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SPORULATION OF ARBUSCULAR MYCORRHIZAL FUNGUS, *Glomus intraradices,* THROUGH ROOT ORGAN CULTURE

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ABSTRACT

This study reports the cultivation of arbuscular mycorrhiza (AM), *Glomus intraradices* spores in Ri-TDNA transformed carrot (*Daucus carota)* roots using *Agrobacterium rhizogenes* MTCC culture 532. The germination and mass multiplication of surface sterilized AM spores was studied at different pH levels, sucrose concentrations and inoculums sources. The multiplication of AM spores were successfully established using dual culture technique in modified Strullu and Romands medium amended with 1.0% sucrose at pH 5.5 and incubation temperature of 27°C under dark condition. The influence of using different inoculum sources such as bunch of spores, infected root bits and spore gel/ agar plug of *G. intraradices* in root organ culture were evaluated during successive generations under *in vitro* conditions. Among these, agar plug with high density of viable spores (initially 50) along with few mycorrhized root apexes guaranteed maximum multiplication of spores (9772±770 numbers) after 16 weeks.

Key words: *Agrobacterium rhizogenes*, AM spores, *Daucus carota*, *Glomus intraradices*, mass multiplication, root organ culture

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi show symbiotic obligate associations with the roots of higher plants. About 80-90% plant species studied so far have shown this symbiotic relationship (Lekberg and Koide, 2005) which, in turn, improves plant growth by increasing resistance to drought and disease or by enhancing the efficiency of nutrient absorption (Douds and Siedel, 2012). Axenic cultures of these fungi cannot be established as these obligate biotrophs rely on plant tissues to complete their life cycle, but it is possible to grow AM fungi in sterile conditions in root organ culture (Bi *et al*., 2004). This not only overcomes the problems of conventional time consuming pot culture methods for mass production of AM fungi, but also limits high cross contamination. The constraints in having pure *in vitro* cultures of AM fungi are due to their firm symbiotic obligate association which is further convoluted by biotrophic and hypogenous nature of the mycobionts occupied. To overcome these challenges, several attempts have been made during the last few years to obtain symbiosis under *in vitro* conditions ((Mathur and Vyas, 2007). The use of root organ culture has proved particularly successful and a list of Glomalean species successfully cultivated on root organ cultures has been documented by Fortin *et al*. (2002).

Agrobacterium rhizogenes, a Gram negative soil bacterium, has been exploited for hairy root induction through its steady transformation of Ri T-DNA (Baranski, 2008). The bacterium offers a capable system in the establishment of long term monoxenic culture of AM fungi (Bidondo *et al.*, 2012). Their modified hormonal balance makes them particularly vigorous and allows copious growth on artificial media (Tepfer and Tempe, 1981). The transformed or hairy roots are also biosynthetically stable for longer periods (Roychowdhury *et al.*, 2013). Carrot (*Daucus carota* L.) and binweed (*Convolvolus sepium* L.) were among the earliest plant species to be transformed using this bacterium (Danesh *et al.*, 2006). The transformed roots were well exploited for culturing the monoxenic species of AM fungi and better understanding of AM symbiosis (Costa *et al.*, 2013). Moreover, it can be used as a quality inoculum and extended to all crops. Since the spore load is less in *in vivo* grown inoculum and a huge quantity of inoculum is needed for direct sown crops, its application is confined to nursery level studies only. Present study was ,therefore, focused to overcome this problem to develop monoxenic culture of *Glomus intraradices* and increase its sporulation potential using dual culture technique.

MATERIALS AND METHODS

The *in vitro* experiments were conducted in the Department of Agricultural Microbiology, TNAU, Coimbatore (India) in 2014 to induce hairy roots from *Agrobacterium rhizogenes* strain 532 inoculated carrot (*Daucus carota*) explants.

Hairy root induction in carrot

Farm fresh carrots were washed with copious amount of water, peeled off carefully with a sterilized scalpel and treated with 0.1% mercuric chloride for 10 min. followed by thorough washing with sterile distilled water. The carrots were dipped in 70% ethanol for 1-2 min., flamed until ethanol was burned off and transversely cut into 0.5 cm thick sections to expose the cambial tissues of the carrots. Bacterial suspension was taken from a 48 h old culture, grown in nutrient agar, and inoculated on the distal face of sections. Preparation of carrot discs were as per the procedures of Becard and Fortin (1988) with slight modifications. Each disc (explant) was placed on half strength Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962) supplemented with 150 µM acetosyringone with the basal sides faced upwards. All the plates containing inoculated carrot discs were incubated at 25°C in tissue culture racks under dark conditions.

Genomic DNA extraction from hairy roots

Hairy roots (approx. 0.5 g) were cut into small pieces and ground with 600 µL extraction buffer (100 mM tris HCl pH 7.5, 1.4 mM NaCl, 2.0 mM EDTA and 2% w/v CTAB) using a mortar and pestle. The homogenate was kept in a water bath at 60°C for 30 min. and centrifuged at 10,000 rpm for 3 min. Equal volume of phenol and chloroform was added to the supernatant and centrifuged at 12,000 rpm for 20 min. To this supernatant, 400 µL chloroform and isoamyl alcohol (24:1) was added followed by centrifugation at 12,000 rpm for 20 min. Then, sodium acetate (50 µL) and ice cold isopropanaol (600 µL) were added to the supernatant, incubated at room temperature and kept overnight at 4°C. The crude DNA was pelleted by centrifugation at 12,000 rpm for 20 min. Pellets were air-dried at room temperature and dissolved in 50 µL 0.1 X TE buffer (1 mM tris HCl pH 8.0 and 0.1 mM EDTA pH 8.0). For each PCR reaction, 2 µL DNA preparation was used as template.

PCR reactions were performed using reaction mixture that contained 2 µL genomic DNA, 2.0 µL 10X PCR buffer (10 mM tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 10 mM each of dNTPs, 5 mM of upstream and downstream primers and 0.15 units *Taq* DNA polymerase. The primer sequences used were: T DNA AgF - 5' GCGCATCCCGAGGCGATG 3', and T DNA AgR - 5' AGG TCTGGCGATCGCGAGGA 3'. The reactions were performed in a final volume of 15 µL.

PCR was performed to amplify *ags* gene of T-DNA. The genomic DNA of *A. rhizogenes* was used as positive control. PCR amplification was performed with a program of initial denaturation at 94°C for 3 min., 30 cycles of denaturation at 94°C for 1 min., annealing at 55°C for 1 min., extension at 72°C for 1 min. and a final extension at 72°C for 7 min. The amplified products were stored at 4°C. Agarose gel electrophoresis was performed as per Sambork *et al.* (1989) to check the quality of DNA and also to separate the products amplified through polymerase chain reaction.

Sub-culturing and mass multiplication of transformed hairy roots

Each excised hairy roots with actively growing apex were placed in petri-plates containing fresh modified Strullu and Romands (MSR) medium (Declerck *et al*., 1998) amended with 0.3% gellangum and 1.0% sucrose and incubated in an inverted position. The multiplication of transformed roots was done by subsequent subculturing of roots for 2-3 generations under optimized *in vitro* conditions (27°C in dark). After sufficient growth or every 3-4 weeks; white, healthy, turgescent non-ramified root apexes with few lateral roots (with a minimum length of 5 to 7 cm) were removed aseptically and transferred to fresh medium before it becomes necrotic. These root organ cultures (ROC) were used to develop monoxenic culture of arbuscular mycorrhizal fungi under *in vitro* conditions (Cranenbrouck *et al.,* 2005). The growth of hairy roots were observed in terms of fresh biomass during 15, 30 and 45 days after placement on medium amended with two different sucrose concentrations (1.0 and 1.5% in MSR medium (pH 5.5).

Extraction of AM spores

Mycorrhizal inoculum (100 g), used as trap culture soil, was obtained from Department of Agricultural Microbiology, TNAU, Coimbatore (India) containing azygospores of *G. intraradices* and colonized root bits which was dispersed in 1000 mL water. The spores were isolated by wet sieving and decanting technique (Gerdemann and Nicolson, 1963). The spores of *G. intra-radices* were differentiated mainly on the basis of morphology and nature of hyphal attachment *viz.*, size, wall layer and colour of spores, hyphal attachment and hyphal thickness (Schenck and Perez, 1987).

Selection and disinfection of AM fungal spores

Morphologically unique spores of *G. intraradices* were chosen individually under a dissecting microscope using a micropipette or dissection needle. Before *in vitro* use, the spores were surface sterilized to eliminate contaminants. Initially the spores were transferred to a filtration apparatus, rinsed 2-3 times with sterile water and then treated with Chloramine T (2%) solution with 2 drops of Tween 20 (0.1 % v/v) added just before use. It was then treated with antibiotics solution of streptomycin sulphate (0.02%) and gentamicin sulphate (0.01%), which was filtered with a 0.2 µm acrodisc membrane filter. Finally they were rinsed with sterile water 3 times (Becard and Piche, 1992).

Effect of varying sucrose concentrations on multiplication of Glomus intraradices spores

The 20 surface sterilized spores of *G. intraradices*, captured in membrane filter (size > 0.2 µm), were aseptically picked and placed on MSR medium having varying sucrose concentrations (1.25, 1.0 and 0.5%) at different pH levels (5.0, 5.5 and 6.0). The petri-plates were sealed and incubated in inverted positions in dark at 27°C. The experiment was conducted in completely randomized design with each treatment replicated six times. The germination of AM spores was assessed using a stereomicroscope (10 X) after one week. The treatment with maximum percentage of germinated spores was selected for spore multiplication. Fresh transformed roots of approximately 8 cm length, with few apexes forming herringbone structure from 3 week old root organ culture, were placed carefully near the vicinity of germinating spores (after 1 week) in petri-plates so that the growing hyphae remained perpendicular to a secondary root. The petri-plates were sealed properly and incubated in dark at 27°C.The spore multiplication was observed periodically at an interval of 4, 8, 12 and 16 weeks (Cranenbrouck *et al.,* 2005). The spore count was measured using grid line intersection method (Giovannetti and Mosse, 1980).

Establishment of monoxenic culture of Glomus intraradices

The petri-plates showing good spore multiplication were selected and single mature spores aseptically transferred to the root organ culture of carrot. Five replications were maintained with single spore inoculation under same *in vitro* conditions. The infection process and spore production was monitored for 12 weeks. The established monoxenic *in vitro* AM cultures of *G. intraradices* were sub-cultured over many successive generations.

Influence of inoculum source of G. intraradices

During successive generations, three types of spore inoculation were tested. The subsequently established continuous culture of AM associated transformed roots were examined for the multiplication of these monoxenic spores. In first method, bunch of spores (50 in number) with extending hyphae from monoxenic culture of *G. intraradices* root organ culture were extracted with a sterile needle and transferred to new petri-plates containing transformed roots. In second method, the mycorrhiza colonized roots carrying 20 spores were placed on MSR medium and tested for spore multiplication. In third method, a section of agar plug/spore gel containing 50 mature spores along with mycorrhizal root apexes were transferred aseptically near the growing tips of fresh transformed roots placed in MSR medium. The spore production was periodically recorded at an interval of 4, 6, 8, 12 and 16 weeks (Cranenbrouck *et al.,* 2005). All the treatments were replicated five times in complete randomized design and plates kept under the same *in vitro* conditions.

Scanning electron microscopy of G. intraradices spores

The morphological characters of spores were observed using scanning electron microscope (SEM-FEI-Quanta 250, Pregon, USA) in the Department of NanoScience and Technology, TNAU, Coimbatore (India). The root bits were cut into small pieces (approx. 0.3 to 0.5 cm long) and placed on one side of the double sided adhesive carbon conducting tape. The tape was then mounted on 8 mm dia. aluminium stub. The stub was kept in sample holder of SEM and scanned for imaging. The sample surface was observed at a magnitude of 200, 400 and 500x and the images recorded under high voltage of 20.0 kV.

Statistical analysis

The data was analyzed using analysis of variance ($P_{0.05}$) as per Panse and Sukhatme (1978). For computation packages like AGDATA, AGRES and Microsoft excel were used.

RESULTS AND DISCUSSION

Hairy root induction in carrot using Agrobacterium rhizogenes

After two weeks of incubation of *A. rhizogenes* inoculated carrot discs at 25°C, a high frequency of *in vitro* roots (hairy roots) were produced in half strength MS medium (Plate 1). The transversely cut discs treated with acetosyringone produced white elongated roots that continued for another week. Carrot is one of the most susceptible plant species for hairy root production (Christey and Braun, 2004). Chilton *et al.* (1982) have reported that wounded carrot explants exude some signals which facilitate the transfer and integration of bacterial Ri T-DNA into plant genome. The efficiency of transformation is highly dependent on the bacterial strain used. These genes encoded in T-DNA are of bacterial origin but have eukaryotic regulatory sequences enabling their expression in infected plant cells. Smith and Dickson (1997) have suggested that trimming carrot discs to expose cambium improves the generation of healthy hairy carrot roots. Acetosyringone promotes the transformation by actively inducing the transfer of T-DNA from *A. rhizogenes* to the plant (Kyung-Hwan *et al.,* 1996).

Molecular confirmation of ags gene transformation by PCR analysis

Molecular analysis of DNA extracted from carrot hairy roots for the transformation of *ags* gene of Ri T-DNA was confirmed through PCR analysis (Plate 2). Incubation of carrot discs at 25°C provided conducive condition for bacterial (agropine) strain to insert maximum copies of *ags* gene present in T-DNA. Confirmation of the transfer of root inducing T- DNA was done after detection of *ags* gene in transformed hairy roots. Analogous primer was also used by Rajkumar and Murugesan (2014) in *Psoralea corylifolia* hairy roots for confirmation. Another study by Saravanakumar *et al*. (2012) reported PCR confirmation of *rol* C gene transfer from *A. rhizogenes* strain R1000 at 557 bp in *Withania somnifera* L. The T-DNA integration of *A. rhizogenes* in plant genome might have lead to the alterations in the physiology and, therefore, induced the phenotypic expressions of the roots which, in other words, termed as induction of hairy roots.

Mass multiplication of hairy roots

The improvement in culture conditions for hairy root proliferation is assessed by increase in weight mainly because most hairy root research focuses on increased root biomass. A significant increase in fresh biomass of transformed roots was observed on 45th day in treatment where 1.0% sucrose was added to MSR media (143% increase over 30th day) than that of 1.5% sucrose in MSR (129% increase) (Fig. 1). The suppression of hairy roots growth in media containing 1.5% sucrose could be attributed to the high osmotic pressure which is in agreement with Udomsuk *et al. (*2009). Sucrose is the most common carbon source used in plant cell, tissue and organ culture. Sucrose acts as a fuel source during plant tissue culture for sustaining photomixotrophic metabolism ensuring optimal development, although other important roles such as carbon precursor or signaling metabolite have also been reported (Baena-González *et al*., 2007). The neoplastic (cancerous) roots produced by *A. rhizogenes* infection are characterized by high growth rate, genetic stability and growth in hormone free media (Srivatsava and Srivatsava, 2007). The aseptically transferred roots on MSR medium showed good proliferation and covered the entire plate in 5-6 weeks (Plate 3). The subsequent sub-culture of root organs done once in 3-4 weeks showed similar growth pattern with regeneration of new lateral roots. Root organ culture of carrot was used throughout the study for *in vitro* production of AM spores through dual culture method.

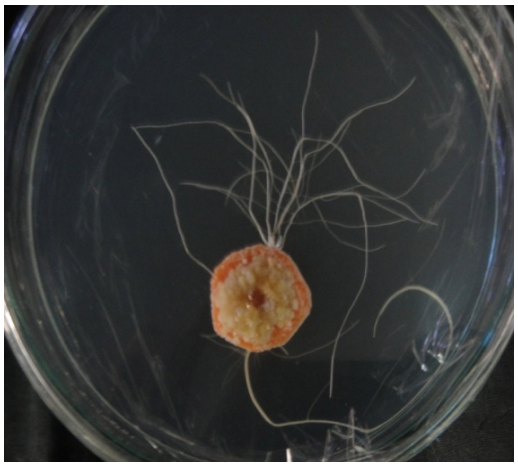
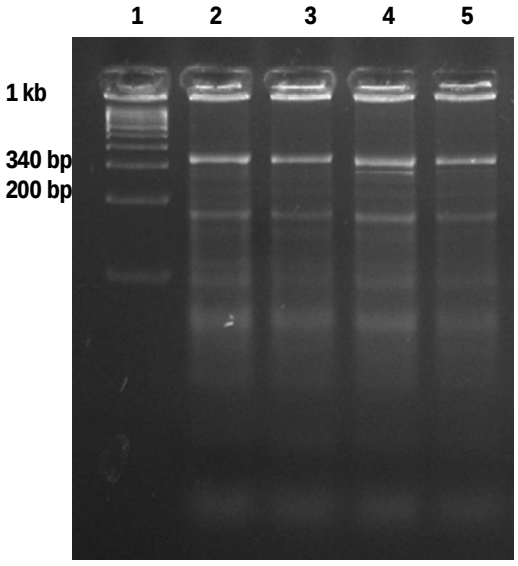


Plate 1: Hairy root induction in carrot



Lane 1 – 100 bp ladder
Lane 2 – *Agrobacterium rhizogenes* strain 532
Lane 3 – *Agrobacterium rhizogenes* strain 2364
Lane 4 – Transformed roots (*A.rhizogenes* 532)
Lane 5 – Transformed roots (*A.rhizogenes* 2364)

Plate 2: PCR confirmation of agropine synthase (*ags*) gene

Establishment of AM symbiosis in hairy roots - Dual culture technique

The surface sterilized (young, densely filled, pale yellow coloured) spores of *G. intraradices*, placed in MSR medium, germinated well (65%) in sucrose (1.0%) at pH 5.5. Following the germination and association of spores with transformed roots, the

germinated spores established good colonization in root organ cultures of carrot. The growth of hairy roots was induced extensive hyphal branching during first four weeks after spore germination. It may probably be due to branching factor (strigolactones), exuded from host roots that lead to the formation of pre-infection hyphal branching structures in AM fungi. Tamasloukht *et al*. (2003) have reported that a semi-purified exudate from carrot root organ cultures was shown to induce the expression of mitochondrial-related genes and, in turn, fungal respiratory activity before intense hyphal branching. In another study, *G. mosseae* exhibited chemotropic growth towards roots at a distance of at least 910 µm in response to host-derived signals, possibly strigolactones as reported by Sbrana and Giovannetti (2005). Pawlowska *et al*. (1999) have reported that the progress in understanding the biology of AM fungi is hampered by the limited number of species that can be successfully propagated and studied *in vitro*. The fungus can, therefore, be propagated *in vitro* using monoxenically formed resting spores and/or colonized root fragments. The number of resting spores formed *in vitro* correlated positively with the length of roots occupied by arbuscular mycorrhizal structures. Hairy root cultures are potentially valuable for the study and cultivation of AM fungi, which do not grow on synthetic medium axenically.

Spores were terminal or intercalary and appeared single or in clusters. The number of spores attained at the end of 12 weeks was 4200 $\pm$ 429 spores in MSR medium with 1.0% sucrose where the germination of spores was high. It was evident to spot higher multiplication rate of spores with higher spore germination. The spores get germinated and colonize hairy roots at negatively geotrophic nature. Diop *et al*. (1992) observed less contamination in culturing of transformed carrot roots due to this nature. Becard and Fortin (1988) have reported that the combination of transformed carrot roots and sterile AMF spores can be used to produce "dual *in vitro* cultures" that provide an efficient method of producing abundant spores (typically over 5000) and mycelia in a 9 cm petri-plate.

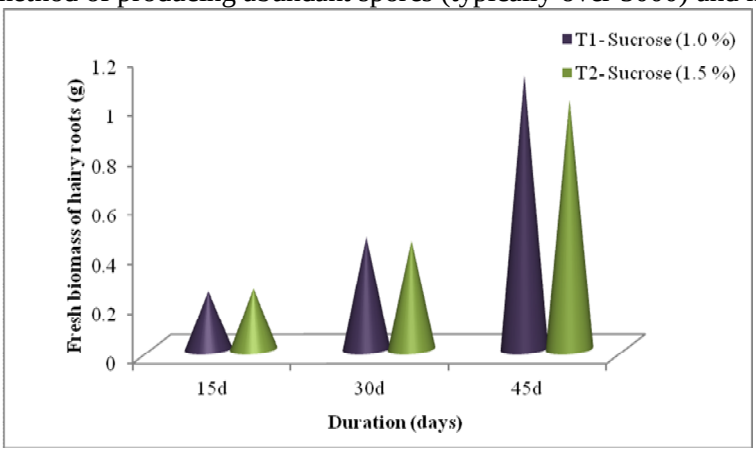


Fig. 1: Influence of sucrose on fresh biomass of *A. rhizogenes* transformed root

Monoxenic culture development of G. intraradices

The process of germination, successful colonization, hyphal extension, mycelial growth, formation of spores and increase in spore multiplication in monoxenic culture plates was almost similar as observed in case of dual culture technique. The smaller spores turned to mature ones with production of newer ones at every observation at a periodic interval of one week. A

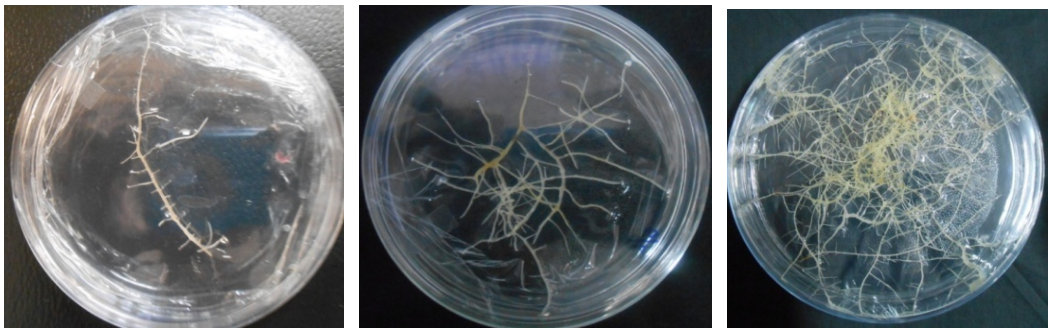


Plate 3: Different stages of regeneration of hairy roots; Placement of transformed roots on MSR medium on initial day (left), followed by good proliferation during first week (mid) and regeneration of hairy roots covering the entire plate during sixth week (right)

significant increase in spore production that reached a maximum of 2550 ± 429 numbers was observed at the end of the experimentation (Table 1). The spore production varied highly between replicates of same treatment. These observations were in conformity with Elsen *et al. (*2003) while working on *in vitro* cultures of various AM fungi specially focusing on *G. intraradices.* According to Voets *et. al.* (2005), the high variability observed could be attributed to the host fungus interaction and to both the fungal and plant physiology. Indeed, partner communication in the pre-symbiotic and symbiotic phase is a complex multi-step process under genetic control, regulated by an intimate molecular dialog between the host root and the obligate symbiont (Becard *et al.*, 2004). In our experiment also, initial root colonization varied within the replicates with some colonization occurring within 2 weeks and in some extended upto 4 weeks which reflects the intensity of spore production. However, Voets *et al.* (2005) opined that it could be leveled off at the maturity stage when the spore size and numbers reaches maximum. It could be related to the supplementation of nutrients from medium to the roots and, in turn, the fungal symbiont supplied carbon for photosynthesis. Thus the auto-trophic culture system was successfully exploited in carrot hairy roots through this dual culture technique.

Table 1: Monoxenic culture of *G. intraradices* in root organ culture

Growth time interval (weeks)	Particulars	No of spores observed
1	Spore germination – 80%	-
2	Initiation of hyphal growth	-
3	Hyphal elongation and appressorium formation	-
4	Mycelial growth attains maximum with initiation of small spores	46 ± 14
5	Increase in spore production	170 ± 52
6	Spore production	400 ± 123
7	Spore production	750 ± 126
8	Spore production	1450 ± 244
10	Spore production	2090 ± 352
12	Spore production	2550 ± 429
S.E.±		262.4
$CD_{0.05}$		537.5

Values represent mean of five experimental units

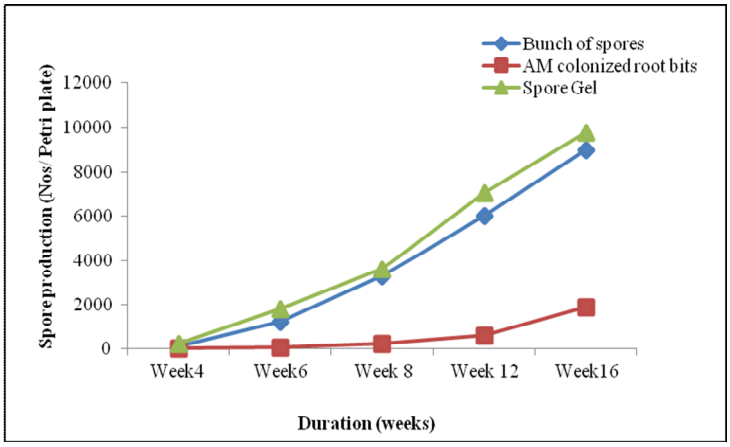
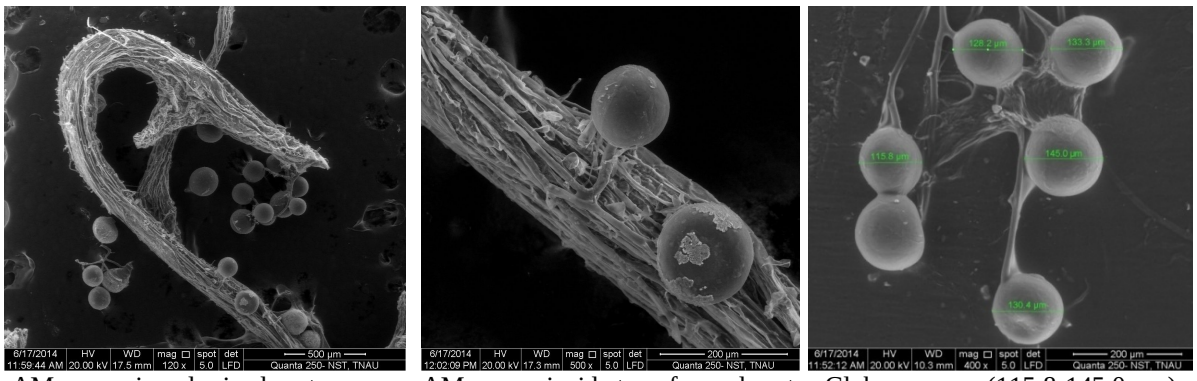


Fig. 2: Influence of source of inoculum on AM spore multiplication



AM spores in colonized root apexes AM spores inside transformed roots Globosespores (115.8-145.0 µm)

Plate 4: Scanning electron microscopic view of *Glomus intraradices* spores developed through root organ culture (ROC)

Influence of inoculum sources on AM spore multiplication

Among the three sources of initial inoculum in root organ cultures, agar plug with high density of viable spores along with mycorrhized root apexes produced maximum spores [9772 ± 770], followed by inoculation of bunch of spores [8990 ± 728] in transformed roots (Fig. 2). The continuous culture is obtained by association of monoxenic mycorrhizal roots and/or spores (Chabot *et al.*, 1992), often attached to extraradical mycelia, to a new, actively growing non-mycorrhizal host root, onto a fresh medium. The bunch of monoxenic spores was largely effective for a range of *Glomus* species (Declerck *et al*., 2000). The second source wherein apical segments of actively growing mycorrhizal roots carrying a few spores with or without extraradical mycelium were used proved least effective. Since the method requires the use of young, actively growing cultures to allow continuous growth of host root (St-Arnaud *et al*., 1996), the continuous culturing may, therefore, be difficult to establish spore multiplication. When culture is old, the medium becomes exhausted and roots become necrotic, thus render mycorrhizal roots difficult to grow and AM fungi difficult to proliferate. The guaranteed multiplication of spores achieved in agar plug method favoured the association of AM spores with transformed roots which generally occurs near the growing apex where the cellular wall is thin and easier to penetrate. The SEM view of *G. intraradices* spores developed through root organ culture (Plate 4) depicted the pure culture of the mycorrhizal species.

Despite vast potential of AM fungi in agricultural, forestry and horticulture, their commercial production is a big limitation in their popularization due to their biotrophic nature. The main drawbacks associated with the conventional pot culture mycorrhizal inoculum propagation method are bulkiness, vulnerability to pest infestation and low density of spore load (Sharma *et al.*, 2000). The widely used method for obtaining AM fungal inoculum has been the use of root segments of plant hosting AMF and /or the infected soil containing AMF propagules (Gaur and Adholeya, 2000). However, these inocula are voluminous, heavy and often contaminated by other organisms (Vallino *et al*., 2009). So the present promising root organ culture technique not only increases the sporulation of AM fungi but also avoids the effects of other rhizosphere microorganisms which can be used in the formulation of a suitable water-soluble AM spore inoculum. Future research is desired to investigate the method of application such as seed treatment of this novel inoculum in direct sown crops.

Conclusion: Present study clearly demonstrated that the root organ culture of carrot produced viable axenic culture of *Glomus intraradices* with enormous sporulation in MSR medium in a short period which is certainly a difficult task in conventional pot culture method. The technique can overcome the problems associated with conventional vermiculite based inoculum and the high density spore inoculum also showed a promising way of exploiting its benefits in the field of agriculture and horticulture.

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