Morpho-Typing and Molecular Diversity of Arbuscular Mycorrhizal Fungi in Sub-Tropical Soils of Coimbatore Region, Tamil Nadu, India

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Abstract The diversity potential of arbuscular mycorrhizal fungi (AMF) in three different tropical soils of southern part of India was assessed by traditional morpho-typing of AMF-spores and by culture-independent nested-PCR of internal transcribed spacer region of ribosomal genes. The population diversity of AMF in soil was strongly correlated with available P_2O_5 in soil. Among the three different soils, black-cotton soil had more diversified AMF species than alluvial and red sandy soils. Pooled data of morpho-typing and sequence-driven analysis revealed that *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora* are the AMF genera present in these soils. The diversity of AMF in soil differs with the mycorrhiza colonizing the plant roots.

Keywords Arbuscular mycorrhizal fungi · Diversity · Internal transcribed spacer · Morpho-typing · Nested-PCR

Introduction

The arbuscular mycorrhizal symbiosis is most commonly occurring underground symbiosis in plant and found in a large majority of terrestrial plants [1]. The mycorrhizal association involved in plant nutrient uptake, protection against soil borne pathogens and improvement of soil stability. As fungal strains may differ in their effect on host plants, detection of arbuscular mycorrhizal fungal (AMF) diversity in soil is very essential for any agro-system [2].

Approximately 150 AMF species have been described by means of morphological features of spores [3]. Several works on this issue revealed that characterization of spore morphology from an ecosystem may not reflect the kind of AMF that are active inside the root [4–6]. Further more, some recently identified lineages of Glomales may go undetected by staining techniques [5]. The detection level obtained by morpho-typing may not suitable for some ecological studies of AMF. Alternatively, molecular techniques based on DNA analysis seem to offer a wide-range of advantages over conventional methods [6].

Indirect (spore isolation from soil) and direct (DNA isolation from roots) methods have been used successfully in assessing AMF diversity within an ecosystem [7]. Spore-based methods rely on identification of morpho-species and record simply the presence or absence of the spores regardless of functional significance. Simple extraction of spores directly from the soil is inadequate and sequential trap cultures must be used to record the full range of species present and to encourage initially non-sporulating fungi to reveal themselves. In contrast, direct molecular analyses reveal the diversity of fungi occupying the root and presumably contributing to the mycorrhizal effect on plant growth. However, the species may or may not correlate with traditional morphospecies and the gene tree approach to taxon recognition is sensitive to sampling bias [8]. Though several methodologies have been followed in understanding the biology of these organisms, molecular marker-based studies are almost missing in India [6, 9]. The morpho-typing coupled with ribosomal based identification of AMF would give more information than the individual approach. Hence the present work was conducted to correlate the morpho-typing and PCR- based molecular typing of AM fungi in three different tropical soils of this region.

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Materials and Methods

Soil Sampling

Three different soil types present in Coimbatore district were collected at fallow period (September, 2008) for this study. Each sample contains three replicates and each replicate is the pooled sample of five different locations of top soils (0–20 cm). After removal of stubbles, pebbles and gravels manually, samples were packed into three lots and stored at 4°C for further use. The samples for physio-chemical properties (Table 1) were further air dried and analysed. The microbiological analysis of the soil was carried out on the same day of sampling to minimize the storage effects.

AM Count

The soil samples were subjected to count and isolate AM spores by following standard wet-sieving and decantation method as described by Gerdemann and Nicolson [10] and expressed as number of spores per 100 g soil. AM spores were observed through stereozoom microscope (Nikon, Japan) and identification of AMF was done as proposed by Hall and Fish [11]. The infective propagules of AMF present in the soil samples were analysed by MPN technique [12].

Trap Culture

All the three soil samples were raised with common AM trap crop, maize (cultivar Co1) up to 45 days under green house condition and the plants were uprooted and washed with sterile water till free of soil, cut into 1 cm bit,

homogenized and estimation of mycorrhizal colonization of the root was done after clearing and staining the roots [13]. Presence of AMF-hyphae or vesicles was taken as positive indication for colonization and the per cent AMF colonization was estimated by examining 100 root bits for each sample.

Mycorrhizal Diversity Analysis by Direct Molecular Method

The homogenized root bits were powdered with liquid-nitrogen and total DNA was extracted using DNAeasy Plant DNA extraction kit (Qiagen, Germany) and DNA concentration was adjusted to 20 ng/μl. The nested-PCR was performed using this DNA as template to amplify full-length of internal transcribed spacer (ITS) region as described by Redecker et al. [14]. The first-step PCR was performed with universal eukaryotic primers NS5 and ITS4 [15] and 100 times diluted first PCR products were used as template for second-step PCR using the F-ITS4, fungal specific reverse primer [16] and NS5, forward primer [14].

PCR amplified ITS region was purified using PCR clean-up kit (GenElute PCR Clean-up kit, Sigma, USA) according to the manufacturer's instruction and cloned using pTZ57R/T vector supplied with T/A cloning kit (Fermentas, USA). All the white clones were subjected for re-amplification of insert by M13 forward and reverse primers (Fermentas, USA) and restricted with *Hae*III enzyme (Fermentas, USA). The restriction reaction was carried out at 37°C for 3 h and resolved the banding pattern using 2% agarose gel electrophoresis. Banding patterns were visualized by ethidium bromide staining and documented in AlphaImager TM1200 documentation and analysis system. The number of similar banding patterns

Table 1 Physio-chemical and microbiological properties of sub-tropical soils used in this study

Properties	Black cotton soil	Red sandy soil	Alluvial soil
pH	8.66 ± 0.02	8.29 ± 0.01	8.47 ± 0.01
EC (dS m ⁻¹)	0.46 ± 0.01	0.50 ± 0.01	0.37 ± 0.01
Texture	Sandy clay	Sandy clay	Clay
Organic C (g/kg)	4.4 ± 0.10	2.6 ± 0.12	3.8 ± 0.10
Available N (kg/ha)	227 ± 6.56	260 ± 7.51	241 ± 6.96
Available P ₂ O ₅ (kg/ha)	34.4 ± 0.99	10.8 ± 0.31	11.8 ± 0.34
Available K ₂ O (kg/ha)	715 ± 20.64	639 ± 18.44	993 ± 28.67
Total bacteria (× 10 ⁶ cfu/g dw soil) ^a	68.23 ± 1.97	20.68 ± 0.60	76.47 ± 2.21
Total fungi (× 10 ⁴ cfu/g dw soil) ^b	64.70 ± 1.87	10.34 ± 0.30	70.58 ± 2.04

Values are mean ± SE of three replicates; the physio-chemical properties were measured according to Jackson [29]

^a Total bacterial count on soil extract agar medium using serial dilution and plating method [30]

^b Total fungal counts on Rose Bengal Agar medium using serial dilution and plating method [31]

were grouped and representative clone from each group was selected for sequencing using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis by Applied Biosystems automated sequencer (Model 3100). The identity of ITS sequence was established by

performing similarity search against GenBank database (<http://www.ncbi.nih.gov/BLAST>) and phylogenetic tree was constructed using Neighbor-joining method [17] using MEGA 4.0 [18] and the tree file was analyzed using same software.

Fig. 1 AM infected maize roots showing intra-cellular hyphal growth (a) and vesicles (b) of AM fungi from three different soils. *S1* Black cotton soil, *S2* Red sandy soil, *S3* Alluvial soil

Results

Physio-chemical characters of the soil samples collected from different agricultural fields of Coimbatore, India revealed that they are distinct in their nutritional and biological properties. Red sandy soil having poor nutrients status than other two soils also reported in microbiological properties. Alluvial soil and black cotton soil recorded maximum bacterial and fungal populations than red soil (Table 1).

Arbuscular mycorrhizal spore count was lesser in black soil, which recorded very high P status. In contrast, AM spore counts as well as infective propagules were recorded higher in red soil which is comparatively poor in nutrients status as well as P status. When these soils were grown with trap crop maize, root colonization was observed in all the soils and not much variation was recorded among the soil types (Fig. 1; Table 2).

The morphological traits which were taken for the morpho-typing of AMF include sporocarp, subtending hyphae, wall layers, spore colour and shape. It was observed that predominantly two types of AM spores viz., *Glomus mosseae* and *Scutellospora* sp. were present in all the three soil samples analysed (Fig. 2; Table 3). *Glomus fasciculatum* and *G. geosporum* were occurring only in black soil and red sandy soils. Alluvial soil recorded the unique presence of *G. albidum*, *Gigaspora gigantea* and *Acaulospora* sp. Totally eight different AM fungi were observed in black soil, out of which six species belongs to *Glomus*, one on *Gigaspora* and one on *Scutellospora*. In red soil, out of five, three belongs to *Glomus* and one each of *Gigaspora* and *Scutellospora*. In alluvial soil, out of five, two are *Glomus*, one each of *Gigaspora*, *Scutellospora* and *Acaulospora*.

Further, the AM diversity was assessed for these soils by direct method in maize roots as trap crop. The nested PCR amplified the ITS regions of ribosomal genes (about 1200 bp) from the fungal DNA present in the root, as there is no PCR amplification in the second PCR, when non-mycorrhizal maize root DNA was used (Fig. 3). After cloning all the PCR products using T/A cloning vector, 30 clones from red sandy soil, 22 from alluvial and 53 from

black cotton soil were obtained. RFLP analysis of these three cloned libraries distinctly grouped into four or five clusters. Alluvial soil and black cotton soil had five clusters, while red sandy soil had only four (Table 4). The representative of each RFLP profile, invariable of soil type were selected for sequencing and such seven clones were selected and sequenced. The closest species and per cent homology with existing sequences are presented in Table 4. The BLAST analysis identified five distinct species of AMF present in the soils, such as *Acaulospora laevis*, *Acaulospora* sp., *Scutellospora heterogama*, *Glomus geosporum* and *Glomus mosseae*. The distribution pattern of these species were almost in accordance with morpho-typing and few species identified by morpho-typing of soil like *G. fasciculatum*, *G. etunicatum*, *G. viscosum*, *G. albidum*, *Gigaspora decipiens* and *G. gigantea* are not detected in maize roots. Further, the phylogenetic tree using Neighbor-joining method was in accordance with already published sequences and matches with the family proposed (Fig. 4). Among the three different soils examined for AMF diversity, the black cotton soil recorded high diversity followed by alluvial soil and red lateritic soil.

Discussion

AMF have considerable impact on plant growth and management of these fungi in sustainable agriculture is essential [19]. As AMF diversity is essential for colonization and formation of symbiotic relation between plant and fungi and each fungal species differ in its host infectivity, studying species richness of AMF for each soil will be helpful for effective AM inoculant development and to provide resource for elite strains. With this background, a small-level survey was made to assess the AMF diversity in the three different agricultural soils that are extensively present in Coimbatore (India) region. The results clearly indicated that nutritionally-poor soil (red soil) had more AMF population. It is well known that P availability is critical to AM symbiosis and always had negative correlation with available P_2O_5 [20, 21], which was noticed in present study also. However, fungal colonization was not

Table 2 AMF population in three different sub-tropical soils of Coimbatore

Soil samples	AMF spores per 100 g soil	AMF infective propagules (MPN count per 100 g soil)	AM fungal colonization in maize ^a (%)
Black cotton soil	135 ± 3.90	5.40 × 10 ³	80 ± 1.33
Red sandy soil	150 ± 4.33	7.00 × 10 ³	78 ± 2.02
Alluvial soil	145 ± 1.89	2.80 × 10 ³	79 ± 2.28

Values are mean ± SE of three replicates

^a The soil grown with maize as trap-crop and the per cent infection by AMF was assessed on 45th day by clearing and staining the roots

Fig. 2 Spore morphology of AM fungi isolated from three different soils. S1, Black cotton soil; S2, Red sandy soil; S3, Alluvial soil. Gc, *Glomus clarum*; Gf, *Glomus fasciculatum*; Ge, *Glomus etunicatum*; Gm, *Glomus mosseae*; Gv, *Glomus viscosum*; Gg, *Glomus geosporum*; Ga, *Glomus albidum*; Gid, *Gigaspora decipens*; Gim, *Gigaspora margarita*; Gig, *Gigaspora gigantea*; Scn, *Scutellospora nigra*; Sc, *Scutellospora* sp.; Ac, *Acaulospora* sp.

merely controlled by soil nutrients [22], and all the soils sown with maize crop recorded uniform infection percentage.

The morpho-typing of AMF spores collected in these soils recorded high-diversity and among the three soils, black-cotton soil recorded maximum types of AMF (eight different types). Similar morpho-typing dependent surveys made

world-wide recorded quite high number (13–20) of species in one type of soils [23]. The major factors limiting the diversity are soil, crop plants and agro-climate of the region [24]. Most of the studies mainly focused the host plant species to study the AMF diversity but the diversity of AMF varies from plant species to species [21, 25, 26]. Hence, the present study, we focused the soil diversity rather than host-plant to get more

Table 3 Morpho-typing and distribution pattern of AMF spores in three different sub-tropical soils

Species identified ^a	Black cotton soil	Red sandy soil	Alluvial soil
<i>Glomus clarum</i>	+	–	–
<i>Glomus fasciculatum</i>	+	+	–
<i>Glomus etunicatum</i>	+	–	–
<i>Glomus mosseae</i>	+	+	+
<i>Glomus viscosum</i>	+	–	–
<i>Glomus geosporum</i>	+	+	–
<i>Glomus albidum</i>	–	–	+
<i>Gigaspora decipens</i>	+	–	–
<i>Gigaspora margarita</i>	–	+	–
<i>Gigaspora gigantea</i>	–	–	+
<i>Scutellospora</i> sp.	+	+	+
<i>Acaulospora</i> sp.	–	–	+

^a The key system generated by Hall and Fish [11] were used for identification

Fig. 3 Amplification of ITS regions of ribosomal DNA from maize roots grown with three different soils by nested PCR. The universal eukaryotic ITS primers (NS5 and ITS4) in PCR-I with maize root DNA as template. The 100 times diluted product of PCR-I was used as template for PCR-II with fungal specific ITS primers (NS5 and F_ITS4). *M* 1 kb and low range DNA markers, *S1* Black cotton soil, *S2* Red sandy soil, *S3* Alluvial soil, *F* *Aspergillus flavus* genomic DNA (as positive control for PCR I and II); *C* Uninfected maize root DNA (negative control for PCR-II)

Table 4 Occurrence of AMF in three different sub-tropical soils by ITS sequencing from maize root

Clone name	Sequence homology	Number of similar clones ^d		
		Black cotton soil	Red sandy soil	Alluvial soil
DLS1	<i>Acaulospora laevis</i>	20	3	2
DLS2	<i>Acaulospora</i> sp.	11	–	–
DLS3	<i>Acaulospora laevis</i>	8	–	–
EBS1	<i>Scutellospora heterogama</i>	8	14	6
MBS1	<i>Glomus geosporum</i>	–	5	5
MBS2	<i>Glomus mosseae</i>	–	8	4
MBS3	<i>Glomus mosseae</i>	6	–	5

^a Species identified based on ITS sequence similarity search against the GenBank database

^b Accession number of the closest species in NCBI BLAST search

^c Per cent similarity of the sequence in BLAST result

^d Similar RFLP profiling clones are grouped after digested with *Hae*III

Fig. 4 Neighbor-joining tree of AM fungi by ITS regions of ribosomal gene amplified from maize roots grown in three different sub-tropical soils. DLS, MBS and EBS are the clones designated and selected for sequencing from RFLP profiling. The GenBank

Accession numbers were given in parentheses. Numbers in the nodes denote bootstrap values from 1000 replicates. The phylogenetic tree was clustered into different families as proposed by Schubler et al. [32]

information about AMF-diversity. The morpho-typing of AMF present in the soils revealed that soil with low P recorded more species than P rich soil. In order to correlate the traditional morpho-typing with recent molecular techniques to identify the AMF-diversity, we used full-length amplification of ITS regions by nested PCR followed by RFLP. As described earlier by several workers [16, 27, 28], the infected AMF in trap crop and its diversity of root-colonized AMF was less than the soil AMF.

It is concluded that AMF diversity varied among the three soils surveyed in present work and P deficit soil harbored more AMF spores than P rich soils, but with less diversity. The morpho-typing and ITS based PCR assay revealed that *Glomus*, *Gigaspora*, *Scutellospora* are predominantly present in three soils. It is also concluded that soil-AMF diversity differed from the roots colonized AMF in maize as trap crop.

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