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Differential Response of Arbuscular Mycorrhizal Sporocarps in Long-Term Trap Culturing

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi belonging to phylum Glomeromycota are recognised biofertilizers, but have limited application in fields in absence of effective methods for their rapid multiplication. Of the several structures produced by AM fungi during their life cycle, sporocarps have received little attention in the context of mass multiplication. In the present study isolated sporocarps belonging to five Glomeromycota species, collected from natural forests of Arravalis in Delhi, India, were used as a source of inoculum for multiplication in long-term trap cultures. The differential response of these sporocarps with reference to their propagation in next generation and in serving as a clean source of spores in long-term trap culturing also helped in clarifying the role played by these structures in the life cycle of AM fungi.

Key words – Arbuscular, AM, biofertiliser, mycorrhizal, sporocarps, trap culturing

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Introduction

Arbuscular mycorrhizas (AM) are symbiotic associations between fungi belonging to phylum Glomeromycota and roots of most land plants. They are recognised biofertilisers that are sources of phosphorus, nitrogen and other micronutrients (Smith & Read, 2008; Asmelash et al., 2016; Berruti et al., 2016; Igiehon & Babalola, 2017). Sporocarps of arbuscular mycorrhizas are relatively simple structures consisting of spore clusters, which may or may not be covered by a peridium (Błaszkowski, 2012). The sporocarps of Glomeromycota are asexual structures, thus represent clusters of genetically identical spores. They are large (350 µ to 1 mm) which makes them visible through unaided eyes (Goto & Maia, 2005; Walker, 2013). The peridium of sporocarps protects the enclosed spores from other soil microorganisms. Sporocarps represent an important structure in the life cycle of AM fungi, upon which most species discovery and description were based in earlier times, before a shift to phylogenetic classification and identification was made by the researchers (Strumer, 2012; Redecker et al., 2013). However, it has not yet been possible to induce sporocarp production in laboratory conditions, although unplanned production of epigeous sporocarps of *Glomus epigeous* have been observed in long-term storage after ten years (Daniels & Menge, 1980).

AM fungi are obligate symbionts and can not be multiplied in absence of host plants (reviewed by Berruti et al., 2016). There are three methods for multiplication of AM fungi in laboratory or greenhouse experiments: substrate based production, substrate free production (aeroponics) and *in vitro* production system (reviewed by Akhtar & Abdulla, 2014). Since mono axenic or in vitro culture methods have been successful only for few limited species, substrate based culture methods remain the most popular method, being applied in 75 per cent of the studies according to Berruti et al. (2016) and Igiehon and Babalola (2017). Spores of AM fungi carry infection by other soil microorganisms on their surface, which limits the success rate of single spore cultures to just 2%. It is yet not known whether single isolated spores are not able to multiply due to some inherent factor or it is because of difficult to remove damaging infection on their surface. The spores in spore clusters or sporocarps, being genetically identical and being protected by peridium from infection by other microorganisms might offer an alternative or additional source for start of culture using genetically similar spores. In addition, the large average sporocarp size (300 to 3000 microns) in comparison to spore size (30 to 300 microns) would make sporocarp collection and picking a simpler task.

Dried roots with fungal hyphae, vesicles, arbuscules and other fungal structures or soils with spores and hyphae constitute other popular sources of inoculum for

multiplication of these fungi (Brundrett et al., 1996). Various AM fungal taxa differ in their ability to be propagated from a given propagule. Propagation through mycelial fragmentation is preferred for species of the Glomeraceae family, whereas spore germination is the preferred method for members of other families (e.g., Gigasporaceae, Acaulosporaceae and Scutellosporaceae) (Berruti et al., 2016). A survey of the prevalent use of different types of inocula of AM fungi revealed that a mixture of different types of fungal propagules (crude inoculum) was used in 68% of the reviewed experiments, spores alone in 14%, spores mixed with hyphae in 4%, and root inoculum in 2% of the cases (Berruti et al., 2016). However sporocarps have not yet been tried for this purpose.

The identity of starter inocula for multiplication and the resultant culture must be confirmed by molecular methods because identification solely based on morphological characters very often leads to wrong identification (Redecker et al., 2013). Mistakes in the identification of AM fungi up to the level of genera have been reported. For example, on the basis of morphological characters, a species of *Acaulospora* was incorrectly named as *Ambispora brasiliensis*, which is incorrect at the generic, familial and even ordinal level (Goto et al., 2008).

In the present study, in order to explore the feasibility of using sporocarps as starter inocula, five different AM fungal sporocarps collected from natural forests of Aravalli in Delhi, India, were used as starting material in trap cultures, and their multiplication, fresh sporocarp production and contamination by other fungi were tracked in culture. Molecular methods were applied to confirm identification of these taxa.

Materials and Methods

Sporocarps belonging to five species of Glomeromycota namely: *Sclerocystis sinuosa* Gerd. & B.K. Bakshi, *Diversispora aurantia* (Blaszk. Blanke, Renker & Buscot) C. Walker & A. Schüßler, *Glomus* sp., *Glomus macrocarpum* Tul. & C.Tul. (1845) and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler, were collected from natural forests of Aravallis in Delhi, India. The technique of wet sieving, decanting and centrifugation (Daniels & Skipper, 1982) was done to isolate these sporocarps from soils. Morphological characters of sporocarps were used for initial identification before the start of trap cultures. The isolates were multiplied using multiple cycles of trap pot cultures in autoclaved soil with maize (*Zea mays* cultivar Narmada from IARI Delhi, India) as a host plant (Walker, 1999). A single isolated sporocarps/ spore clusters was placed on an aseptically grown, 5-day old root of maize seedling under dissecting microscope and the inoculated seedling was transferred to autoclaved soil for further growth.

There were ten replicates for each species. After every 3 months of the growing the plants, the soil in the pots was checked for AM fungal colonisation, spore and sporocarp production. After every 3 months of growth crop in each pot was checked for per cent AM fungal colonization in roots by root clearing and staining in Trypan Blue (Brundrett et al., 1996). Each slide was also checked for presence of contaminating structures such as other fungal hyphae, bacterial cell, spore etc., AM fungal spore density (no./ 50 g soil) and sporocarp (no./50 g soil) production was determined with wet sieving, decanting and centrifugation (Daniels and Skipper 1982). Spores isolated from cultures were checked for presence of contamination under compound microscope. Pure spores were clean and translucent and could easily be distinguished from infected spores. This continued until three consecutive crops were grown in the same pot for one and half years. No additional nutrients were supplied to these soils and tap water was used for watering.

For morphological identification of progeny, initial observations of spores and sporocarps (colour, shape, etc., in water) were recorded under a stereomicroscope (Nikon E100). Measurements (spore diameter, wall thickness, hyphal thickness and thickness at the attachment point) were made using a compound microscope (Nikon E200). Once the morphological data on the sporocarps and spores was generated, they were characterized up to the level of species using the manual for the identification of Glomeromycota (Blaszkowski, 2012). Some species were also characterized by the monograph (Hall, 1984), the INVAM (http://invam.caf.wvu.edu) and www.phylogeny.com websites.

Molecular characterisation could be done for species that produced sufficient clean uncontaminated spores and sporocarps in cultures and yielded genomic DNA in sufficient quantities. As the fungi grew within roots, and mycelia of different species get mixed up, genomic DNA extraction and amplification for sequencing is not practicable. Clean spores (up to 10) were collected in 0.5 ml tube and crushed thoroughly using a sterile pipette under the dissecting microscope. To each

sample, 100 µl of PCR buffer (Biorad) was added and mixed thoroughly. Genes encoding a fragment of small subunit (SSU) rRNA, internal transcribed spacer (ITS) and large subunit (LSU) rRNA were amplified using 'Krüger primers' (Krüger et al., 2012) with forward primer-5'-GTAGTCATATGCTTGTCTC-3' and reverse primer being 5'-GAAACCTTGTTACGACTT-3'. PCR was performed in the final volume of 50µl containing 1µl of DNA complex, forward primer 200 ng, reverse primer 200 ng, dNTPs (2.5mM each) 2 µl, 10x Taq pol assay buffer 10 µl, Taq polymerase (3µ/µl) 0.5 µl and water 34.5 µl. Amplification was performed as follows: 1x5 mins 90°C, 35x30 sec 94°C, 35x30 sec 55°C, 35x180 sec 72°C, 1x10 min 72°C (final extension) on Biorad T100 thermal cycler. The control contained no template DNA. For visualisation of PCR product, 10 µl of the amplification products were separated electrophoretically in 0.8% agarose gel and stained with ethidium bromide. Cloning and sequencing were done at Chromus Biotech Pvt. Ltd. The sequence data were initially identified by similarity searches in NCBI Blast. A total of 219 sequences showing >= 99% similarity to the new sequences (all Glomeromycota), were downloaded. Multiple sequence alignment using MAFFT was done at the EMBL-EBI server (http://www.ebi.ac.uk/). Maximum likelihood phylogenetic analyses were conducted using RAxML ver. 7.2.8 (Stamatakis et al., 2008) using 1000 bootstraps and the GTRGAMMA model for both bootstrapping and tree inference, through the CIPRES web-portal. ITOL (http://itol.embl.de/) was used for drawing the final trees.

Results

Of the five taxa collected (*Sclerocystis sinuosa*, *Diversispora aurantia*, *Glomus* sp., *Glomus macrocarpum* and *Rhizophagus intraradices*), the first three species had well-defined peridium with completely enclosed spores (Fig. 1), while the other two bore spore clusters without peridium (Fig. 2).

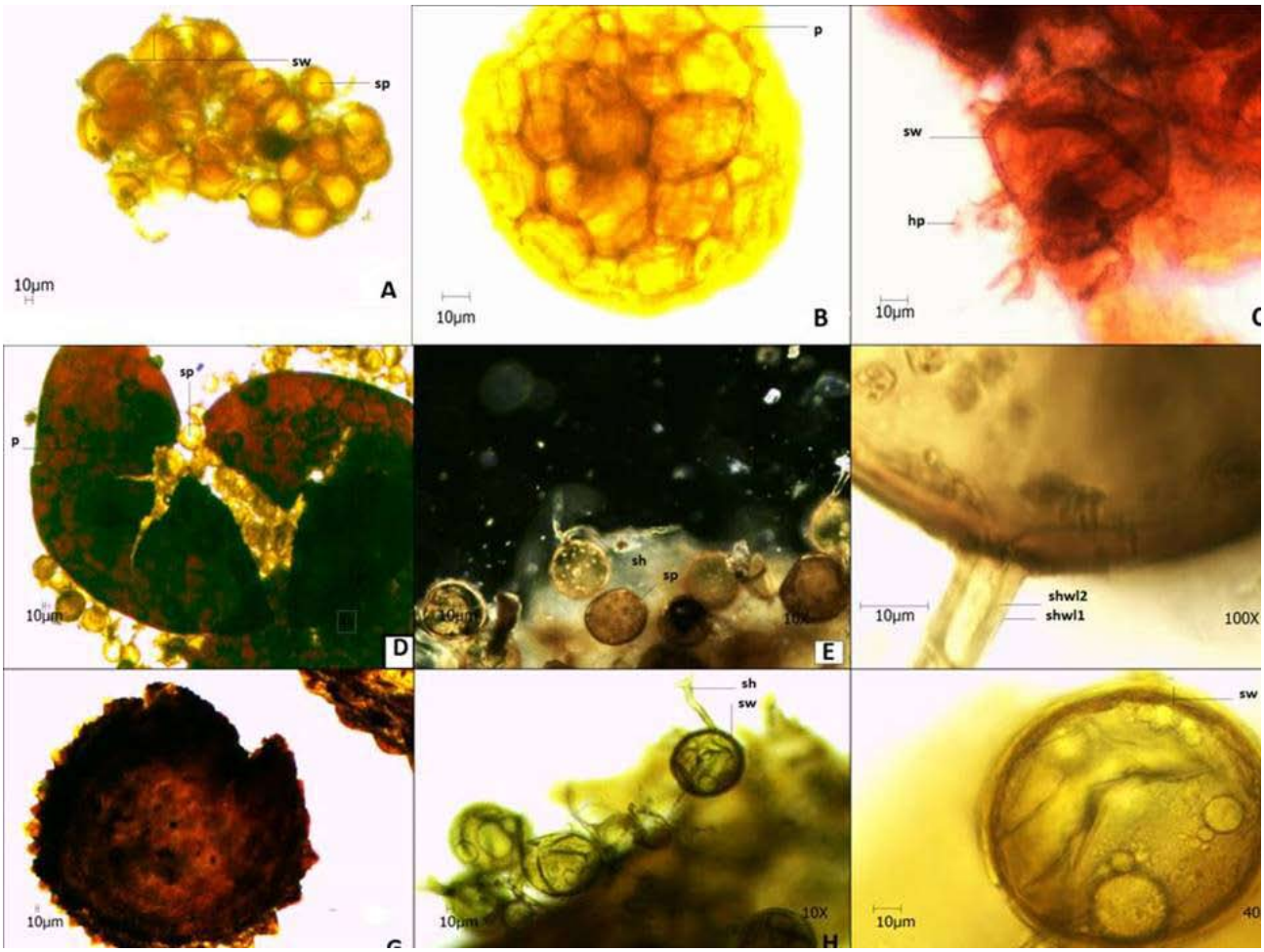

Fig. 1: Sporocarpic fungi isolated from Aravalli Ranges, Delhi. A-C *Sclerocystis sinuosum*; D-F, *Diversispora aurentia* and G-I, *Glomus* sp. (sp- spore, p-peridium, sw-sporewall, sh- subtending hyphae shw1 and shw2 - subtending hyphae wall layers)

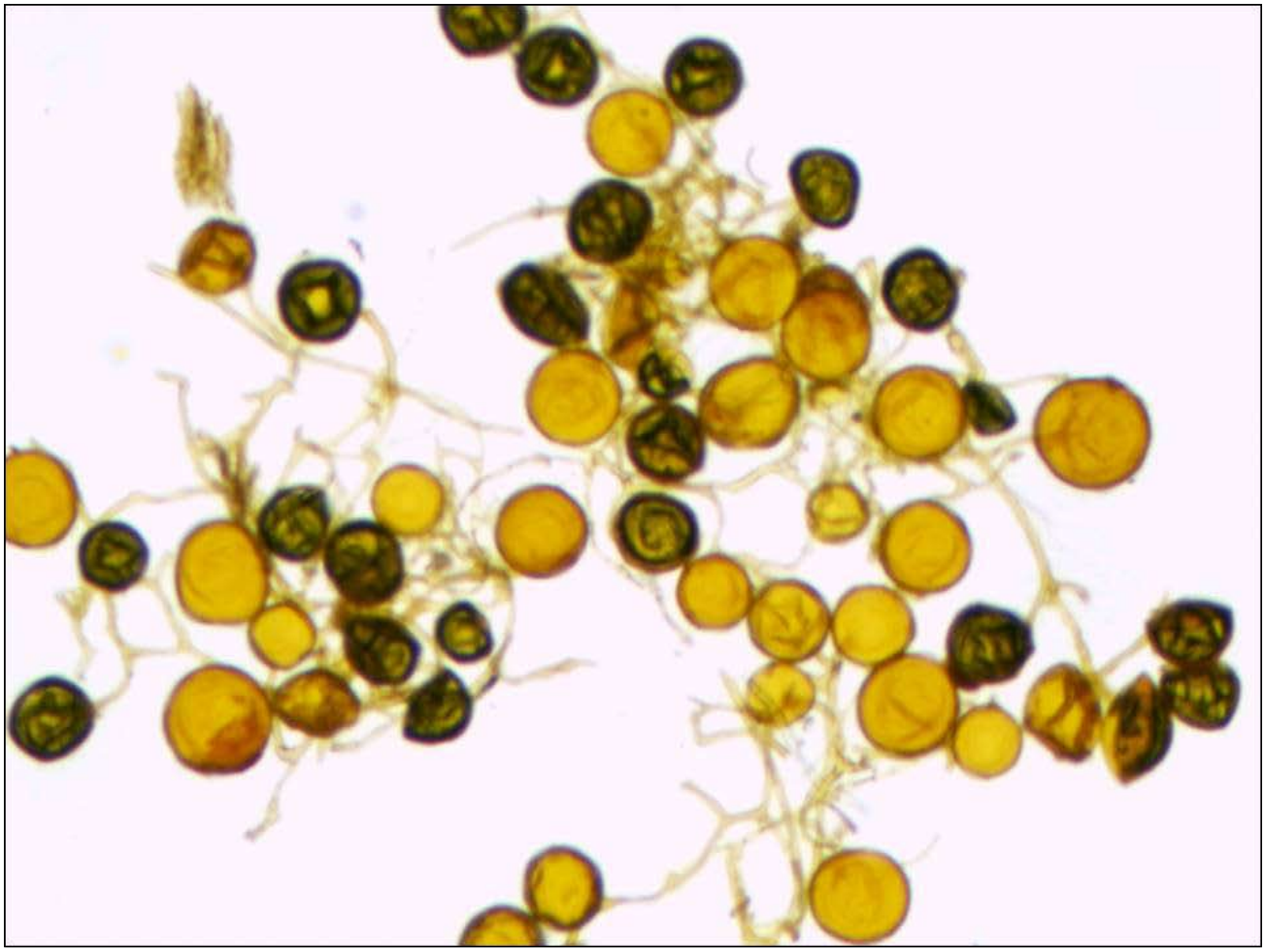

Fig. 2: Spore clusters of *Glomus macrocarpum*

The present study revealed that spore clusters or sporocarps of these AM fungi could be used for multiplication. The different species sampled varied in their response to the use of the sporocarp as a starter inoculum for colonisation, multiplication, spore production and sporocarp production in trap cultures (Table 1). *Sclerocystis sinusum* multiplied very well and produced young sporocarps along with fresh spores after the first generation. However, *Diversispora aurentia* produced typical glomoid spores and good colonisation in maize roots but no sporocarps were produced in trap cultures. Both *Glomus macrocarpum* and *Rhizophagus intraradices* multiplied very well in trap cultures and produced fresh spores but very few spore clusters were seen in both the trap cultures.

Rhizophagus intraradices after the first generation of trap culturing displayed contamination by other soil fungi including *Glomus fasciculatum* and *Glomus mosseae* that could be controlled by selective inoculation of spores collected from these pots for further propagation. Both, *Sclerocystis sinuosa* and *Diversispora aurentia,* showed no contamination from beginning until the third generation. *Glomus macrocarpum* also showed some contamination (4-12%) (Table 1).

Phylogenetic analyses allowed confirmation of the identification of *Glomus macrocarpum* and *Rhizophagus intraradices* (Fig. 3). However, sufficient DNA could not be extracted from spores of either *Sclerocystis sinuosa* or *Diversispora aurentia* for amplification. Sequences confirmed as belonging to the *Glomus macrocarpum* and *Rhizophagus intraradices* with NCBI -BLAST and were deposited in NCBI (GenBank KX485375.1; KX485374.1; MF401587; KX430040.1; Table 2). The data matrix and complete tree are available with the author on e-mail request.

Table 1. Assessment of AM fungi in three harvests of pot cultures raised using sporocarps as starter material. Data presented on roots colonized (%), average number of spores, sporocarps or spore clusters /50 g of soil isolated. Numbers in parentheses indicate percentage contamination.

Name of fungi	1st harvest			2nd harvest			3rd harvest			NCBI accession number
	Sporocarp number	Spore number	Roots colonized	Sporocarp number	Spore number	Roots colonized	Sporocarp number	Spore number	Roots colonized	
Sclerocystis sinuosa	0.2	5 (0)	29(0)	0.5	8(0)	25(0)	1	28(0)	28(0)	–
Diversispora aurantia	0	40(0)	18(0)	0	49(0)	18(0)	0	100(2)	19(0)	–
Glomus macrocarpum	0	52(15)	45(5)	0.2	45(7)	47(4)	0.2	49(9)	47(12)	KX485375. 1KX485374. 1MF401587
Rhizophagus intraradices	0	49(45)*	42(29)	0.2	46(5)	47(0)	0	25(9)	15(5)	KX430040.1

Cultures were started again with spores obtained in first generation

Table 2. NCBI accession number of sequences used in computing phylogenetic analysis. Sequences isolated in present study are shown in bold.

S.No	Species Name	Accession Number
1	Uncultured Glomeraceae	KF939988, KF939986
2	*Glomus macrocarpum*	FR750537, FR750371, FR750366, FR750364, **KX485375.1, KX485374.1**, **MF401587**
3	*Acaulospora koskei*	KP191476, KP191474, KP191475, KP191473
4	*Rhizophagus intraradices*	JN417517, HF968925, HE817882, **KX430040.1**
5	*Glomus diaphanum*	AJ972462, AJ972459, AJ972458, AJ972457
6	*Glomus aggregatum*	GQ205063, GQ205062
7	*Glomus clarum*	GQ205081, FN423696
8	*Glomus dimorphicum*	KJ934791
9	*Glomus heterosporum*	AY285870, AY285871
10	*Glomus invermaium*	HG969392, HG969390, HG969386, HG969384, HG969382, HG969381, HG969380, HG969379, HG969374
11	*Glomus microaggregatum*	HG425991
12	*Ambispora leptoticha*	AB048682, AB048681, AB048680, AB048679, AB048678, AB048677, AB048676, AB048675, AB048674, AB948673, AB048672, AB048671, AB048668, AB048667, AB048658, AB048656, AB048654, AB048648, AB048647, AB048645, AB048644, AB048642, AB048641, AB048640, AB048639, AB048638, AB048636, AB048635, AB048633, AB048631, AJ567807, AJ012201
13	*Ambispora gerdemannii*	JF439210
14	*Archaeospora schenckii*	FR750023, FR750022, FR750021, FR750020
15	*Archaeospora trappei*	FR750038, FR750037, FR750036, FR750035, FR750034, AJ243420, AJ243419
16	*Dentiscutata heterogama*	FR750167
17	*Sclerocystis sinuosum*	AJ437106, AJ437105
18	Rhizophagus fasciculatus	X96842
19	Entrophospora infrequens	U94714, U94713, JN194173
20	Scutellospora calospora	JF439140, JN194176
21	Corymbiglomus tortuosum	JF439095, JF439096, JF439094
22	Redeckera fulvum	AM418547, AM418546, AM418545, AM418548

Contd....

S.No.	Species Name	Accession Number
23	Racocetra persica	JN194177, AJ410740, AJ410739
24	Racocetra coralloidea	AF004686
25	Diversispora epigaea	FR686939, FR686938, FR686941, FR686940
26	Pacispora scintillans	FM876832, FM876831
27	Racocetra fulgida	FR750145, FR750144, FR750143,FR750142, FR750141, FR750140,FR750139, FR750138, FR750137,FR750136, FR750135, FR750134
28	Claroideoglomus etunicatum	AY236330, AY236329, AY236328, AY236327, AY236325, AY236324, AY236323, KC841655, KC841653, KC841652, KC841651, KC841650, KC841648, KC841646, KC841645, KC841644, KC841643, KC841641, KC841639, KC841640
30	Funneliformis caledonium	GQ388723, FJ769289, JQ048839, JQ048831, JQ048830, JQ048778, JQ048777, Q048776, JQ048775,JQ048774, JQ048773, JQ048772,JQ048771, JQ048770, GQ205091, GQ205090, GQ205089, GQ205088, AY035651, FN547498, FN547499, FN547497, FN547494, FN547495, FN423698,AY035647, AY035646, AY035642
31	Glomus intraradices	FM865548, FM865547, FM865607,FM865606, FM865605, FM865604,FM865603, FM865601, FM865600,FM865599, FM865598, FM865597,FM865587, FM865586, FM865585, FM865584, FM865583, FM865582,FM865581, FM865580, FM865579,FM865578, FM865577, FM865576, FM865575, FM865574, FM865573 FM865572, FM865571, FM865570, FM865569, FM865568, FM865567, FM865566, FM865565, FM865563, FM865564, FM865562, FM865561, FM865560, FM865546, FM865545
34	*Funneliformis mosseae*	KF836946, GQ388715, DQ400129, FR750032, JF439112, **new sequence (not submitted)**

Discussion

Production of sporocarps has not been studied after the 1980s when *Glomus epigeous* (Daniels & Menge, 1980) and *Glomus aggregatus* were first reported to produce sporocarps in trap cultures. The present study thus extends the list of species where sporocarp has been produced in trap cultures, adding *Scleocystis sinusum* to the list. Secondly, it attempts to track the generation of spores and sporocarp to three generations in trap cultures in five new species, which has not been reported earlier.

Although this was not the initial intent, the present study highlights the following functions that sporocarps may be performing in the life cycles of AM fungi. Firstly, the results support the likelihood of a protective function that prevents decay due to other microbial infection. Sporocarps in the case of *Sclerocystis sinusum* and *Diversispora aurentia*, which showed no contamination even after the third generation of trap culture, were compact structures that, presumably, decreased the scope for contamination by other fungi. On the other hand, *Glomus macrocarpum* and *Rhizophagus intraradices,* where spore clusters had no peridium, showed contamination by other fungi. Secondly, sporocarps may also be serving as perennating structures, as in the case of *Glomus* sp. with a thick peridium, where spores did not germinate.

Diversispora aurentia did not produce sporocarps in trap cultures even after the third generation of culturing, but we could not determine what triggers the germination of spores from sporocarps; nor could we determine what enables the ~~other~~ AM fungi ~~and~~ to produce sporocarps in culture.

Our study was unique as we tried to confirm identification of AM fungi in different trap cultures using molecular methods. Sequences could be identified from two isolates only and were confirmed to be *Glomus macrocarpum* and *Rhizophagus intraradices*.

In conclusion, the study adds to the biology of AM fungi in the following ways: Firstly, it extends the list of species where sporocarp production has been reported in laboratory conditions as trap cultures. Secondly, it attempts to track the generation of spores and sporocarp to three generations in trap cultures in five new species which has not been reported earlier and finally the application of molecular methods for confirmation of the identity of the progeny of spores in next generations of cultures is tried for the first time. Finally, the study throws light on the functions that sporocarps may be performing in the life cycle of AM fungi.

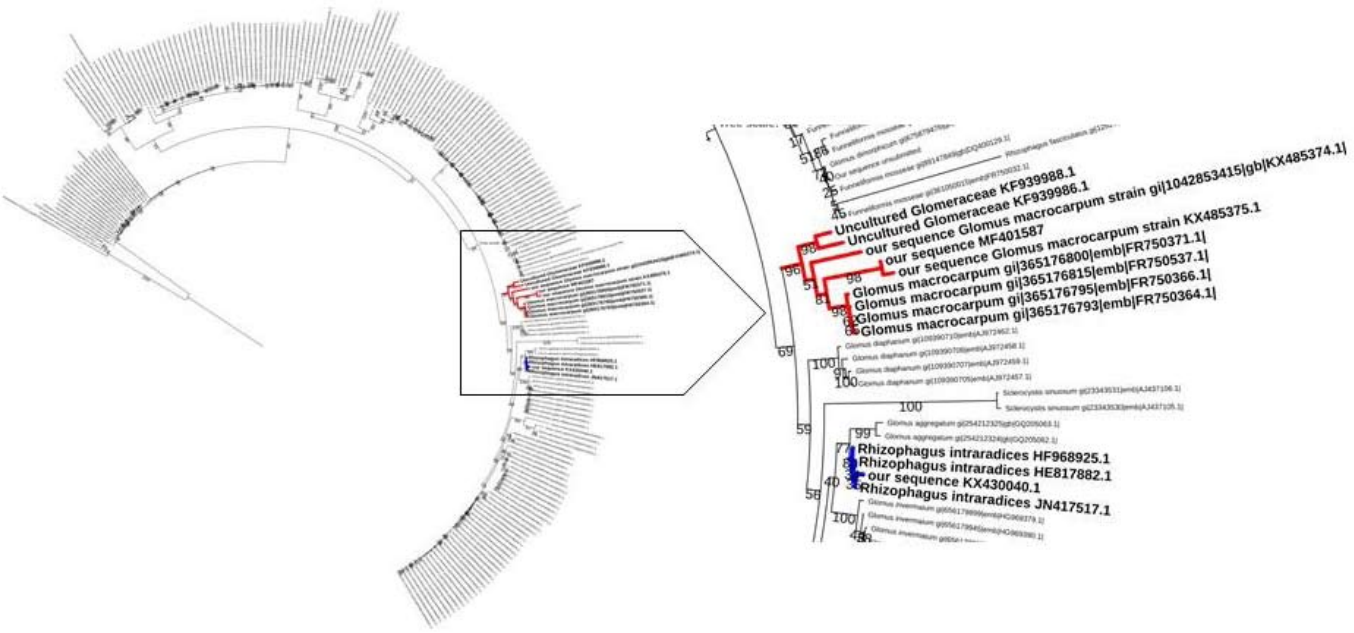

Fig. 3: Maximum likelihood phylogenetic tree based on nuclear small subunit full (SSU)–5.8S–large subunit (LSU) rDNA of sequences isolated in present study (shown in bold), sequences isolated from NCBI which showed identity >= 99% and selected sequences downloaded from NCBI which spanned the same genomic DNA and belonged to same species of Glomeromycota. Multiple sequence alignment with MAFFT was done at EMBL-EBI server (http://www.ebi.ac.uk/). All maximum likelihood phylogenetic analyses were computed through the CIPRES web-portal with RAxML ver. 7.2.8 (Stamatakis et al., 2008) using 1000 bootstraps and the GTRGAMMA model for both bootstrapping and tree inference. Bootstrap values are given for branches among different NCBI accession numbers. The scale bar indicates the number of substitutions per site. Branches with < 60% bootstrap support were collapsed to polytomies. ITOL (http://itol.embl.de/) was used for drawing final trees. The data matrix and complete tree are available with the author.

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