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ISOLATION AND SELECTION OF EFFICIENT AM FUNGI SPORES FROM SUGARCANE RHIZOSPHERE FOR *IN VITRO* AM INOCULUM PRODUCTION

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ABSTRACT : Arbuscular mycorrhizal fungi (AMF) are soil fungi which exhibits a symbiotic relationship with the roots of host plants. In the present study, sugarcane rhizosperic soil samples were collected from six different locations of Tamil Nadu. Rhizospheric soil samples and roots were analyzed for relative abundance of arbuscular mycorrhizal fungi. Based on morphological and taxonomic characterization, the most predominant AM spores present in sugarcane rhizosphere was identified as *Glomus intraradices*. The colonization potential and spores propagation capacity of the fungi in maize (Zea *mays* L.) under open pot culture method was 65±1.24 and 121±2.99 respectively. Four different substrates *viz.*, MSR medium, MS medium, whites medium and 0.75% water agar were used for testing the germination of *Glomus intraradices* spores. Among the different substrates used, MSR medium recorded the maximum per cent of spore germination (80 per cent) and the longest germ tube elongation (63 mm) followed by MS medium at 30 days after incubation maintained at 27° C under darkness. This efficient AM spores which survives naturally can be used for monoxenic *in vitro* inoculum production.

Key words : Sugarcane rhizosphere, Glomus intraradices, Maize, Root colonization, spores surface sterilization, MSR medium, germination test.

INTRODUCTION

The continuous increase in global population, with the limitations in the world's supply of natural resources and extensive use of chemical fertilizers deteriorating the environment presents a major challenge to the agriculture today. Contrary to the chemical fertilizers, organic manures and bioinoculants are less expensive and helps in achieving high productivity without harming the environment. In order to implement such a plan, the judicious use of nature's own biofertilizers such as arbuscular mycorrhizal fungi (AMF) can be advocated as they are beneficial fungi associated with roots of the host plant, including the important agricultural and horticultural crops. AMF enhances the nutrient availability especially phosphorus, augment water uptake and induces resistant against diseases and boost the yield (Lekberg and Koids, 2005) Among a wide range of host species, plants included in the Poaceae family is considered as one of the best host for AM fungi (Powell, 1984). Sugarcane is a cash crop belonging to this family which is grown all over India and accounts for 60% of the national contribution. The plants like sugarcane needs association of AM fungi to overcome 'P' deficiency due to its long standing periods in the same rhizosphere soil and exhausting the 'P' content. (Prabhudoss, 2011). In a survey, forty two AM fungal species of five genera namely, *Glomus, Acaulospora, Scutellospora*, *Gigaspora* and *Ambispora* were discovered from the rhizosphere soil. *Glomus* was found to be the most dominant genus in the AM fungi (Radhika and Rodrigues 2010). AM fungi successfully colonizes a wide range of plant species and are considered as non-host specific (Evelin *et al.,* 2009). For AM colonization and sporulation, the type of substrate, composition of potting mixture and physiology of the host plant are important (Mehrotra and Mehrotra, 1999). Spores are the most important propagules for most AMF and used as starting inoculums for *in vivo* and *in vitro* AM fungi mass multiplication because of their ability to germinate and colonize quickly (Tommerup, 1983). Spore germination may be affected under *in vitro* cultivation methods by many factors *viz.,* the need for a dormancy period (Safir *et al,*. 1990), root exudates from transformed hairy root, temperature, pH, light, growth substrate composition and as well as by presence of contaminating bacteria on the surface of the spores. Paula *et al.* (1990 a) observed that the rate of germination and mycelial growth of AMF increased 90% when the water-agar medium was supplemented with AM spores. Germination is also affected by temperature and the ideal temperature for germination of *Acaulospora laevis* was 20^{0}C, while the optimal range for hyphal growth was 15 to 25ºC. Another important factor for germination of AMF is the

pH (Hepper 1984). Spores of *A. laevis* maintained a few weeks at 6ºC, were exposed to different pH levels, and germination was higher in low pH. Some species produce multiple germ tubes under *in vitro* condition which were related to strategies for fungal survival (Logi *et al.,* 1998). Alok (1997) reported that the germination of spores is mainly influenced by medial gelling agent. Therefore, this study was made to isolate and identify the efficient AMF species which can be used for *in vitro* AM inoculum development.

MATERIALS AND METHODS

Soil sample collection

The preliminary investigation of AMF were carried out by sampling the soil from rhizosphere of sugarcane grown in long run at six locations, *i.e.*, Chithamanikuppam, Cuddalore (dist). Orathur, Chidambaram (dist). Mandharakuppam, Neyveli (dist). Sathamangalam, Vrithachalam (dist). Attur, Salem (dist). Rasipuram, Namakkal (dist). Five representative samples were collected in each location.

Morphological study

Morphological study of AMF was conducted by isolating the spores from soil samples through wet-sieving and decanting technique (Gerdemann and Nicolson, 1963). Based on morphological appearances, most predominant spores are golden or dark brown spores, double cell walled spores and smooth oval shaped spores were observed under stereo microscope (Labomed,USA).

Spores propagation

The *Glomus intraradices* fungi used for this experiment was isolated from sugarcane rhizosphere and multiplied using maize (*Zea mays* L. var-NK6240) as host plant. The plants were grown on soil-sand mix (2:1 w/w) substrate which was sterilized in an autoclave at 15 lb for 30 minutes to kill the indigenous AMF propagules present for avoiding cross contamination. After 60 days of incubation, the soil samples were collected from pots and the spores were isolated by wet-sieving and decanting technique followed by 60 % w/v sucrose gradient centrifugation method (Daniels and Skipper 1982). The mycorrhizal roots were removed from pot culture for estimating AM colonization by root clearing and staining technique (Phillips and Hayman 1970).

Estimation of AM root colonization

Roots of maize uprooted on 60 days after sowing were assessed for AM fungal colonization by following root clearing and staining technique developed by Phillips and Hayman (1970). The total percentage of root colonization was determined by using the following formula:

$$\text{Root colonization (per cent)} = \frac{\text{No. of root bits having colonization}}{\text{Total number of root bits observed}} \times 100$$

Spores surface sterilization

The *Glomus intraradices* spores were surfaced sterilized according to Becard and Piche (1992), by screening for 10 min in 2 per cent Chloramine-T and Tween 20 (0.1 per cent), followed by 30 min in Streptomycin 200 mg/lit and Ampicillin 200 mg/lit and finally rinsed several times with sterile distilled water. If spores are not to be used immediately, they should be stored at 4°C in ringer solution.

Spore germination test

The following substrates were used for germination test *viz.,* 1 per cent water agar, MS medium, (Murashige and Skoog, 1962) MSR medium, (Declerck *et al*., 1996) and white's media (Becard and Fortin, 1988). They were poured into 90 mm diameter Petri plates and allowed to set and each substrate was replicated five times. Every plate had 10 surface sterile spores distributed evenly and the plates were incubated at 27°C in the dark. Plates were observed under stero microscopic once in a week until 30 days of incubation.

RESULTS

Assessment of colonization potential of *Glomus intraradices*

The root colonization potential of isolated *Glomus intraradices* AM fungus under greenhouse condition was 65 per cent (fig. 1 & 2) and the maximum spore count (121 /100g of soil) was observed in sand mixture substrate after 60 days of inoculation. Plants subsequently grown without AMF had no sign of AM colonization. The results clearly indicated that the isolated *Glomus intraradices* spore is alive and viable with ability to colonize roots and multiply quickly at sixty days of inoculation,

Selection of suitable substrate for spore germination

After 30 days of incubation under darkness, germination and germ tube growth of *Glomus intraradices* was influenced by different substrates used. Among them, the highest spore germination percentage (80 %) and germ tube growth (63 mm) was recorded in MSR medium followed by MS salt substrate (Table 1). Control plate without sterilization was contaminated by bacteria and fungi within 7 days of incubation.

Root colonization potential of AM fungus in maize plants grown in soil and sand mixure substrate

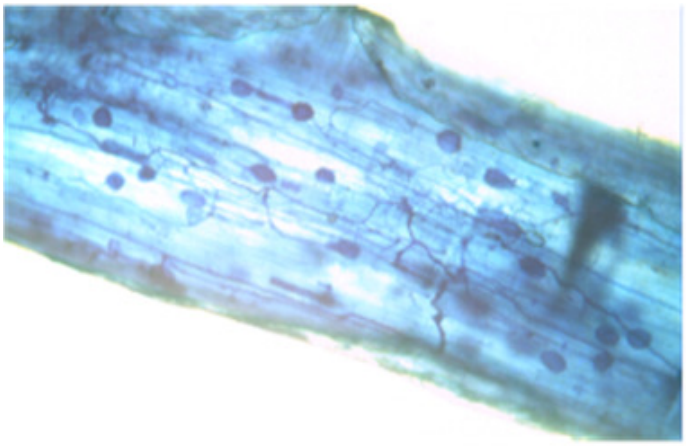


Fig. 1 : *I – G. intraradices.* (x100).

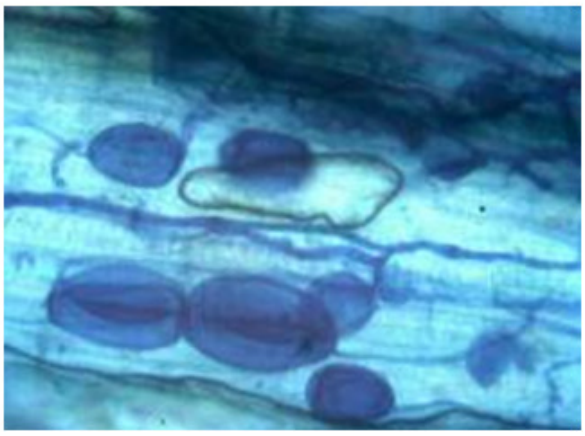
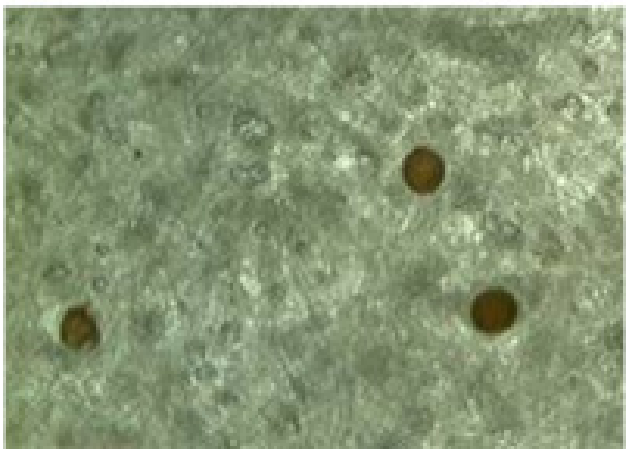
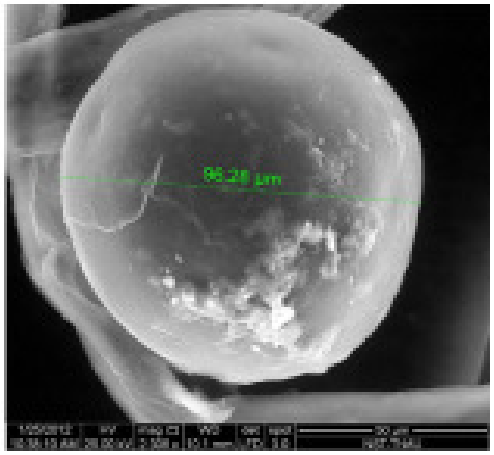


Fig. 2 : Vesicles of *G. intraradices* within root (x450).



A



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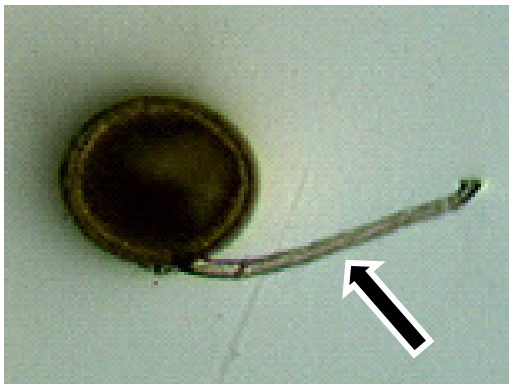

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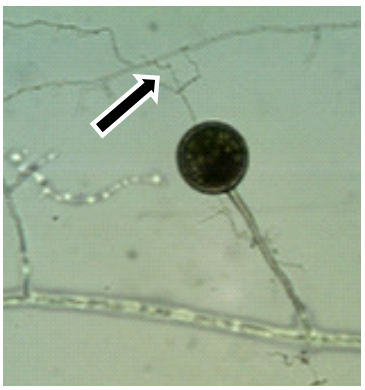

E

A- Surface sterilized isolated *Glomus intraradices* (40X)
B- Scanning Electron Microscope (*SEM*) view of *Glomus intraradices* (96 µm)
C- *Glomus intraradices* germination on MSR substrate (400 X)
D- *Glomus intraradices* germ tube growth (100X)
E- Multiple hyphal branching from *Glomus intraradices* (100 X).

Table 1 : Germination percentage and germ tube growth on *Glomus intraradices* in different substrate.

Different substrate	Totalno. of spores	No of spore germination	Germination percentage	Contamination %	Germ tube growth (mm)
Control*	10	00	00	100±2.90	00
Water agar (0.75%)	10	4±0.11	40±1.16	60±1.74	40±1.16
MS Medium	10	5±0.14	50±1.45	50±1.45	45±1.30
MSR Medium	10	8±0.23	80±2.52	20±0.58	63±1.83
White's Medium	10	2±0.05	20±0.58	80±2.32	34±0.98

*without sterilization of spores.

DISCUSSION

AMF spores were isolated from sugarcane rhizosphere at six different locations. Based on the morphological characterization, majority of spores were smooth, golden brown, double cell walled, and elliptical or oval shaped, 96 µm size which is ideal taxonomic character for *Glomus intraradices.* Morton and Bentivenga, 1994 reported that the taxonomic identification of *Glomus intraradices* species is based upon morphological characteristics of their spores size (40 - 140 ìm), color, and spore wall character. *Glomus intraradices* colonization pattern was of *Arum*- type and the first infection unit was the formation of cylindrical shape hyphae with in maize root followed by vesicles and arbuscular production. Dickson *et al.* (2007) reported that the two major colonization patterns of AM fungi was *Arum*- and *Paris*- type named after the plants (*Arum maculatum*, *Paris quadrifolia*) in which they were first observed. Intercellular hyphae, vesicles and intracellular arbuscules is the characteristic feature of the *Arum*- type. The *Paris*- type is characterized by intercellular hyphae, coils and arbusculate coils (Dickson, 2004). Reports indicates that most of the cultivated plants produce *Arum*-type morphology, whereas *Paris*- type tends to dominate plants of natural ecosystems. In this study, spore propagation result showed a higher viability of isolate *Glomus intraradices* using *Zea may*s as a host plant with higher per cent of root colonization (65±1.24) and fast multiplication of spores (121±2.99/ 100g of soil) in soil sand based substrate. Marleen *et al.* (2011) opined that a number of plant species such as onion and leek (*Allium spp*.), maize (*Zea mays* L.), and Bahia grass (*Paspalum notatum Flugge*) are commonly used for the large-scale production of AM fungi. These plants offer several advantages, among which a short life cycle, adequate root system development, a good colonization level by a large range of AM fungi, In this study, the germination and germ tube growth of isolated *Glomus intraradices,* spores was influenced by the substrate. Among the substrates, the MSR medium showed 90% spores germination with 63 mm germ tube growth after 30 days of incubation. Since MSR medium is composed of all major and minor nutrients supplemented with vitamin solution and the pH of media is also low (5.5), the germination and growth was better in the media than the other media studied. The same trend was observed by Heppe 1984 when spores of *Acaulospora laevis* were exposed to different pH levels, the germination was higher in low pH. The nutrient concentrations in the media influences colonization potential of AM fungi. Compared to MS medium, the MSR medium had 410 mg/L of KH_2PO_4 and this would have contributed to enhanced germination and germ tube growth of AM fungi. Siqueira *et al.* (1985) suggested that addition of a small amount of phosphorus to the agar medium may enhance germination and growth of germ tube. Even up to 500 mg/L concentration, the germination of some AMF spores was not inhibited (Bartolome and Schenck,1994). In MSR medium solidified with 3 % clarigel (HIMEDIA), the spore germination was the maximum due to agar quality but in other substrates, bacto agar was used as gelling agent. Alok *et al.* (1997) concluded that the media gelling agent mainly influences the germination of spores based on agar purity. The control, without surface sterilization showed 100 % contamination while surface sterilized plates showed less than 1 per cent of contamination. Walley and Germida (1996) reported that the spores must be surface sterilized to eliminate all contaminants because they may carry bacteria between wall layers.

CONCLUSION

This study clearly indicated that the *G. intraradices* spores isolated from the rhizosphere of sugarcane showed higher viability and showed high potential for root colonization and fast multiplication in open pot culture. Under *in vitro* conditions, the surface sterilized spores had good germination and produced numerous germ tube within 30 days. This *G. intraradices* spores is highly suitable for *in vitro* commercial production of the AM inoculum.

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