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REGULAR ARTICLE

STUDY ON LIFE CYCLE OF ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS INTRARADICES* USING *IN VITRO* CULTURING TECHNIQUE

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SUMMARY

In this study, life cycle of arbuscular mycorrhizal fungus, *Glomus intraradices* isolated from wheat rhizospheres of Damghan region studies using carrot (*Daucus carota* L.) transformed hairy roots. Mycorrhizal spores and roots were used as inocula. Spore germination was observed 3-5 days after surface sterilization as well as co inoculation with transformed roots. Fungal growth was also recorded 2-10 days after inoculation. Fungal germinating hyphae branched and produced radical shape network 2.5mm in diameter. The first contact between fungus mycelium and roots occurred 1-3 days after germination. 7 days after fungus- host contact, several secondary spores or vesicle like structures observed which were similar to true spores except of their size (20-30µm diameter). The first true spore formed 25 days after contact and then number of spores increased exponentially. These spores were hyaline and whitish at first but then turned to brownish yellow. After 12 weeks, 1000-2500 spores could be recorded in each plate.

Key words: Arbuscular mycorrhizal fungi, *Glomus intraradices*, *In vitro* culture, Life cycle

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1. Introduction

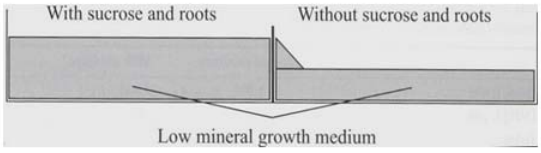
Arbuscular mycorrhizal (AM) fungi found in rhizosphere of several vascular plants and have important roles on sustainable agriculture as well as agricultural ecosystems management [1]. These fungi could be able to colonize host plants by their three sources including spores, mycorrhizal roots and extraradical mycelia [2]. There are obvious differences among fungal families and genera in life cycle and ecology [3]. Fungi in Glomeraceae and Acaulosporaceae families could be able to colonize host plants by 3 mentioned sources while in Gigasporaceae, the only inoculum sources are spores [3, 4]. Spore formation depended on different factors such as seasonality [5, 6], Host plant and fungal species [7, 8], Physiological changes in host plant [9], Nutrient levels [10] as well as interaction with other soil microorganisms [11, 12]. There are so many efforts in order to get pure isolates of arbuscular mycorrhizal fungi but most of them faced to problems and failed due to biotrophic nature of these fungi. *In vitro* culturing of these fungi is very important especially for studying on host plant growth and taxonomic studies [13]. First time, Mosse used of root tissue culture method for obtaining pure culture [14]. Also, *In vitro* culturing of AM fungal species using carrot (*Daucus carota* L.) transformed hairy roots by R_i plasmid of *Agrobacterium rhizogenes* (Ricker) Conn. is also another method [15]. Using this method, several fungal species could be propagated *In vitro* such as *Gigaspora margarita, G. gigantea, Glomus fasciculatum, G. intraradices, G. versiforme and G. caledonium* [16-20]. Using this method, it could be possible to study on molecular as well as biochemical aspects of arbuscular mycorrhizal symbiosis. Unfortunately there have not been studies on

In vitro culturing of these fungi isolated from rhizosphere. The main purpose of this study is possibility of *In vitro* culturing of *G. intraradices* isolated from wheat rhizospheres using transformed hairy roots system as well as study on fungal life cycle under laboratory conditions.

2. Materials and Methods

Soil samples were collected from wheat rhizospheres in Damghan region during May-June 2009. Fungal spores isolated from soil samples using wet sieving and centrifugation in sucrose solution method [21, 22], and then microscopic slides prepared for species identification. Fungal spores as well as mycorrhizal roots were used as inocula. For this purpose, trap cultures established using clover and 30-50 fungal spores in order to get more and healthy number of spores as well as mycorrhized roots. Trap pot cultures kept for 5 months in greenhouse condition and treated with Long Ashton nutrient solution [23]. Clover roots stained for observing colonization by fungal structures [24]. After that, fungal spores as well as mycorrhized roots from trap cultures used as inoculum. 150-200 healthy and young spores of *G. intraradices* were isolated from trap culture soils using wet sieving method but without centrifugation in sucrose solution and kept in sterile distilled water in 4°c for 2 weeks before inoculation in order to breaking spore dormancy [25]. 10-20 mycorrhized root segments, 5cm each were also selected, washed 2-3 times with sterile distilled water and kept in 50ml Falcon tubes in 4°c. For surface sterilization, spores washed 3 times, 5min each with sterile distilled water, treated with Chloramin-T (2%) and 2-3ml Tween 20 for 10min, then, washed again 3 times, 5min each by sterile water and treated 10min by Streptomycin sulphate (0.02%) and Gentamycin (0.01%) solutions. After that, spores washed 5min by sterile water and kept in plates for inoculation. For mycorrhized root segments, Falcon tubes ultrasonicated 2 times, 1min each, then, roots washed 3 times, 5min each by sterile water, sterilized by ethanol (95%) for 10sec, washed again by water, treated with Calcium hypochlorite (6%) for 1-2 min, washed with sterile water and then sterilized and kept similar to fungal spores. Collected spores and root segments cultured in MSR medium [19, 26] with sucrose and incubated at 27°c in order to get free contaminated inoculum. Carrot transformed hairy roots were also prepared by Danesh *et al.*, 2006 method [27]. Two segmented plates used in this study for *In vitro* culturing of fungal species which had sucrose only in one segment as carbon source [28] (Fig. 1). Plates with MSR medium amended and without sucrose prepared, then, carrot transformed root segments cultured in sucrose amended parts of the plates and incubated 7-10 days inversely at 27°c. After that, 4-5 sterilized fungal spores or 1-2 sterilized root segments inoculated adjacent to transformed root branches and incubated again in same conditions. Plates checked every 48h for germination and growth of fungal propagules. Also, time of spore germination, first contact between fungal inoculum and host roots as well as first spore formation and spore numbers were recorded in each plate.

Fig 1- Two-segmented plates used for *In vitro* culturing of *G. intraradices* proposed by St-Arnaud *et al.*, 1996

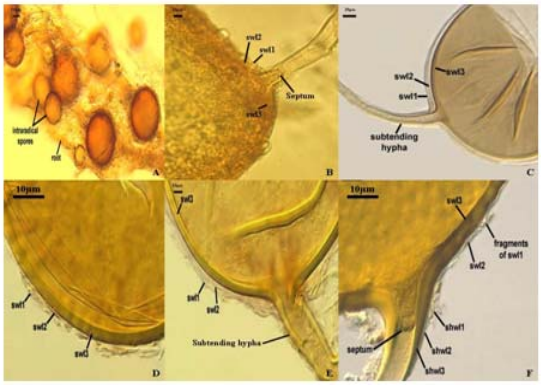


3. Results and Discussion

Following isolation and identification of species in collected soils from wheat rhizospheres in Damghan region, *G. intraradices* was identified on 65% total samples. Spores usually observed in clusters, rarely individually around root segments.

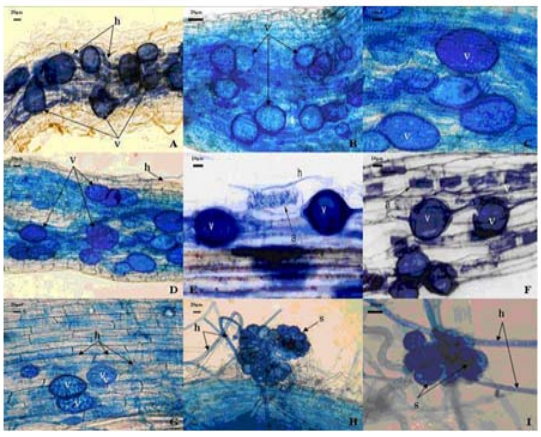
They were globose or subglobose, (85-)119(-157)µm in diameter, consisting 3 layers in wall (Swl1, Swl2, Swl3). Swl1 is hyaline, mucilaginous, 0.5-2µm in diameter and evanescent. Swl2 is also hyaline, 0.7-2.8µm and linked to Swl1. Swl3 is laminated and (1.8-)4.4(-6.7)µm in diameter. Hyphal attachment is cylindrical and straight, (5.5-)10.5(-15)µm width and consisting of 3 layers in wall. Hyphal pore is also closed by septum or may be opened (Fig 2).

Fig 2- Morphological characteristics used for identification of *G. intraradices*. A: Spore clusters formed in host plant roots (10X). B-F: Spore wall layers (Swl1, Swl2, Swl3), hyphal attachment and septum (B, E-40X; C-20X; D, F-100X)



Study on clover root staining obtained from trap cultures showed that all roots were colonized by fungal species. Different structures could be observed in roots including fungal mycelia, vesicles, arbuscules as well as intraradical spores but vesicles and mycelia were more abundant structures could be found in all treatments (Fig.3).

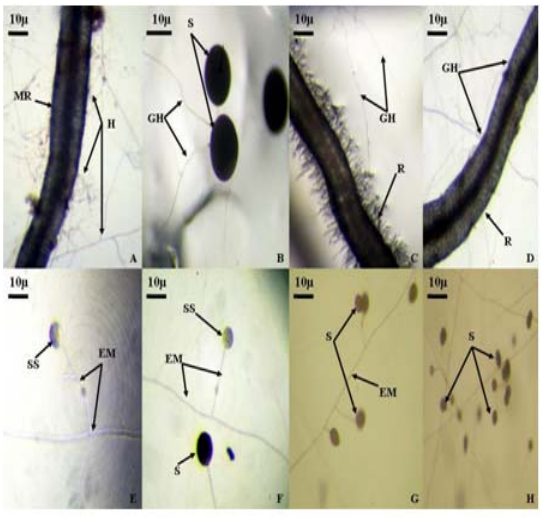
Fig 3- Fungal structures including vesicles (v), hyphae (h), arbuscules (a) and spores (s) observed in clover roots after staining



The best method for isolation of fungal spores from trap culture pots for *In vitro* culturing is wet sieving method without centrifugation in sucrose solution since sucrose even in low concentration in contact with spores resulted in structural changes as well as decreasing or even losing germination ability. Also, incubation of spores at 4°c for 2 weeks is necessary in order to breaking spore dormancy and their stimulation for germination [25]. However, cold treatment depends on fungal species. For example, some *Gigaspora* species such as *G. rosea* does not need this treatment [16]. After surface sterilization as well as inoculum incubation, only less than 5% of

spore treatments and less than 15% of root segments treatments showed contamination which removed immediately. These findings were in agreement with other studies [17, 26]. Spores germinated 3-5 days after co inoculation, produced germinating hyphae which were grown, branched and formed radical shape network 2.5mm in diameter [29, 30]. Root segments were also grown 2-10 days after inoculation. The first contact between fungus mycelium and roots occurred 1-3 days after germination. Once fungal hyphae reached in vicinity of transformed roots, its growth pattern changed, their growth became fast and in a straight way toward hairy roots. Hyphae branched in all directions and formed more extensive extraradical mycelia. If fungal mycelia did not contact with host roots or if host signals could not be recognized by fungi, hyphal growth would be stopped after some days [31]. 7 days after fungus-host contact, secondary spores or vesicle like structures formed on extraradical mycelia which were 20-30µm in diameter. These structures were smaller than true spores and formed almost terminally on short hyphal branches. Fungal mycelia reached to middle barrier of plate and after 1-2 weeks delay passed through and entered to non-sucrose amended section. Once entering mycelia, their growth would be increased exponentially and covered plate in 2 weeks. After that, true spores formed on hyphal branches around 25 days after contact. These spores were hyaline whitish color but turned to brownish yellow after several weeks. These spores were 85µm in diameter which larger than secondary structures but with the same morphology. So, after 4th weeks, spore production could be observed in culture medium and increased exponentially. From the end of 8th to 12th week, this trend could be recorded. After that, roots turned from white color to brownish yellow showed the end of growth and subsequently, spore growth were also stopped. Since the spores size was too small, recording their numbers was also difficult but after 12 weeks, 1000-2500 spores could be counted. Spore age, their physiological conditions as well as time of spore isolation from soil are the most important factors on dormancy [32, 33]. The culture medium used in this study (MSR medium) is very similar to modified White medium (MW) in respect of macro elements. Their difference is due to their micro elements as well as vitamin concentration. MSR medium does not have Iodine, Myo-Inositol and Glycin but MW medium does not have Pentothenate, Biotin and Cyanocobalamin. Germination of *Glomus* spores and hyphal growth are not mostly depended on host plant [34]. However, there are several reports recently on effects of Flavonoids as stimulators or suppressors of spore germination [17, 35]. Among them, Quercetin is one of the most stimulators.

Fig 4- Procedures of propagation and *In vitro* culturing of *G. intraradices*. A: Growth of fungal hypha (H) from root segments (MR) (40X). B: Fungal germinated spore (S) and germinating hypha (GH) production (100X). C-D: Hyphal growth toward host roots (R) and their contact (40X). E: Secondary spores formation (SS) on extraradical mycelia (EM) (40X). F-H: Secondary spores (SS) and true spores (S) formation on extraradical mycelia (EM) (40X)



Also, new substances from Sterols, Diacylglycerols, Phospholipids and free fatty acids produced during spore germination [36, 37]. More or less amounts of humidity are one of the most important suppressors on spore germination [38]. Reports showed that neutral pH could increase germination [39] while acidic pH could decrease [38]. On the other hand, optimum temperature for spore germination is 20-30°c. [40]. Other factors such as spore storage materials [37], low amounts of light [41] and host plant root exudates [42] are effective on hyphal growth and branching patterns. Spore germination, hyphal growth and new spore formation procedures in this study have been shown in Figure 4. Results were in agreement with findings of other researchers [17,26,28].

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