



## Review

# ***In vitro culture of arbuscular mycorrhizal fungi: advances and future prospects***

**Tahir Abdoulaye DIOP**

Université Cheikh Anta DIOP, Faculté des Sciences et Techniques, Département de Biologie végétale, B.P. 5005, Dakar Sénégal/Laboratoire de Microbiologie des Sols, Centre de Recherches ISRA/IRD/UCAD, B.P 1386, Dakar, Sénégal. E.mail: Tahir.Diop@ird.sn.

Accepted 25 August, 2021

**Arbuscular mycorrhizal (AM) fungi are ecologically important for most vascular plants for their growth and survival. AM fungi are obligate symbionts. In recent years, there have been many attempts to cultivate *in vitro*. Some relevant results indicate efforts are not far from successful growth of AM fungi independent of a plant host. This paper describes the methods to cultivate root organs, and to select and purify AM fungal inocula (intraradical and extraradical forms). Axenic and monoxenic cultures of AM fungi are summarized and we propose a methodology to recover and achieve continuous culture of AM fungi without loss of infectivity. The *in vitro* systems have proved to be a valuable tool to study fundamental and practical aspects of AM symbiosis, complementing the *in vivo* experimental approaches. Finally, we give some proposals of *in vitro* AM researches to exploit more the potentialities of AM symbiosis.**

**Key words:** Arbuscular mycorrhizal fungi, continuous culture, root organ cultures, intraradical and extraradical forms, *in vitro*.

## INTRODUCTION

Arbuscular-mycorrhizal (AM) fungi are integrated components of most of terrestrial plants. The nutrient exchange and other benefits due to AM symbiosis are sufficient to research conditions of their preservation without contaminations. *In vitro* culture of AM fungi was achieved for the first time in the early 1960s (Mosse, 1962). Since this pioneering work, several efforts aimed at culturing the axenic or monoxenic of AM fungi (Becard and Piché, 1992; Chabot et al. 1992a; Diop 1990, 1995; Declerk et al 1996a) using different sources of inoculum have been successful. *In vitro* culture system is a valuable tool for the study of AM fungi. The purpose of this review is to summarize the recent results on *in vitro* culture of AM fungi, propose a methodology for

continuous culture of these obligate biotrophic microorganisms and indicate areas for further researches.

## ROOT ORGAN CULTURES

The use of excised roots as host partner in AM symbiosis was first proposed by Mosse and Hepper (1975). Isolated root can be propagated continuously in different solid and liquid media with high reproducibility. Clonal roots of some 15 plants have been established (Butcher, 1980), and this list has been enlarged during the last decades. Initiation of isolated roots requires pregermination of

seeds previously surface sterilized with classical disinfectants (sodium hypochlorite, hydrogen peroxide), then thoroughly washed in sterile distilled water. Germination of seeds occurs after 48 h at 28°C in the dark on water agar or moistened filter papers. The tips (2 cm) of emerged can be transferred to a rich medium such as modified White medium (Bécard and Fortin, 1988) or Strullu and Romand medium (Strullu and Romand, 1987). The pH of the medium is adjusted to 5.5 before autoclaving. Fast-growing roots are cloned by repeated subcultures.

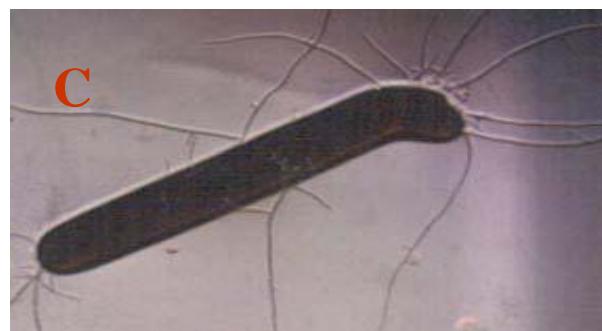
Transformation of roots by the soil-borne microorganism *Agrobacterium rhizogenes* has provided a new way to obtain mass production of roots in a very short time. A 2-day old loopful of a bacterial suspension is used to inoculate sections of root organs. Genetically modified caroot (*Daucus carota L.*) roots by *A. rhizogenes* show profuse roots two to four weeks later. Then, the tips are aseptically cultivated on rich medium (Bécard and Fortin, 1988). Several subcultures (3 to 4) are necessary in this medium enriched with antibiotics such as carbenicillin or ampicillin, to obtain free living roots without bacteria. A clonal culture derived from a single root is then established.

*A. rhizogenes* inserts in transformed roots copies of T-DNA (transfer DNA) which occurs in a large plasmid of *A. rhizogenes* (Chilton et al., 1982). Therefore, transformed roots have a quick, vigorous and homogenous growth in relative poor substrates without supplementation of hormonal substances. The negative geotropism of transformed roots facilitates contacts with hyphae of AM fungi (Bécard and Fortin, 1988; Mugnier and Mosse, 1987). Tepfer (1984) indicated they can survive for a long time without subculture, and Diop (1990) observed less contaminations in culture of transformed carrot roots due to their negative geotropism.

## FUNGAL INOCULA

All types of AM propagules (isolated spores or vesicles, mycelia, sheared mycorrhizal roots) are virtually able to initiate AM symbiosis (Figure 1). Chlamydospores of *Glomus* sp (Mosse and Hepper, 1975; Mugnier and Mosse, 1987) and non-sporocarpic azygospores of *Gigaspora margarita* (Bécard and Fortin, 1988; Bécard and Piché, 1989a; Diop et al., 1992) are obviously the preferred inoculum starter even though dormancy and strain mutation may occur under greenhouse conditions. The preference is due to availability of facility to recover, sterilize and to germinate these propagules.

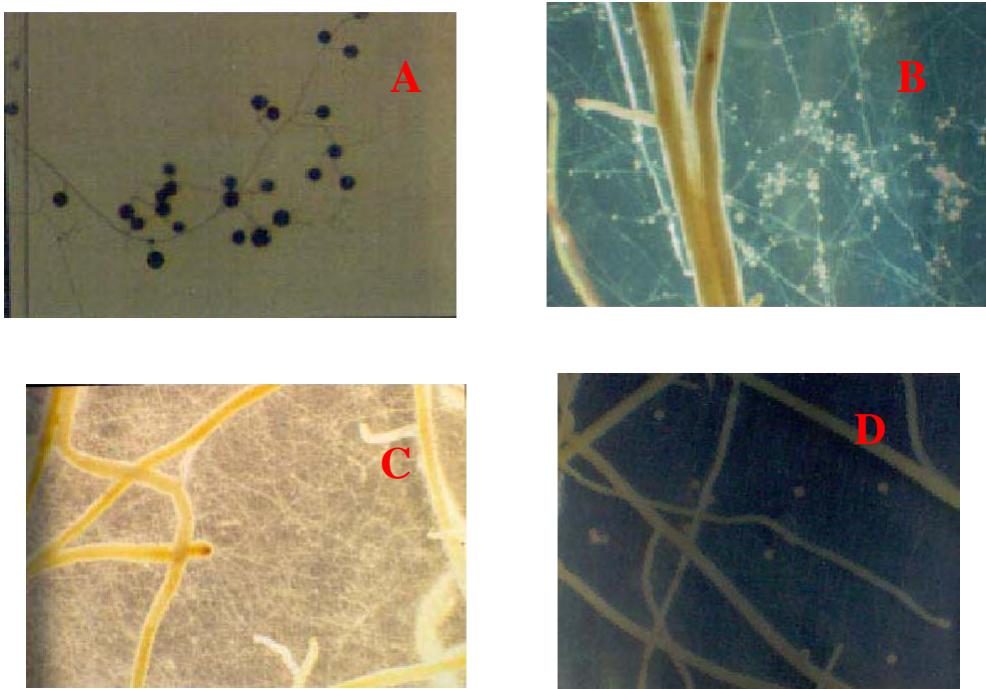
Considering the inability of some AM fungi to produce spores (Johnson, 1977; McGee, 1989), the use of intraradical forms of AM fungi seems to be a good opportunity to establish AM symbiosis. As intravesicles in mycorrhizal roots act as reserves and propagules, they have a higher inoculum potential than other AM propagules such as spores and hyphae (Magrou, 1946;



**Figure 1.** Germinating patterns of AM propagules on water agar: vesicle of *Glomus fasciculatum* (A), spore of *Gigaspora margarita* (B), Mycorrhizal root piece of *Glomus intraradices* (C).

Bierman and Linderman, 1983; Strullu and Romand, 1986; Mosse, 1988). Several authors have exploited the intraradical forms of AM fungi to achieve mass production of *Glomus* sp. (Diop, 1995; Diop et al., 1994, Strullu et al., 1996; Declerk et al., 1996a; 1998).

For all AM propagules, proper selection and efficiency of sterilization process are keys of the success of axenic or monoxenic AM fungal cultures. Isolated spores are often surface sterilized using the two -steps procedure of Metz et al. (1979) as modified by Bécard and Fortin (1988). AM sheared inocula are surface sterilized according to Diop et al. (1994) method. Then, vesicles are easily isolated by lacerating heavily colonized roots. Basically, the surface sterilization involve baths in chloramines T (2%) solution with traces of a surfactant (Tween 20/80) and antibiotics such as streptomycin or gentamycin. Sterilized AM propagules must be stored at 4°C until use.



**Figure 2.** Different patterns of development of AM fungi produced in dual culture with isolated roots: *Glomus intraradices* (A), *Glomus versiforme* (B), *Glomus aggregatum* (C) and *Gigaspora margarita* (D).

## INDEPENDENT GROWTH

Pure culture of AM fungi is still a big challenge for microbiologists. The pre-symbiotic growth of AM is characterized by formation of a so-called running hyphae. After some weeks without additional host partner, growth of germinated AM propagules stops the hyphae septate and the cytoplasm is retracted. Several factors such as nutrition (Hepper, 1983), chemical treatments (Gianinazzi-Pearson et al., 1989; Bécard and Piché, 1989a), and genetical factors (Burggraaf and Beringer, 1989; Bécard and Pfeffer, 1993; Bianciotto and Bonfante, 1993) have been studied to explain the lack of pure growth of the extraradical phase of AM fungi. Low amounts of natural or synthesized flavonoids positively stimulated fungal growth (Morandi, 1989; Chabot et al., 1992b). Otherwise, certain amino acids (Hepper and Jacobsen, 1983), volatile compounds, particularly carbon dioxide and root exudations, promote hyphal elongation (Carr et al., 1985; Elias and Safir, 1987; Bécard and Piché, 1989b).

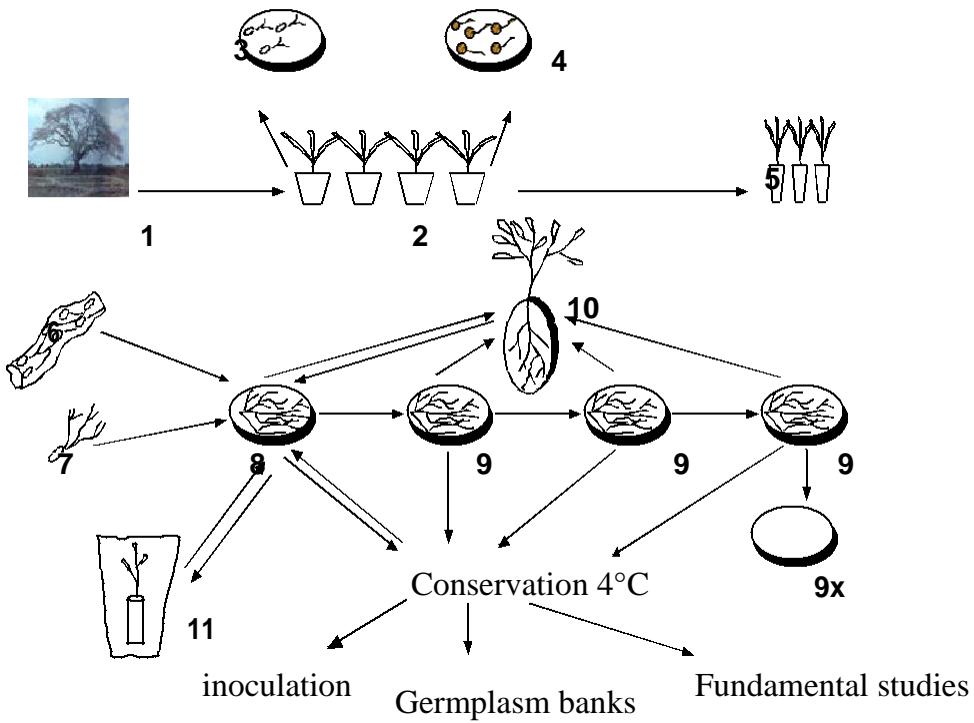
Hildebrandt et al. (2002) mentioned the presence of slime-forming bacteria identified *Paenibacillus validus* on surface sterilized spores of *Glomus intraradices*. These bacteria stimulate the growth of *G. intraradices* up to spore formation in the absence of any plant tissue. Similar results on presymbiotic sporulation have been found with other *Glomus* sp (Diop, unpublished data). The authors explain this unusual sporulation by possible

utilization by the fungi of a chemical component (or components) secreted by the bacteria. Recently Buee et al. (2000) isolated a semi-purified fraction called “Branched factor” from 8 mycotrophic plant species that are very active in stimulation of germination and nuclear division of *Gigaspora* sp.

Sheared-root of *G. versiforme* and *G. intraradices* develop on water agar medium extensive extraradical structures up to sporulation without supplemented living host partner during three months (Diop et al., 1994b). The richness of the intraradical forms of AM in lipid contents may support this growth irrespective of the decrease metabolic activity of the root explants by both surface sterilization and the shearing process. The results confirm that the quality rather than the quantity of root exudates is more involved in the stimulation of hyphal elongation (Carr et al., 1985; Elias and Safir, 1987).

## MONOXENIC CULTURES

The entire vegetative development of AM fungi in monoxenic cultures is followed by several works using either transformed or non transformed roots (Bécard and Piché, 1992; Fortin et al. 2002). Figure 2 shows morphological features of different AM fungi cultivated in vitro in association with isolated roots. A non-exhaustive list of AM fungi cultivated monoxenically has been given



**Figure 3.** Protocol of extraction, purification, cultivation and preservation of AM fungi.

(Fortin et al., 2002). The modified White medium (Minimal medium M) (Bécard and Fortin, 1988) and the modified Strullu and Romand medium (MSR medium) (Diop, 1995) are the most suitable medium to standardize the *in vitro* culture of AM fungi. The long term behaviour of *G. margarita* on Ri-TDNA transformed roots of carrot showed 80% of the fungal infections units were produced during the period of root aging (Bécard and Fortin, 1988; Diop et al., 1992).

Developing countries will benefit more AM symbiosis using the monoxenic system, which is cheaper than greenhouse culture (Diop et al., 1994a). We propose a methodology for recovering, for purifying, for cultivating tropical AM fungi (Figure 3). AM fungi can be isolated from surface soil layers to soil depths greater than 35 m (1, see Figure 3) (Diop, 1995). They are multiplied in association with a mycotrophic plant under greenhouse conditions (2). Isolated spores or vesicles can be monoxenically cultivated in presence of isolated roots (3,4) or on entire plant host (5). Intraradical forms of AM fungi (6, 7) are used as starter inocula to have monoxenic cultures (8) on M medium (Bécard and Fortin, 1988) or on MSR medium (Diop, 1995). Regular subcultures every 4 months with total transmission of AM symbiosis (9) allowed to obtain different fungal generations in continuous cultures. Probable loss of infectivity is avoided by introducing plant in the *in vitro* system (10). The use of Sunbags (11) in *in vitro* system is an other alternative to obtain large scale AM inoculum without contaminations. The axenic AM propagules are conserved at 4°C in the

dark for several months or use for fundamental or inoculation practices.

The possibility of continuous culture and cryopreservation have resulted in an international collection of *in vitro* AM fungi (websites: <http://www.mbla.uclac.be/ginco-bel> and <http://res2.agr.ca/ecorc/ginco.can/>).

## FUNDAMENTAL AND PRACTICAL STUDIES

Although *in vitro* culture is an artificial system, it may be a valuable tool to study fundamental and practical aspect of AM symbiosis, complementing the experimental approaches. Development of extraradical mycelium under aseptic conditions is often accompanied by the production of so-called arbuscule-like structures (ALS) (Bago et al., 1998a) or branched absorbing structures (BAS) (Bago et al., 1998b). The authors suggest the possible implication of the ALS/BAS in the exchange sites between AM fungi and host plants.

Diop (1995) established a bank of germplasms of AM monoxenically cultivated in association with isolated tomato or transformed carrot roots. The produced propagules (spores, hyphae, infected roots) were able to germinate and to reinfect new plants efficiently. Encapsulation stabilize the biological properties of mycorrhizal roots and isolated vesicles or spores (Strullu et al., 1991; Declerk et al., 1996b). This immobilization also preserve the infectivity of AM propagules under *in vitro* or *in vivo* assays.

AM fungi can contribute to root disease suppression though mechanisms are not well understood (Linderman, 1994). The most obvious effect of AM fungi has been attributed to amelioration of nutrient uptake (P and others), resulting in more vigorously growing plants better able to ward off or tolerate root disease. St-Arnaud et al. (1995a) proposed a compartmentalized *in vitro* system to elucidate interactions between *G. intraradices* and the root pathogen *Fusarium oxysporum* f.sp *chrysanthemi*. Significant negative correlations were found between conidia production and *G. intraradices* hyphae or spore concentrations. McAllister et al. (1994) found no *in vitro* interactions between spores of *G. mosseae* and *Trichoderma koningii* or *Fusarium solani*. Life cycles of *G. intraradices* and the burrowing nematode, *Radopholus similes*, were achieved in monoxenic cultures (Elsen et al., 2001). The AM fungus increased protection of the root by reducing the nematode population by 50%.

## FUTURE PROSPECTS

The comparisons between *in vivo* and *in vitro* systems for production of spores would be very much in favour for the axenic systems (Fortin, personal communication). However the effectiveness of these propagules under adverse conditions remains unclear. Some AM fungi lose their infectivity after several successive *in vitro* subcultures (Plenchette et al., 1996). Therefore it will be necessary to evaluate inoculum potential of different generations of AM propagules in continuous monoxenic cultures. In order to preserve permanent fungal biodiversity, *in vitro* and *in vivo* collections must always be maintained.

The encapsulation of AM fungi produced monoxenically in alginate beads offers the possibility to diversify inoculation process. It will be useful to incorporate in beads containing AM fungi, some stimulatory compounds such as flavonoids (Bécard and Piché, 1989; Gianinazzi-Pearson et al., 1989) or synergic microorganisms (Hildebrandt et al., 2002).

The development of arbuscule-like structures only in a few dual cultures (Karandashov et al., 2000) poses the questions whether they have taxonomic significance or they are produced in soils. The compartmentalized *in vitro* system (St-Arnaud et al., 1995b) may help to better understand the mechanisms involved in interactions between AM fungi and pathogenic/nonpathogenic rhizosphere microorganisms and also the metabolism of AM fungi.

## ACKNOWLEDGEMENTS

The author wishes to thank the International Foundation for Science (Contract C/2896), the FNRAA project (AP28/SS310800), and the Inco-Enrich project (contract ICA4-CT-2000-30022) for financial support.

## REFERENCES

- Bago B, Azcon-Aguilar C, Piché Y (1998a). Architecture et développement dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic culture conditions. *Mycology* 90:52-62.
- Bago B, Azcon-Aguilar C, Goulet A, Piché Y (1998b). Branched absorbing structures (BAS) : a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. *New Phytol.* 139:375-388.
- Bécard G, Fortin JA (1988). Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytol.* 108:211-218.
- Bécard G, Piché Y (1989a). Fungal growth stimulation by CO<sub>2</sub> and roots exudates in vesicular-arbuscular mycorrhizal symbiosis. *Appl. Environ. Microbiol.* 55:2320-2325.
- Bécard G, Piché Y (1989b). New aspects of the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus *Gigaspora margarita*. *New Phytol.* 112:77-83.
- Bécard G, Piché Y (1992). Establishment of vesicular-arbuscular mycorrhiza in root organ culture : review and proposed methodology. In *Techniques for the study of mycorrhiza*. Edited by Norris J, Read D, Varma A. Academic Press, New York. pp. 89-108.
- Bécard G, Pfeffer PE (1993). Status of nuclear division in arbuscular mycorrhizal fungi during *in vitro* development. *Protoplasma* 174:62-68.
- Bianciotto V, Bonfante P (1993). Evidence of DNA replication in an arbuscular mycorrhizal fungi during *in vitro* development. *Protoplasma* 176:100-105.
- Biernan B, Linderman RG (1983). Use of vesicular-arbuscular mycorrhizal roots, intraradicle vesicles and extraradicle vesicles as inoculum. *New Phytol.* 95:97-105.
- Buee M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000). The pré-symbiotic growth of arbuscular mycorrhizal fungi is reduced by a branching factor partially purified from plant root exudates. *Am. Phytopathol. Soc.* 6:693-698.
- Burggraaf AJP, Beringer JE (1989). Absence of nuclear DNA synthesis in vesicular-arbuscular mycorrhizal fungi during *in vitro* development. *New Phytol.* 11:25-33.
- Caar GR, Hinkley F, Le Tacon F, Hepper C M, Jones M GK, Thomas E (1985). Improved hyphal growth of two species of vesicular-arbuscular mycorrhizal fungi in the presence of suspension-cultured plants cells. *New Phytol.* 101:417-426.
- Chabot S, Bécard G, Piché Y (1992a). Life cycle of *Glomus intraradices* in root organ culture. *Mycologia* 84:315-321.
- Chabot S, Bel-Rhlid R, Chênevert R, Piché Y (1992b). Hyphal growth promotion *in vitro* of the VA mycorrhizal fungus, *Gigaspora margarita* Becker and Hall, by the activity of structurally specific flavonoid compounds under CO<sub>2</sub> enriched conditions. *New Phytol.* 122:461-467.
- Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempé J (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. *Nature* 295:432-434.
- Declerk S, Strullu DG, Plenquette C (1996a). *In vitro* mass production of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri T-DNA transformed carrot roots. *Mycol. Res.* 100:1237-1242.
- Declerk S, Strullu DG, Plenquette C, Guillemette T (1996b). Entrapment of *in vitro* produced spores of *Glomus versiforme* in alginate beads : *in vitro* and *in vivo* inoculum potentials. *J. Biotechnol.* 48:51-57.
- Declerk S, Strullu DG, Plenquette C (1998). Monoxenic culture or the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem : a proposed methodology for germplasm collection. *Mycologia* 90:579-585.
- Diop TA (1990). Méthodes axéniques de production d'inocula endomycorhiziens à vésicules et à arbuscules : étude avec le *Gigaspora margarita*. MSc Thesis, University of Laval, Quebec.
- Diop TA (1995). Ecologie des champignons mycorhiziens à vésicules et à arbuscules associés à *Acacia albida* (del) dans les zones sahéliennes et soudano-guinéennes du Sénégal. Thèse de docteur en Biologie et Physiologie végétales, Angers, France.
- Diop TA, Bécard G. and Piché Y. 1992. Long-term *in vitro* culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed root of carrot. *Symbiosis* 12:249-259.

- Diop TA, Plenchette C, Strullu DG (1994a). Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. *Mycorrhiza*, 5:17-22.
- Diop TA, Plenchette C, Strullu DG (1994b). *In vitro* culture of sheared mycorrhizal roots. *Symbiosis* 17:217-227.
- Elias KS, Safrir GR (1987). Hyphal elongation of *Glomus fasciculatus* in response to root exudates. *Appl. Environ. Microbiol.* 53:1928-1933.
- Elsen A, Declerck S, De Waele D (2001). Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dixenic culture. *Mycorrhiza* 11:49-51.
- Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughan AP, Piché Y (2002). Arbuscular mycoorhiza on root-organ cultures. *Can. J. Bot.* 80:1-20.
- Gianinazzi-Pearson V, Branzanti B, Gianinazzi S. (1989). *In vitro* enhancement of spore germination and early hyphal growth of vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. *Symbiosis* 7:243-255.
- Hepper CM (1983). Limited independent growth of a vesicular arbuscular mycorrhizal fungus *in vitro*. *New Phytol.* 93:537-542.
- Hepper C, M, Jacobsen I (1983). Hyphal growth from spore of the mycorrhizal fungus *Glomus caledonius* : effects of amino-acids. *Soil Biol. Biochem.* 15:55-58.
- Hildebrandt U, Janetta K, Bothe H (2002). Towards growth of arbuscular mycorrhizal fungi independent of a plant host. *Applied and environmental microbiology* Vol 68, N° 4 pp. 1919-1924.
- Johnson P N (1977). Mycorrhizal Endogonaceae in a New Zealand forest. *New Phytol.* 78:161-170.
- Karandashov V, Inna K, Heidi-Jayne H, Eckhard G (2000). Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots. *Mycorrhiza* 10:23-28.
- Linderman RG, (1994). Role of VAM fungi in biocontrol. In : Pfleger FL, Linderman RG (eds) *Mycorrhiza and plant health*. APS Press, St Paul, Minn, pp 1-25.
- Magrou J (1946). Sur la culture de quelques champignons de mycorhizes à arbuscules et à vésicules. *Rev. Gen. Bot.* 53:49-77.
- McAllister CB, Garcia Romera I, Godeas A, Ocampo JA (1994). *In vitro* interactions between *Trichoderma koningii*, *Fusarium solani* and *Glomus mosseae*. *Soil Biol. Biochem.* 26:1369-1374.
- McGree PA (1989). Variation in propagules numbers of vesicular-arbuscular mycorrhizal fungi in a semi-arid soil. *Mycol. Res.* 92:28-33.
- Mertz SM, Heithaus JJ III and Bush RL (1979). Mass production of axenic spores of the endomycorrhizal fungus *Gigaspora margarita*. *Trans. Br. Mycol. Soc.* 72:167-169.
- Morandi D (1989). Effects of xenobiotics on endomycorrhizal infection and isoflavonoid accumulation in soybean roots. *Plant Physiol. Biochem.* 27:697-701.
- Mosse B (1962). The establishment of vesicular arbuscular mycorrhiza under aseptic conditions. *J. Gen. Microbiol.* 27:509-520.
- Mosse B (1988). Some studies relating to "independent" growth of vesicular-arbuscular endophytes. *Can. J. Bot.* 66:2533-2540.
- Mosse B and Hepper CM (1975). Vesicular-arbuscular infections in root-organs cultures. *Physiol. Plant Pathol.* 5:215-223.
- Mugnier J, Mosse B (1987). Vesicular- arbuscular infections in *Ri T-DNA* transformed roots grown axenically. *Phytopathology* 77:1045-1050.
- Plenchette C, Declerck S, Diop TA and Strullu DG (1996). Infectivity of monoxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with *Ri T-DNA* transformed carrot root. *Appl. Microbiol. Biotechnol.* 46:545-548.
- St-Arnaud M, Hamel C, Vimard B, Caron M., Fortin JA (1995b). Altered growth of *Fusarium oxysporum* f.sp. *chrysanthemi* in an *in vitro* dual culture system with the vesicular arbuscular mycorrhizal fungus *Glomus intraradices* growing on *Daucus carota* transformed roots. *Mycorrhiza* 5 :431-438.
- St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995a). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an *in vitro* system in absence of host roots. *Mycol.. Res.* 100:328 –332.
- Strullu DG, Romand C (1986). Méthode d'obtention d'endomycorhizes à vésicules et à arbuscules en conditions axéniques C.R. Acad. Sci. Ser. III Sci. Vie 303:245-250.
- Strullu DG, Romand C (1987). Culture axénique de vésicules isolées à partir d'endomycorhizes et ré -association *in vitro* à des racines de tomate. C. R. Acad. Sci. Paris Sér. III, 305:15-19.
- Strullu DG, Romand C, Plenchette C (1991). Axenic culture and encapsulation of the intraradical forms of *Glomus* spp. *World J. Microbiol. Biotechnol.* 7:292-297.
- Strullu DG, Diop TA, Plenchette C (1997). Réalisation de collections *in vitro* de *Glomus intraradices* (Schenk et Smith) et de *Glomus versiforme* (Karsten et Berch) et proposition d'un cycle de développement. C. R. Acad. Sci. Paris, 320:41-47.
- Tepfer D (1984). Transformation of several species of higher plants by *Agrobacterium rhizogenes* : sexual transmission of the transformed genotype and phenotype. *Cell* 37:959-967.