more efficient in AM fungal colonization, and the subsequent spore formation capacity was significantly higher than the AM fungi *G. deserticola* inoculated natural roots of *K. pinnata* and *A. cepa*, respectively (Table 1). Therefore, due to the formation of more percentage of colonization, and more number of spores than the hosts with natural roots as a source for multiplication, our system with induced rootlets of *L. purpureus* provides a simple way to propagate *G. deserticola* spores to prepare spores without microbial contamination and that spores can be isolated for pure DNA preparation for further molecular analysis.

**PCR amplification of ITS region from *G. deserticola* spores and *L. fraterna*—Total DNA extracted from *G. deserticola* was subjected to PCR

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**Table 1**—Percentage of mycorrhizal colonization, and total number of spore production—a comparative study in three different host plants.

<table>
<thead>
<tr>
<th>Host plants</th>
<th>Percentage of VAM fungal colonization at 10 days interval of post inoculation</th>
<th>Total number of spores per 1 g base on 90th day of post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0% 0% 0% 0%</td>
<td></td>
</tr>
<tr>
<td><em>Lablab purpureus</em> (induced roots)</td>
<td>20 ± 1 37 ± 1 62 ± 1 80 ± 2</td>
<td>29 ± 1</td>
</tr>
<tr>
<td><em>Kalanche pinnata</em> (natural roots)</td>
<td>10 ± 2 21 ± 1 30 ± 3 50 ± 4</td>
<td>12 ± 2</td>
</tr>
<tr>
<td><em>Alium cepa</em> (natural roots)</td>
<td>15 ± 1 24 ± 2 35 ± 2 56 ± 3</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

The data presented was an average value of 3 independent experiments, each experiments with 15 experimental host plants. The standard deviation indicates the significance of the data.
using primers ITS1/ITS4, yielded a product of ~500 bp as revealed by agarose gel electrophoresis (Fig. 2A, B).

Based on PCR-amplification of the ITS-region of G. deserticola genomic DNA using ITS1/ITS4 primers, resulted in a single band of ~500 bp. It was reported previously that the primers ITS1 and ITS4, specifically amplified the ITS region of 10 species of ectomycorrhizal fungi, Amanita jacksonii, Boletus piperatus, Hebeloma cylindrosporum, L. amethystina, L. bicolor, L. laccata, L. proxima, Paragyrodon sphaerosporus, Pisolithus tinctorius, and Suillus sinuspaulianus, respectively.

In this study, in addition to G. deserticola, we used L. fraterna, an ecto-mycorrhizal fungi, as a control. PCR amplification of the ITS-region from the genomic DNA of L. fraterna with primers ITS1/ITS4 yielded a fragment of ~500 bp as revealed by agarose gel electrophoresis (Fig 2A).

Our findings showed that the size of the ITS-region in L. fraterna was ~500 bp to that of reported other Laccaria genus. However, it differed at species levels from the so far reported ectomycorrhizal fungi. The ITS-region showed two variable non-coding regions that were rested within the rDNA repeat between the highly conserved small sub-unit 5.8S and large sub-unit rRNA genes. Several features make ITS-region as a convenient target region for the molecular identification of a fungus. Generally, in fungi, the entire ITS region is often between 500 to 800 bp and can readily be amplified with universal primers that are complementary to sequences within the rRNA genes. Moreover, the ITS region is often highly variable among morphologically distinctive fungal species.

We developed a reliable new method to multiply microbial contamination free AM spores to study molecular characterization of the fungi, using the induced root system of L. purpureus. In addition, we have found that specific primers amplified the ITS-regions, (ITS1 and ITS4) from G. deserticola and L. fraterna. It is a new report on the identification of ITS-regions in endomycorrhizal fungi G. deserticola, and L. fraterna, an ectomycorrhizal fungi.

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