



Research Article

STUDIES ON IDENTIFICATION OF ARBUSCULAR MYCORRHIZAL FUNGI - MORPHOLOGICAL AND DNA FINGERPRINTING TECHNIQUE

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(Received: October 10, 2015; Accepted: November 19, 2015)

ABSTRACT

The aim of this study was to investigate the identification of four different species of arbuscular mycorrhizal fungi namely, *Gigaspora margarita*, *Glomus mosseae*, *Glomus fasciculatum* and *Glomus leptoticum*. The identification was performed by the combination of morphological and molecular techniques. Random Amplified Polymorphic DNA assay [RAPD] were used to evaluate the genetic similarities between four different AMF species. The mycorrhizal spores were isolated from different location in Chennai and multiplied by Trap Culture Method. *Allium cepa* was used as host plant in Trap culture. Genomic DNA of 4 different species was isolated by cetyltrimethyl ammonium bromide (CTAB) method. The isolated DNA sample was amplified with four different random primers. The RAPD analysis was applied to determine DNA polymorphic differences and relatedness.

Keywords: Mycorrhizal spores, *Allium cepa*, PCR, SDS-PAGE, RAPD Analysis.

INTRODUCTION

Arbuscular mycorrhizal Fungi are ubiquitous forms of mycorrhiza and they are distributed over all types of soil in various habitats (Vestbery, 1995). Exchange of nutrients take place between plant and mycorrhizal fungi (Wardle and van der Putten, 2002). AMF is surviving better in extreme environmental conditions (Sylvia and Williams, 1992). Identification of AMF Spore is based on its morphological characters (Sturmer and Morton, 1997) and they are identified with the presence of arbuscules and vesicles (Smith and Read, 2008). The mycorrhizal hyphae produce arbuscules inside the cell and vesicles outside the cell (Tisdale *et al.*, 1995). The rhizosphere part of plant roots is colonized by AMF spores (Mosse, 1986). Mycorrhizal association is carried by the host plant and fungus and other environmental factors (Harley and Smith, 1983). AMF colonization shows resistance against plant diseases (Hooker *et al.*, 1994) and it

also decrease the extent of root diseases (Bagyaraj, 1984; Hooker *et al.*, 1994). Molecular techniques are suggested to identifying the phylogenetic relationship using ribosomal genes of AMF spores (Redecker *et al.*, 1997). PCR based method is used for detecting the colonization of mycorrhizal fungi with plant roots (Vandenkoornhuyse *et al.*, 2001). DNA markers are developed to identify the mycorrhizal fungi (Ram Reddy *et al.*, 2005). RAPD method is used to determine the DNA polymorphic differences between two organisms (Welsh and McClelland, 1990; Williams *et al.*, 1990). The aim of the present study was to isolate four different species of arbuscular mycorrhizal fungi namely, *Gigaspora margarita*, *Glomus mosseae*, *Glomus fasciculatum* and *Glomus leptoticum*. The RAPD technique was attempted to analyse the DNA polymorphism and homogeneity among the isolated AMF spores.

MATERIALS AND METHODS

Isolation, identification and multiplication of AMF spores

Soil sample was collected from ECR road, Old Mahabalipuram road, Kovalum, Maduravayil and Tambaram in Chennai, Tamil Nadu, India from the rhizosphere of plant roots. The AMF spores were isolated using wet sieving and decanting technique (Gerdemann and Nicholson's, 1963). AMF spores were sorted through dissecting microscope and confirmed as *Gigaspora margarita*, *Glomusmosseae*, *Glomusfasciculatum* and *Glomusleptoticum* based on size, shape, colour and hyphal attachment (Walker, 1983). The identified spores were multiplied by trap culture technique (Morton et.al., 1993). *Allium cepa* was used as a host plant for the multiplication of AMF spores.

Molecular characterization of AMF

Mycorrhizal DNA was extracted from the spores by the CTAB method (Ausubel et al., 1994). CTAB extraction buffer was added to the root samples. The mixture was incubated for 60 mins at 65°C and 4.5 mL of Chloroform/ Isoamyl alcohol (24: 1) was added and rocked gently for 10 min and centrifuged at 10,000 rpm for 10 min at room temperature. Top aqueous layer was pipetted into 10 mg / mL RNase enzyme and to the content equal volume of phenol: chloroform / Isoamyl alcohol was added, mixed gently and centrifuged at 10,000 X g for 10 min at room temperature. The top aqueous layer was mixed with 0.6 volumes of ice cold isopropanol. The precipitated DNA was washed with 70 % ethanol, dried at 65 °C and dissolved in 100 µL of T₁₀ E 0.1 buffer. After DNA extraction the concentrations of DNA in the samples were estimated at determination of absorbance at 260 nm. The purity of the DNA samples was checked by the absorbance ratio of A 260 nm / A 280 nm (Becker et al., 1996).

Random amplified polymorphic DNA (RAPD) analysis

The difference among the AMF isolates was analyzed by RAPD-PCR (Lee et al., 1988). Amplification of DNA fragments was carried out in thermal cycler. PCR reaction mixture contains four random primers - CTGCCGCCAC, AGGGGTCTTG, AGGTGACCGT and GGACCCAACC, 1.5 units of Taq polymerase enzyme, 10 X PCR Amplification buffer (50 mM KCL, 100 mM Tris pH 8.0, 15 mM MgCl), 10 mM dNTP, Template DNA, Distilled water. The temperature

cycles were 94°C for 5 minutes, followed 94 °C for 1 minute, 32 °C for 2 min, 72 °C for 2 mins (35 cycles), 4 °C for 10 minutes. The RAPD PCR products (20 µL) were ran on 2 % agarose gel electrophoresis with ethidium bromide stain along with the nucleic acid molecular marker as the standard.

RESULT:

Table 1: Number of AMF Spores

Area	Number of AMF Spores/ Kg
ECR road	225
Old Mahabalipuram road	158
Kovalum	125
Maduravayil	97
Tambaram	83



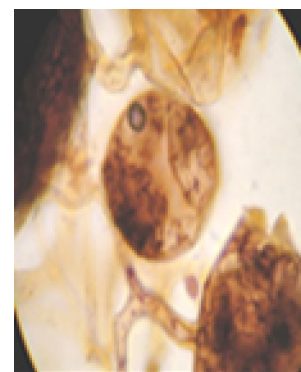
Gigaspora margarita



Glomus mossae



Glomus fasciculatum



Glomus leptoticum

Figure 1: The AMF spores

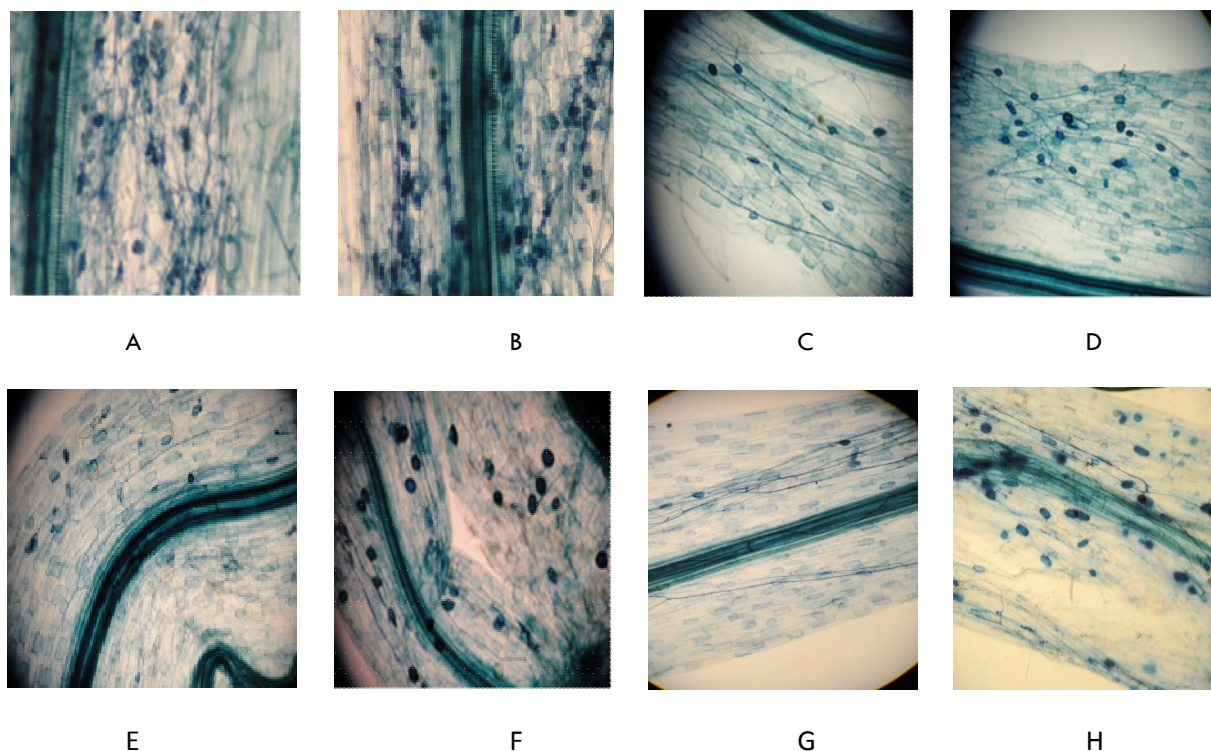
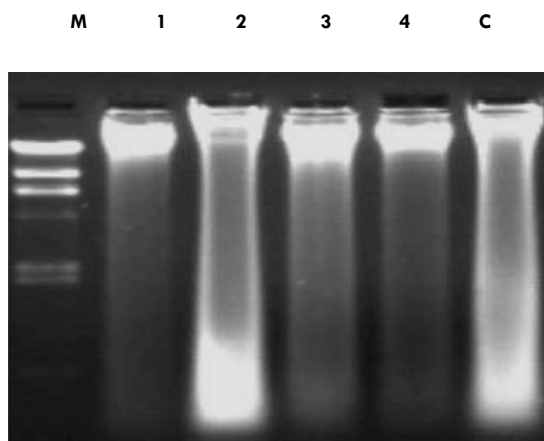


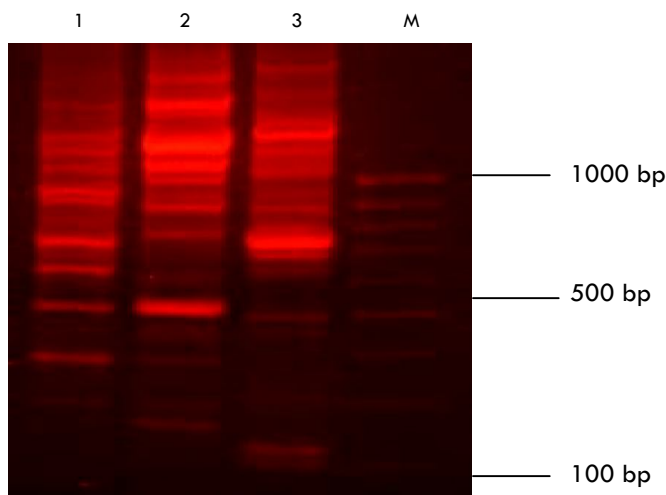
Figure 2

A-Association of *Gigaspora margarita* at 15 days, B- Association of *Gigaspora margarita* at 30 days C-Association of *Glomus mosseae* at 15 days, D- Association of *Glomus mosseae* at 30 days, E- Association of *Glomus fasciculatum* at 15 days, F- Association of *Glomus fasciculatum* at 30 days, G- Association of *Glomus leptoticum* at 15 days and Association of *Glomus leptoticum* at 30 days.



M- λ DNA/Hind III digest marker; 1- Genomic DNA from *Allium cepa* roots associated with *Gigaspora margarita*; 2- Genomic DNA from *Allium cepa* roots associated with *Glomus mosseae*; 3- Genomic DNA from *Allium cepa* roots associated with *Glomus fasciculatum*; 4- Genomic DNA from *Allium cepa* roots associated with *Glomus leptoticum*; C- Control DNA from uninfected onion roots.

Figure 3: Genomic DNA isolation by CTAB method



1- RAPD of *Glomus mosseae*, 2- RAPD of *Glomus fasciculatum*, 3- RAPD of *Glomus leptoticum*, M- 1 Kb Marker

Figure 4: Random Amplified Polymorphic DNA analysis

Gigaspora margarita

Colour- varies from hyaline to white, spore size – varies from 260-480µm. It contains a suspensor with a width of 27-58µm and 1 wall group; the auxillary cells are clustered or spiny.

Glomus mossae

Colour varies from yellow to dark brown shape varies from globose to ellipsoid,walls are thick and hyaline and brownish yellow.Size ranges from 60-320µm, thick outer membrane is attached to a funnel shaped hyphae.

Glomus fasciculatum

Colour varies from yellow to golden yellow, spore size - varies from 60- 105µm and it contains a subtending hyphae,spore wall is smooth and membranous. Shape varies from globose to sub globose.

Glomus leptoticum

Colour varies from yellow to off white, Indistinct, size- varies from 48- 262µm, spore form singly or in clusters, a subtending hyphae is present,spore contents are enclosed with in a membrane.

DISCUSSION

In my present study the rhizosphere soil samples were collected from distinct part of Chennai and the total number of spores isolated from various regions gets varied (Kehri and Chandra, 1988). The data of total number of spores that were collected per 1 kg of soil is tabulate in (Tab. 1).

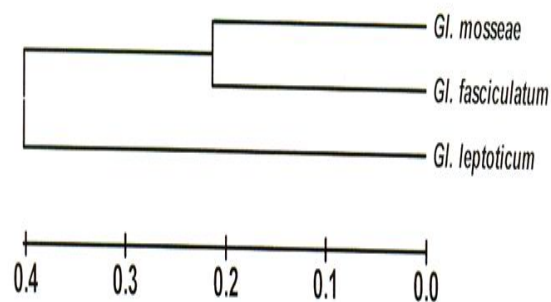


Figure 5: Dendrogram Cluster Analysis

Based on morphological character the AMF spore colour, size, shape and extended hyphae the spores were identified as *Gigaspora margarita*, *Glomus mosseae*, *Glomus fasciculatum* and *Glomus leptoticum* (Fig.1 and 2). RAPD method was used to examine the similarity and polymorphism between 4 different AMF spores. The Genomic DNA fragments of *Glomus mosseae*, *Glomus fasciculatum* and *Glomus leptoticum* was amplified using random primers except *Gigaspora margarita* and it was difficult to extract DNA and to amplify the DNA from the *Gigaspora margarita* and the extracted DNA was used as a template (Fig. 3). Annealing temperature during RAPD analysis was 32°C. At that temperature, *Gigaspora margarita* did not respond for RAPD. Polymorphic bands were used to analyse the genetic similarity and genetic polymorphism between each pair of species (Fig. 4 and 5). The RAPD study shows 34.48 % similarity between *Glomus mosseae* and *Glomus fasciculatum* when compare to other pairs. The polymorphism range was higher (79.31%) in *Glomus mosseae* and *Glomus leptoticum* and it indicates higher percentage of homogeneity among other group of mycorrhiza. The maximum numbers of amplified fragments were obtained from *Glomus fasciculatum* and *Glomus leptoticum*.. Further research work is required to isolate pure AMF gene to perform gene Sequencing.

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