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Araştırma Makalesi/*Research Article (Original Paper)*

Using *In Vitro* Culturing Technique for Studying Life Cycle of Arbuscular Mycorrhizal Fungus (AMF) *Glomus intraradices*

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Abstract: In this study, life cycle of arbuscular mycorrhizal fungus, *Glomus intraradices* isolated from wheat rhizospheres of Damghan region in the Iran was observed using carrot (*Daucus carota* L.) transformed hairy roots *in vitro*. Mycorrhizal spores and roots were used as inoculum. 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 2.5 mm 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 were similar to true spores except of their size (20-30 µm 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.

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

İn Vitro Kültür Tekniği Kullanılarak Arbusküler Mikorhizal Fungus (AMF) *Glomus intraradices*'in Yaşam Döngüsü'nün İncelenmesi

Özet: Bu çalışmada, İran'ın Damghan bölgesinde bulunan buğday bitkisinin rizosfer bölgesinden izole edilen ve havuç (*Daucus carota* L.) bitkisinin saçak köklerine inokule edilen arbusküler mikorhizal fungus *Glomus intraradices*'in laboratuvar koşullarında yaşam döngüsünün incelenmesi amaçlanmıştır. Çalışmada AMF inokulumu olarak mikorhizal spor ve kökler kullanılmıştır. İnokulasyon ile birlikte yüzey sterilizasyonun yapılmasından 3-5 gün sonra, havuç bitkisinin saçak köklerinde spor çimlenmesi gözlenmiştir. İnokulasyondan 2-10 gün sonra ise fungal gelişimin olduğu kaydedilmiştir. Fungal çimlenmede, hifler dallanarak, 2.5 mm çapında kök şeklinde ağ oluşturmuşlardır. Fungus miselyumu ve bitki kökleri arasındaki ilk temas, fungus sporunun çimlenmesinden 1-3 gün sonra meydana gelmiştir. Fungus-konukçu temasından 7 gün sonra ise büyüklükleri dışında (20-30 µm çapında) diğer özellikleri gerçek sporlara benzeyen spor ve vesikel benzeri yapılar gözlenmiştir. İlk gerçek spor oluşumu, fungus-konukçu temasından 25 gün sonra gerçekleşmiş, daha sonra sayıları katlanarak artmıştır. Bu sporların rengi başlangıçta şeffaf ve beyazımsı iken daha sonra sarımsı kahverengine dönmüştür. İnokulasyondan 12 hafta sonra da her bir petrideki spor sayısı 1000-2500 olarak kaydedilmiştir.

Anahtar kelimeler: Arbusküler mikorhizal fungus, *Glomus intraradices*, *İn vitro* kültür, Hayat döngüsü

Introduction

Arbuscular mycorrhizal (AM) fungi exist in rhizosphere of several vascular plants and have important roles in sustainable agriculture as well as agricultural ecosystems management (Bethelenfalvay and Schuepp 1994). These fungi could be able to colonize host plants by their three sources including spores, mycorrhizal roots and extraradical mycelia (Smith and Read 1997). There are obvious differences among fungal families and genera in life cycle and ecology (Brundrett et al. 1999). 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 (Brundrett et al. 1999; Klironomos et al. 2002). Spore formation depended on different factors such as seasonality (Gemma and Koske 1988; Bever et al.

2001), host plant and fungal species (Bever et al. 1996; Bever 2002), physiological changes in host plant (Sylvia and Schenck 1983), nutrient levels (Douds and Schenck 1990) as well as interaction with other soil microorganisms (Paula et al. 1991; Bakhtiar et al. 2001). 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 (Hepper 1984). First time, Mosse used toot tissue culture method for obtaining pure culture (Mosse 1962). 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 (Mugnier and Mosse 1987). 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* (Chabot et al. 1992; Douds and Becard 1993; Declerck et al. 1996; 1998). 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.

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 (Gerdemann and Nicolson 1963; Jenkins 1964), 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 in pots filled with sterilized sand as well as clover seeds as host plant. 30-50 fungal spores mixed with soil near the seeds in order to get more and healthy number of spores. Trap pot cultures kept for 5 months in greenhouse condition and treated with Long Ashton nutrient solution (Hewitt 1966). Clover roots stained for observing colonization by fungal structures (Philips and Hayman 1970). After that, fungal spores as well as mycorrhizal 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 (Fortin et al. 2002). 10-20 mycorrhized root segments, 5 cm each were also selected, washed 2-3 times with sterile distilled water and kept in 50 ml Falcon tubes in 4°C. For surface sterilization, spores washed 3 times, 5 min each with sterile distilled water, treated with Chloramin-T (2%) and 2-3 ml Tween 20 for 10 min, then, washed again 3 times, 5 min each by sterile water and treated 10 min by streptomycin sulphate (0.02%) and gentamycin (0.01%) solution. After that, spores washed 5 min by sterile water and kept in plates for inoculation. For mycorrhized root segments, Falcon tubes ultrasonicated 2 times, 1 min each, then, roots washed 3 times, 5 min each by sterile water, sterilized by ethanol (95%) for 10 sec, washed again by water, treated with Calcium hypochlorite (6%) for 1-2 min, washed with sterile water and then sterilized and kept similar that fungal spores. Collected spores and root segments cultured in MSR medium (Strullu and Romand 1986; Declerck et al. 1996) with sucrose and incubated at 27°C in order to get free contaminated inoculum. Carrot transformed hairy roots were also produced by Danesh et al. (2006) method. Two segmented plates used in this study for *in vitro* culturing of fungal species which had sucrose only in one segment as carbon source (St Arnaud et al. 1996) (Figure 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 48 h 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.

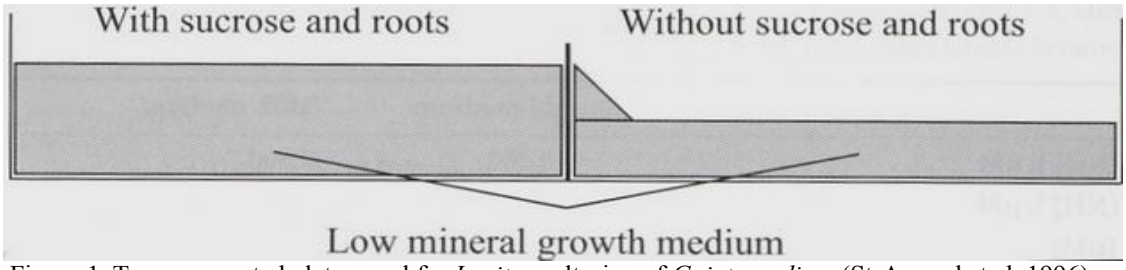

Figure 1. Two-segmented plates used for *In vitro* culturing of *G. intraradices* (St-Arnaud et al. 1996).

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, 0.5-2 µm in diameter and evanescent. Swl2 is also hyaline, 0.7-2.8 µm 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 (Figure 2).

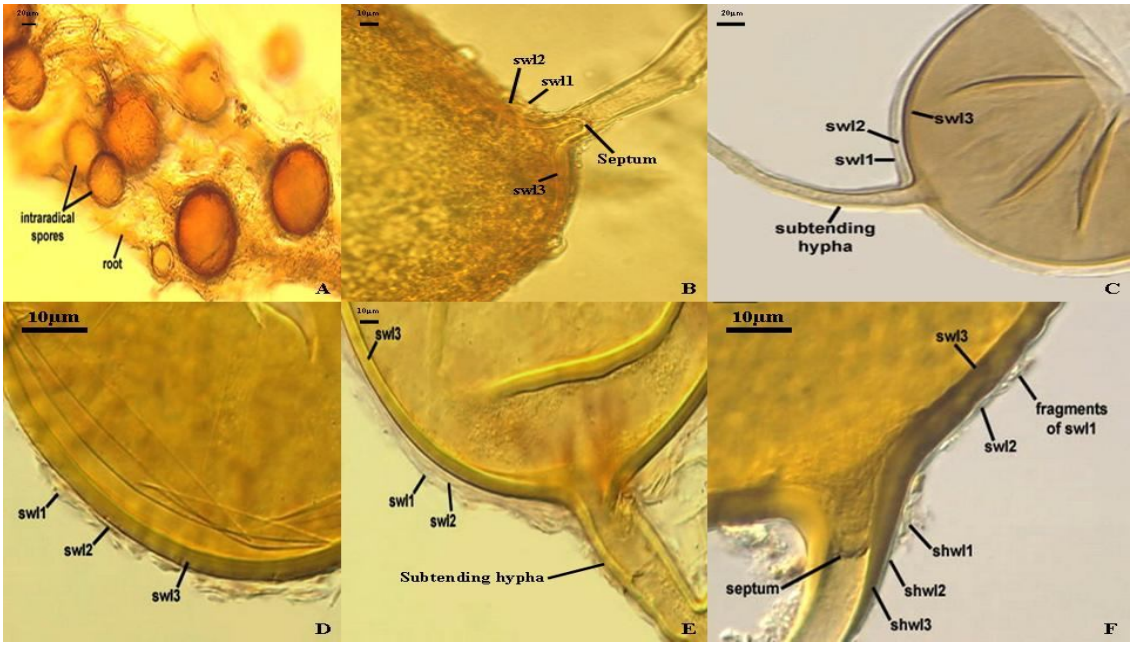

Figure 2. Morphological characteristics used for identification of *G. intraradices*. A: Spore clusters formed in host plant roots (10X). B-F: Spore wall layers (Swl1, Swl2, Swl3), hyphal attachment and septum (B, E-40X; C-20X; D, F-100X)

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 (Figure 3).

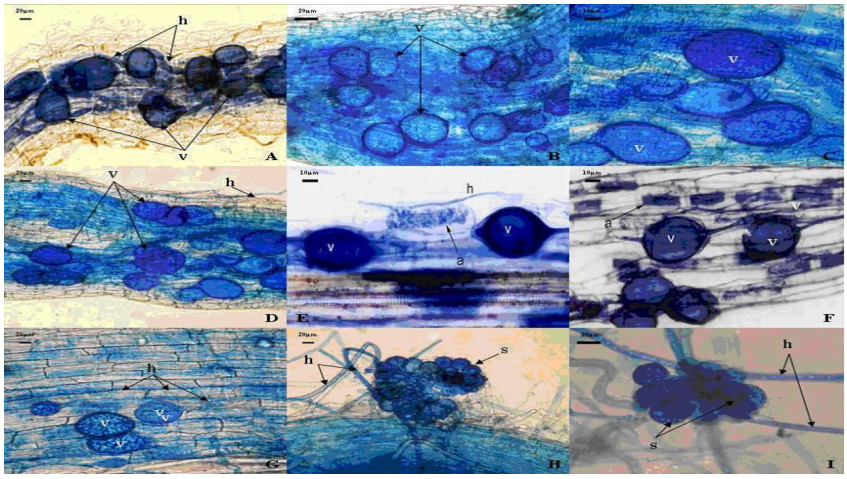

Figure 3. Fungal structures including vesicles (v), hyphae (h), arbuscules (a) and spores (s) observed in clover roots after staining

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 (Fortin et al. 2002). However, cold treatment depends on fungal species. For example, some *Gigaspora* species such as *G. rosea* does not need this treatment (Becard and Fortin 1988). After surface sterilization as well as inoculum incubation, only less than 5% of spore treatments and less than 15% root segments treatments showed contamination which removed immediately. These findings were in agreement with other studies (Strullu and Romand 1986; Chabot et al. 1992). Spores germinated 3-5 days after co-inoculation, produced germinating hyphae which were grown, branched and formed radical shape network 2.5 mm in diameter (de Souza and Berbara 1999; Declerck et al. 2000). 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 (Becard and Piche 1989). 7 days after fungus-host contact, secondary spores or vesicle like structures formed on extraradical mycelia which were 20-30 µm 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 85 µm in diameter which larger than secondary structures but with the same morphology. So, after 4^{th} weeks, spore production could be observed in culture medium and increased exponentially. From the end of 8^{th} to 12^{th} 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 (Tommerup 1983; Hardie 1984). 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 germination hypha growth are not mostly depended on host plant (Giovannetti and Sbrana 1998). However, there are several reports recently on effects of Flavonoids as stimulators or suppressors of spore germination (Tsai and Phillips 1991; Chabot et al. 1992). Among them, Quercetin is one of the most stimulators. Also, new substances from Sterols, Diacylglycerols, Phospholipids and free fatty acids produced during spore germination (Gaspar et al.

1994; Sancholle et al. 2001). More or less amounts of humidity are one of the most important suppressors on spore germination (Siqueira et al. 1985). Reports showed that neutral pH could increase germination (Gunasekaran et al. 1987) while acidic pH could decrease (Siqueira et al. 1985). On the other hand, optimum temperature for spore germination is 20-30°C (Sheikh and Sanders 1988). Other factors such as spore storage materials (Sancholle et al. 2001), low amounts of light (Nagahashi et al. 2000) and host plant root exudates (Giovannetti et al. 1993) are effective on hyphal growth and branching patterns. Spore germination, hyphal growth and new spore formation procedures in this study showed in Figure 4. Results were in agreement with findings of other researchers (Strullu and Romand 1986; St Arnaud et al. 1996).

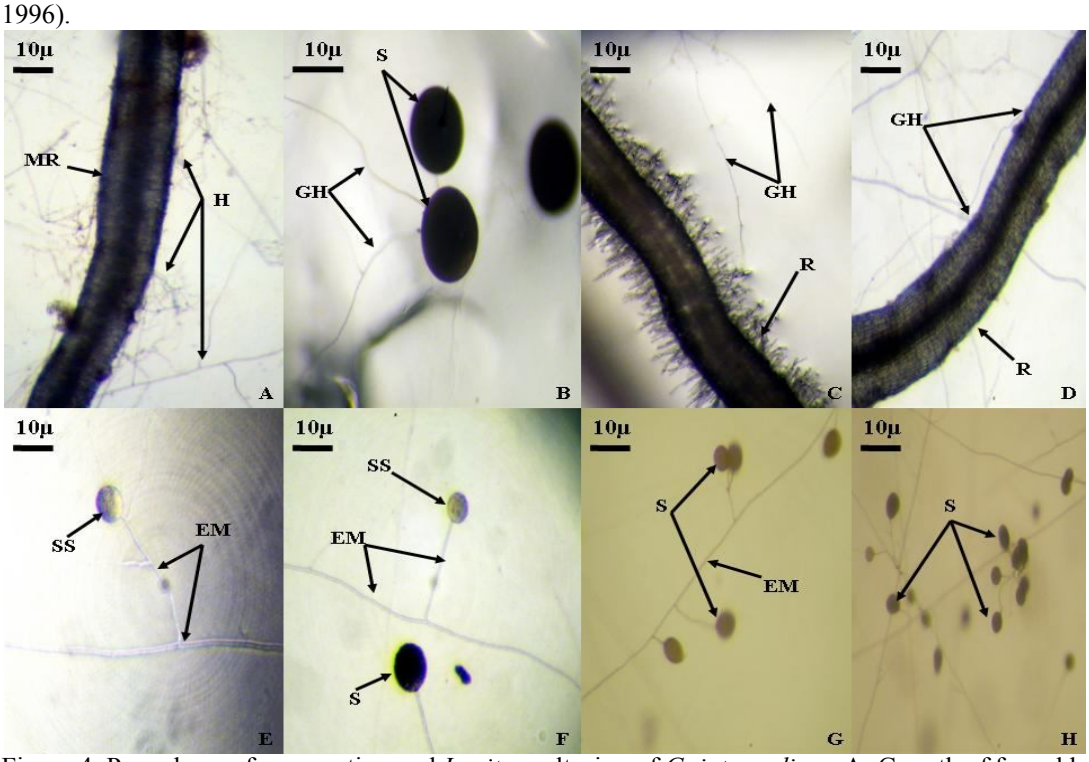

Figure 4. Procedures of propagation and *In vitro* culturing of *G. intraradices*. A: Growth of fungal hypha (H) from root segments (MR) (40X). B: Fungal germinated spore (S) and germinating hypha (GH) production (100X). C-D: Hyphal growth toward host roots (R) and their contact (40X). E: Secondary spores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

In conclusion, obviously, the use of *In vitro* cultivation of AM fungi has become an essential tool for the study of mycorrhizal interactions as well as their practical uses. One of the greatest challenges we are facing is to achieve the *In vitro* cultivation of most, if not all glomalean fungi. For example in this technique, different isolates of *Glomus intraradices*, under the same set of conditions, produce largely different numbers of spores, a phenotypic variation very easy to identify. The protocol presented here aims to give researchers the possibility to initiate and maintain their own isolates. The benefits derived from this technique is not only restricted to the study of symbiotic interactions, but also permits the increase in knowledge in morphology, taxonomy, phylogeny and biochemistry fields.

References

Bakhtiar Y, Iller D, Cavagnaro T, Smith S (2001). Interactions between two arbuscular mycorrhizal fungi and fungivorous nematodes and control of the nematode with fenamifos. Appl. Soil. Ecol. 17: 107-117.

Becard G, Fortin JA (1988). Early events of vesicular arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. 108: 211-218.

Becard G, Piche Y (1989). Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl. Environ. Microbiol. 55: 2320–2325.

Bethelenfalvay GJ, Schuepp H (1994). Arbuscular mycorrhizas and agrosystem stability, pp. 171-131, In: Impact of Arbuscular Mycorrhizas in Sustainable Agriculture and Natural Ecosystems, Gianinazzi S and Schuepp H (eds), Birkhauser Verlag, Basel.

Bever JD, Morton JB, Antonovics J, Schultz PA (1996). Host dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. J. Ecol. 84: 71-82.

Bever JD, Schultz PA, Pringle A, Morton JB (2001). Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why. Bioscience. 51: 923-931.

Bever JD (2002). Negative feedback within a mutualism: host-specific growth of mycorrhizal fungi reduces plant benefit. In: Proceedings of the Royal Society of London, B, Biological Sciences 269: 2595-2601.

Brundrett MC, Abbott LK, Jasper DA (1999). Glomalean mycorrhizal fungi from tropical Australia. I. Comparison of the effectiveness and specificity of different isolation procedures. Mycorrhiza. 8: 305-314.

Chabot S, Becard G, Piche Y (1992). Life cycle of *Glomus intraradix* in root organ culture. Mycologia. 84: 315-321.

Danesh YR, Goltapeh EM, Alizadeh A, Modares Sanavi M (2006). Optimizing carrot hairy root production for monoxenic culture of arbuscular mycorrhizal fungi in Iran. J. Biol. Sci. 6(1): 87-91.

de Souza FA, Berbara RLL (1999). Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia. 91: 343–350.

Declerck S, Cranenbrouck S, Dalpe Y, Seguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000). *Glomus proliferum* sp. nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia. 92: 1178–1187.

Declerck S, Strullu DG, Plenchette C (1996). *In vitro* mass production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycol. Res. 100: 1237-1242.

Declerck S, Strullu DG, Plenchette C (1998). Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia. 90: 579-585.

Douds D, Becard G (1993). Competitive interaction between *Gigaspora margarita* and *Gigaspora gigantea in vitro*. In: Proceedings of the Ninth North American Symposium on Mycorrhizae, Guelph, ON, Canada, August 8-12.

Douds DD, Schenck NC (1990). Increased sporulation of vesicular arbuscular mycorrhizal fungi by manipulation of nutrient regimes. App. Environ. Microbiol. 56: 413-418.

Fortin JA, Becard G, Declerck S, Dalpe Y, St. Arnaud M, Coughlan AP, Piche Y (2002). Arbuscular mycorrhiza on root-organ cultures. Can. J. Bot. 80(1): 1-20.

Gaspar L, Pollero RJ, Cabello MN (1994). Triacylglycerol consumption during spore germination of vesicular-arbuscular mycorrhizal fungi. J. Am. Oil. Chem. Soc. 71(4): 449-452.

Gemma JN, Koske RE (1988). Seasonal variation in spore abundance and dormancy of *Gigaspora gigantea* and in mycorrhizal inoculum potential of a dune soil. Mycologia. 80: 211-216.

Gerdemann GW, Nicolson TH (1963). Spore of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Trans. Brit. Mycol. Soc. 46: 235- 244.

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993). Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytol. 125: 587–593.

Giovannetti M, Sbrana C (1998). Meeting a non-host: the behaviour of AM fungi. Mycorrhiza. 8: 123–130.

Gunasekaran P, Sundaresan P, Ubalthoose N, Lakshmanan M (1987). Effect of pH, temperature and nutrients on the germination of a vesicular arbuscular mycorrhizal fungus, *Glomus fasciculatum in vitro*. In: Proceeding of Indian AS-Plant Science 97: 231-234.

Hardie K (1984). Germination of *Glomus mosseae* spores isolated from stock pots of different ages. Trans. Brit. Mycol. Soc. 83: 693-696.

Hepper CM (1984). Isolation and culture of VA mycorrhizal (VAM) fungi. pp. 95-112, In: VA Mycorrhiza, Powell CL and Bagyaraj DJ (eds). CRC Press, Boca Raton, FL.

Hewitt EJ (1966). Sand and water culture methods used in the studies of plant. Nutrition Technical Communications, Vol. 22. Commonwealth Agricultural Bureau, London, United Kingdom, p. 430–434.

Jenkins WR (1964). A rapid centrifugal technique for separating nematodes from soil. Pl. Dis. Rep. 48: 692.

Klironomos JNN, Hart MM (2002). Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza. 12: 181-184.
Mosse B (1962). The establishment of vesicular arbuscular mycorrhiza under aseptic conditions. Gen. Microbiol. 27: 509-520.
Mugnier J Mosse B (1987). Vesicular-arbuscular mycorrhizal infections in transformed Ri T-DNA roots grown axenically. Phytopath. 77: 1045-1050.
Nagahashi G, Douds D, Buee M (2000). Light-induced hyphal branching of germinated AM fungal spores. Pl. Soil. 219(1-2): 71-79.
Paula MA, Reis VM,. Dobereiner J (1991). Interactions of *Glomus clarum* with *Acetobacter diazotrophicus* in infection of sweet potato (*Ipomoea batatas*), sugarcane (*Saccharum* spp.) and sweet sorghum (*Sorghum vulgare*). Biol. Fert. Soils. 11: 111-115.
Philips JM, Hayman DS (1970). Improved procedures clearing root and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infections. Trans. Brit. Mycol. Soc. 55: 158-161.
Sancholle M, Dalpe Y, Grandmougin-Ferjani A (2001). Lipids of mycorrhizae. pp. 63–93. In: The Mycota IX fungal associations. Hock B (ed). Springer-Verlag, Berlin and Heidelberg.
Sheikh NA, Sanders FE (1988). Effect of temperature on germination of mycorrhizal spores and infection in roots. Biologia. 34: 223-236.
Siqueira JO, Sylvia DM, Gibson J, Hubbell DH (1985). Spores, germination and germ tubes of vesicular-arbuscular mycorrhizal fungi. Can. J. Microbiol. 31: 965-972.
Smith SE, Read DJ (1997). Mycorrhizal Symbiosis, 2nd ed., Academic Press, London.
St Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an *in vitro* system in the absence of host roots. Mycol. Res. 100: 328–332.
Strullu DG, Romand C (1986). Methode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. C.R. Acad. Sci. 303: 245–250.
Sylvia DM, Schenck NC (1983). Application of superphosphate to mycorrhizal plants stimulates sporulation of phosphorus-tolerant vesicular-arbuscular mycorrhizal fungi. New Phytol. 95: 655-661.
Tommerup IC (1983). Spore dormancy in vesicular-arbuscular mycorrhizal fungi. Trans. Brit. Mycol. Soc. 81: 37–45.
Tsai SM, Phillips DA (1991). Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores *in vitro*. Appl. Environ. Microbiol. 57: 1485–1488.