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Minireview

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

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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 lost 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 p1ants 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 thouroughly 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 carrota 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.

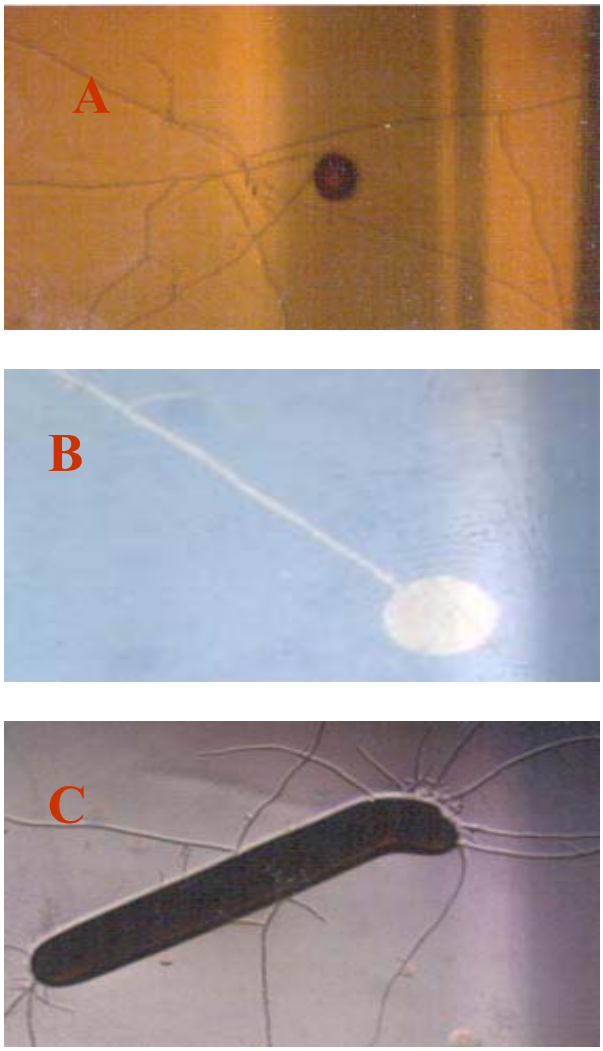

Figure 1. Germinating patterns of AM propagules on water agar: vesicule *of Glomus fasciculatum* (A), spore of *Gigaspora magarita* (B), Mycorrhizal root piece of *Glomus intraradices* (C).

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; 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 sterilize 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 lacering 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.

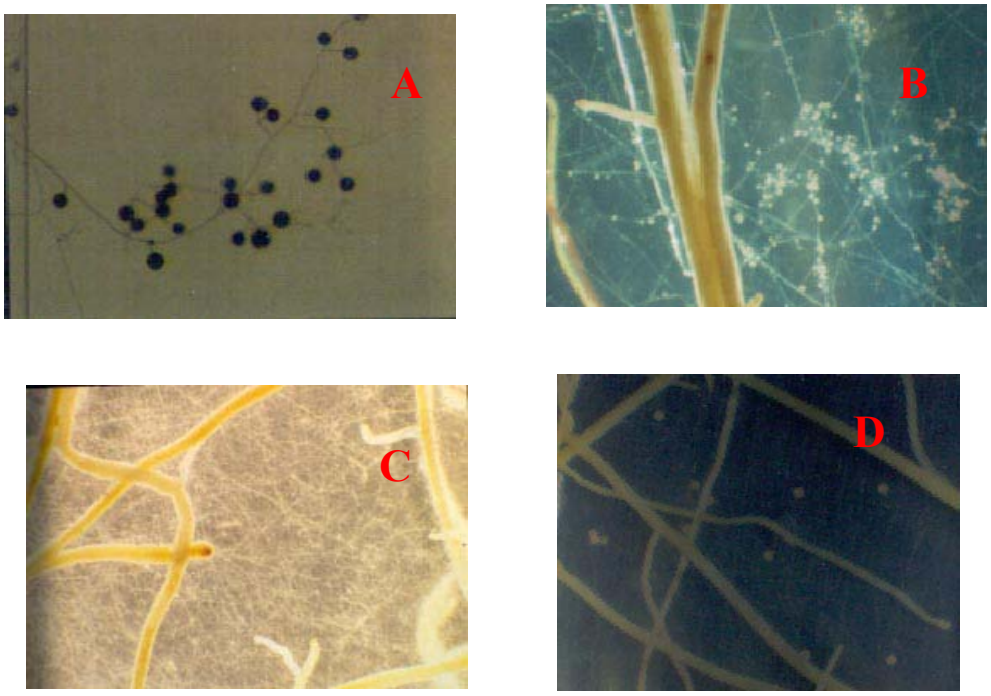

Figure 2. Different patterns of development of A AM fungi produced in dual culture with isolated roots: *Glomus intraradices* (A), *Glomus versiforme* (B), *Glomus aggregatum* (C) and *Gigaspora margarita* (D).

INDEPENDENT GROWTH

Pure culture of AM fungi is still a big challenge for microbiologists. The pre-symbiotique 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).

Hildebrant 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

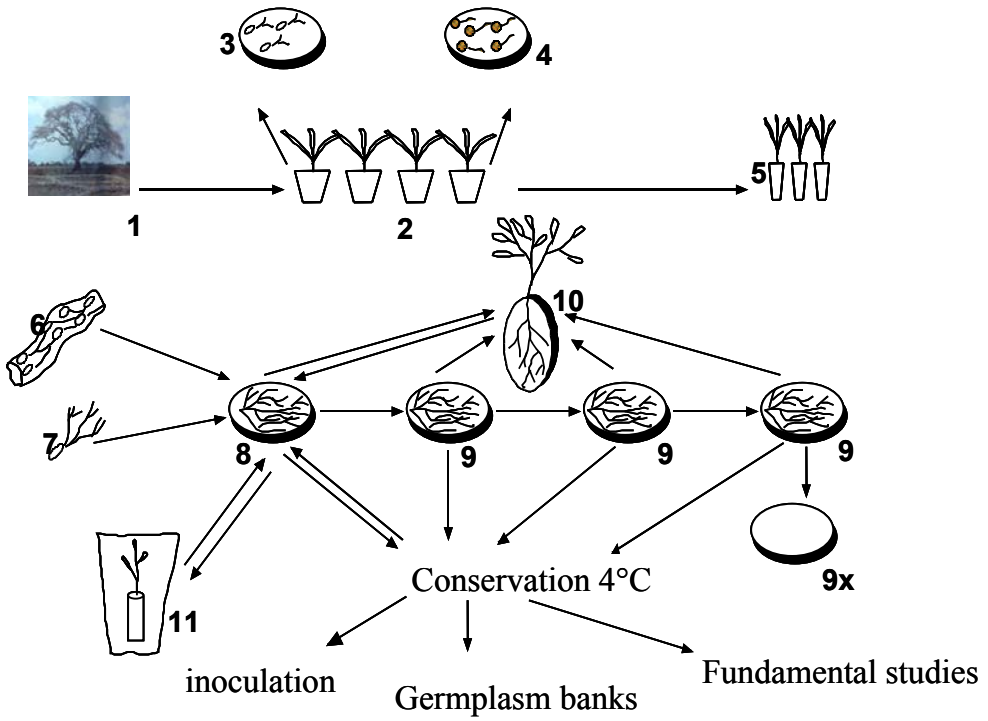

Figure 3. Protocol of extraction, purification, cultivation and preservation of AM fungi.

(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 an 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 inculation 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 *chrysanthem*i. 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 continous 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 (Hildebrant 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.

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