Mini Review

In vitro Cultivation of Arbuscular Mycorrhizal (AM) Fungi

K M Rodrigues and B F Rodrigues

Department of Botany, Goa University, Goa 403 206 India. E-mail: felinov@gmail.com

Abstract

Arbuscular mycorrhizal (AM) fungal association is essential for most of the vascular plants for their growth and survival. The obligate biotrophic nature of AM fungi makes this association intricate. Several attempts have been made for cultivation and mass inoculum production of this plant beneficial symbiosis. The use of root organ culture (ROC) technique has proved to be particularly successful. This technique has greatly influenced our understanding on various aspects of AM symbiosis. This article provides an update on the developments made in the *in vitro* AM fungal inoculum production and the range of AM fungal species being cultivated using ROC. The method by which ROC of AM fungi have been cultivated is described along with the culture media used, choice of transformed host root to study the interaction, selection and sterilization of different AM fungal propagules and the use of continuous cultures to preserve the colonizing potential of the *in vitro* produced AM fungal inoculum. The morphological features of AM fungal cultures developed under *in vitro* conditions and how these have improved our understanding on this symbiosis are also discussed. Some of the potential uses and limitations of this system are also highlighted.

Key words: arbuscular mycorrhizal fungi, root organ cultures, AM fungal propagules, extra-radical mycelium

Citation: Rodrigues KM and Rodrigues BF. 2013. *In vitro* cultivation of arbuscular mycorrhizal (AM) fungi. *J Mycol Pl Pathol* 43(2): 155-168.

Introduction

Arbuscular mycorrhizal (AM) fungi are soil organisms with a worldwide distribution that form root symbiosis with most plant families. Their functional importance in natural and semi-natural ecosystems is commonly accepted with regard to enhanced plant productivity and diversity as well as increased plant resistance to biotic and abiotic stresses (Smith and Read 2008). Nowadays, these fungi are receiving attention because of their increasing range of application in agriculture, horticulture and forestry, as well as in environmental reclamation, increasing crop yield and health, and limiting the use of agrochemicals (Johansson et al 2004).

Arbuscular mycorrhizal fungal propagules exist as spores, living hyphae, isolated vesicles, mycorrhizal root segments or colonized soil (Diop et al 1994a). Root segments and spores isolated from open-pot culture (Gilmore 1968) of AM-inoculated plants have been the usual source of AM inoculum for research purposes (Ferguson and Woodhead 1982). However, this type of inoculum occupies a large space in production and is prone to contamination even with good phytosanitary care (Ames and Linderman 1978). Production of propagules under aseptic conditions remains one of the most promising methods of obtaining high quality pathogen-free inoculum that is required for research purposes.

The conventional method used to study the life cycle of AM fungi *in situ* is to associate them with root organ culture (ROC) (Fortin et al 2002). The establishment of *in vitro* ROC has greatly increased our understanding of various aspects of the AM symbiosis by allowing non destructive *in vivo* observations throughout the fungal life cycle and its potential for research and inoculum production is gaining importance. The cultivation of AM fungi in association with the Ri T-DNA transformed roots has enabled new possibilities in the study of the extra-radical mycelium of AM (Fortin et al 2002). In this mini review, an effort has been made to highlight various *in vitro* cultivation systems of AM fungi along with different hosts, culture media and types of AM fungal propagules used to initiate monoxenic cultures. The use of mycorrhizal ROC has allowed the elucidation of many aspects of this intimate symbiotic plant-fungal association. Although the host plant is replaced by Ri T-DNA transformed roots, the fungus is able to colonize and sporulate. The development of spores, morphologically and structurally similar to those produced in pot cultures, and the ability of the *in vitro* produced propagules to retain their viability to colonize and initiate new mycorrhizal symbiosis indicates that the fungus is able to complete its life cycle. Thus the success achieved by *in vitro* culture of AM species using Ri T-DNA transformed roots indicates that this technique can be exploited for large scale inoculum production.

System Description

Since Mosse and Hepper (1975) first established cultures of AM fungi using excised roots, tremendous improvements have been made in the use of Ri T-DNA transformed roots (Mugnier and Mosse 1987), in the manipulation of the culture media to induce sporulation

(Bécard and Piché 1992), and in the development of a bi-compartment system that allowed the production of root-free AM fungal mycelium and spores (St-Arnaud et al 1996). This improvement has enabled studies in sporulation dynamics (Declerck et al 2001), spore ontogeny (Pawlowska et al 1999), stimulation of germination and hyphal growth by CO_2 (Bécard and Piché 1989a), regulation of hyphal growth and branching by root exudates (Nagahashi et al 1996), reactions to compounds from the host and non-host roots (Schreiner and Koide 1993), uptake, transfer and metabolic fate of ^{13}C-labeled metabolites (Pfeffer and Shachar-Hill 1996), response of AM fungi to cell wall-associated phenolics (Douds et al 1996) and flavonoids (Morandi et al 1992), lipid metabolism (Bago et al 2002), transport of mineral nutrients to roots (Dupré de Boulois et al 2005) and isolation of microbe free AM fungal mycelium and spores for molecular analysis (Pawlowska and Taylor 2004).

Using the split-plate method, Douds (2002) demonstrated that AM fungi continue to sporulate after medium from the distal compartment has been partially replaced, and glucose provided to the proximal compartment, resulting in repeated harvests from the same Petri plate culture. Different production systems have been derived from the basic ROC in Petri plates. Tiwari and Adholeya (2003) and Adholeya et al (2005) cultured root organs and AM fungi in small containers, by which large-scale production was obtained. Large-scale cultivation of AM fungi has also been performed in an airlift bioreactor (Jolicoeur et al 1999), in a mist bioreactor with perlite as the substrate (Jolicoeur 1998), and in a bioreactor containing solid medium (Fortin et al 1996). In the patented container-based hydroponic culture system of Wang (2003), the root organs and AM fungus were periodically exposed to a liquid culture medium. Gadkar et al (2006) further developed a container in which a Petri plate containing ROC was used to initiate fungal proliferation in a separate compartment filled with sterile expanded clay balls. In parallel to the systems based on excised roots, Voets et al (2005) and Dupré de Boulois et al (2006) developed two *in vitro* culture systems based on autotrophic plants. In the system developed by Voets et al (2005), the shoot developed outside the Petri plate while the roots and AM fungus were associated inside the Petri plates that were filled with a suitable gelled medium, resulting in more than 12,000 spores per Petri plate after 22 weeks of culturing. In another system (Dupré de Boulois et al 2006), the shoot developed in a sterile tube vertically connected to the top of a Petri plate in which the AM fungus and roots developed. The cultures were then placed in growth chambers to provide controlled environmental conditions adequate for plant growth and ~1,600 spores were obtained in a period of 12 weeks in the root compartment of a bi-compartmental Petri plate. A derived plant *in vitro* production system has recently been detailed in a patent proposal (Declerck et al 2009) where each pre-inoculated *in vitro* produced plant (Voets et al 2009) is individually introduced into a sterile growth tube. A nutrient solution circulates in this closed system flowing onto the mycorrhizal roots. These studies have thus greatly improved our earlier understanding of AM fungi propagation processes and life cycles (Strullu et al 1997). Other potential uses for this system are the production of pure, concentrated inoculum and sterile fungal tissue for genetic and physiological studies.

Advances in the development of *in vitro* systems have opened new prospects in the study of the AM symbiosis. Research areas such as fungal colony architecture, physiology, biochemistry, cytology and molecular biology, traditionally affected by the intrinsic problems presented by culturing AM in soil, have especially benefited from this *in vitro* revolution (Declerck et al 2005).

AM Fungal Species Cultivated on ROC

Up till now, several Glomaceae and a few Gigasporaceae genera have been successfully cultivated *in vitro* on ROC (Table 1) and are maintained in international culture collections (Declerck and Dalpé 2001).

Acaulospora rehmii is the first Acaulosporaceae representative to have been successfully cultivated *in vitro.* Given that the long-term goal of all such research is the establishment of AM fungi in axenic culture, it is essential to establish many individual species in dual culture to enable investigation on particular aspects of the symbiosis.

Culture Media

Two media frequently used to culture AM fungi on ROC are the minimal (M) medium (Bécard and Fortin 1988) and the modified Strullu Romand (MSR) medium (Strullu and Romand 1986, modified by Declerck et al 1998). Both these media contain micro- and macro-nutrients as well as vitamins and sucrose (Cranenbrouck et al 2005). Both media are solidified with a gelling agent such as PhytaGel and GelGro. The successful development of fungal isolates into sustainable culture has been achieved using minimal M medium (Bécard and Fortin 1988). While this medium has been widely used for the study of AM fungi *in vitro*, it appears unsuitable for the culture of other AM fungal species (Douds 1997). Manipulation of medium composition and pH to suit new fungal isolates could lead to a better understanding of factors affecting the complex biology underlying the symbiosis. MSR medium lacking sucrose promoted higher *in vitro* germination rates in *R. irregularis* (D'Souza et al 2013). ROC systems in

Table 1. Arbuscular mycorrhizal (AM) species cultivated on Root Organ Culture

AM species	Reference
Acaulospora rehmii Sieverd and Toro	Dalpé and Declerck 2002
Gigaspora rosea Nicolson & Schenck	Bago et al 1998b
Gi. margarita Becker & Hall	Miller-Wideman and Watrud 1984; Diop et al 1992; Gadkar and Adholeya 2000
Gi. gigantea (Nicolson & Gerd.) Gerd. & Trappe	Gadkar et al 1997
Gi. decipiens Hall & Abbott	Fernández Bidondo et al 2012
Glomus etunicatum Becker & Gerd.	Schreiner and Koide 1993
G. versiforme (Karst.) Berch	Diop et al 1994a; Declerck et al 1996a
G. deserticola Trappe, Bloss & Menge	Mathur and Vyas 1995
G. fistulosum Skou & Jakobsen	Nuutila et al 1995; Gryndler et al 1998
G. clarum Nicolson & Smith	De-Souza and Berbara 1999; Rodrigues and Rodrigues 2012
Funneliformis caledonius (Nicolson & Gerd.) Walker & Schuessler	Hepper 1981; Karandashov et al 2000
F. geosporus (Nicolson & Gerd.) Walker & Schüβler	Declerck et al 1998
F. mosseae (Nicolson & Gerd.) Walker & Schuessler	Douds 1997
Rhizophagus irregularis (Blaszk., Wubet, Renker & Buscot) Walker & Schuessler	Chabot et al 1992a; St-Arnaud et al 1996
R. fasciculatus (Thaxter) Walker & Schüβler	Declerck et al 1998
*R. proliferus (*Dalpé & Declerck) Walker & Schuessler	Declerck et al 2000
Sclerocystis sinuosa Gerd. & Bakshi	Bi et al 2004

bioreactors (Jolicoeur et al 1999) and containers (Gadkar et al 2006) were performed with liquid M medium. In the compartmented culture system (Gadkar et al 2006), glucose-soaked cotton rolls were supplied to the ROC and AM fungus, while the compartment containing expanded clay was filled with a layer of liquid M-medium without sugars and vitamins. Similar to the *in vivo* hydroponic culture systems, sufficient aeration of the liquid medium is needed in the *in vitro* solution culture techniques (Jolicoeur et al 1999). Whole-plant *in vitro* culture systems were conducted on the MSR medium lacking sucrose and vitamins (Dupré de Boulois et al 2006) that were similarly solidified with either Phytagel or GelGro. The addition of vitamins and sucrose is not required in whole-plant *in vitro* culture systems as the autotrophic plant provides sugars obtained by photosynthesis and metabolizes the vitamins required for plant growth.

AM Host Root

Ri T-DNA transformed roots have been used effectively in studying the interaction between various plant hosts and AM fungi. ROC was first developed by White and coworkers (White 1943; Butcher 1980) who used excised roots on synthetic mineral media supplemented with vitamins and a carbohydrate source. However, profuse root growth, characterized by the formation of numerous lower order branches, has been obtained in relatively few plant species. The formation of lower order roots is essential for increase in root biomass and the establishment of continuous cultures. ROC was first performed successfully by Mosse and Hepper (1975) using an *in vitro* system based on a dual culture of spores and excised roots of *Trifolium* (clover) species. Mugnier and Mosse (1987) obtained similar results using *Daucus carota* L. (carrot) roots genetically transformed by *Agrobacterium rhizogenes* Conn. Later, Strullu and Romand (1986, 1987) showed that it was also possible to re-establish mycorrhiza on excised roots of *Fragaria* × *Ananassa* Duchesne (strawberry), *Allium cepa* L. (onion), and *Solanum lycopersicum* L. (tomato), using the intra-radical phase (vesicles or entire mycorrhizal root pieces) of several *Glomus* species as inoculum. The *in vitro* large scale production of *R. irregularis* spores was initially attempted on ROC (Declerck et al 2001) and later extended to plant systems (Voets et al 2009). This system of dual culture allowed abundant production of spores of *G. margarita* (Diop et al 1992). A natural genetic transformation of plants by the ubiquitous soil bacterium *A. rhizogenes* (Riker et al 1930) induces a condition known as 'hairy roots'. This stable transformation (Tepfer 1989) produces Ri T-DNA transformed plant tissues that are morphogenetically programmed to develop as roots. A modified hormonal balance encourages vigour and allows profuse growth on

artificial media (Tepfer 1989). Carrot and *Convolvulus sepium* L. (bindweed) were among the earliest species to be transformed using *A. rhizogenes* (Tepfer and Tempé 1981). These Ri T-DNA transformed roots have since served in a wide range of fundamental and applied studies on AM symbiosis. ROC is commonly initiated on carrot root tissue and its use has permitted an increase in spore production of *F. mosseae* (Mugnier and Mosse 1987). In recent years however, different excised roots, notably of *Cichorium intybus* L. (chicory) (Fig. 1a) and *Medicago truncatula* Gaertn. (barrel medic), have been successfully used to culture AM fungi (Boisson-Dernier et al 2001; Fontaine et al 2004). It has been revealed that a change of root clone impacts AM fungal spore production (Tiwari and Adholeya 2003). Voets et al (2005) used *Solanum tuberosum* L. (potato) and obtained production of ~12,000 spores in 12 weeks of cultivation. Fernández et al (2009) carried out *in vitro* monoxenic symbiosis between *R. irregularis* and transformed *Glycine max* (L.) Merr. (soybean) roots (TSRs) and showed that TSR cultures were able to support the growth and characteristic development of the fungus. Pratap Chandran et al (2011) co-cultivated transformed roots *Canavalia* species with *G. microcarpum* Tul. & Tul. in Petri plate and observed 60% AM colonization on the 20th day. Other hosts, such as tissue cultured banana (*Musa acuminata* Colla) were found suitable for association (Koffi et al 2009) but were less effective for large scale production of spores.

AM Fungal Inocula

Many species and strains of AM fungi have been cultured in the ROC system. However, only a few species are fast growers and colonizers, able to produce thousands of *in vitro* propagules in a few months and thus have potential in large scale production. In most cases, two types of fungal inocula viz. extra-radical spores or propagules from the intra-radical phase (mycorrhizal root fragments and isolated vesicles) of the fungal ontogeny can be used to initiate monoxenic cultures (Fig. 1b, c, & d). Cultures of AM fungal species that do not produce vesicles (*Scutellospora* and *Gigaspora* species) are systematically produced using spores, which are usually large and germinate vigorously. Recently, sporocarps of *F. mosseae* have also been used in an attempt to establish *in vitro* cultures (Budi et al 1999). The intra-radical forms of AM fungi have been less commonly used as starter material despite being a potentially good source of inoculum. Strullu and Romand (1987) demonstrated that intra-radical vesicles and hyphae within root pieces or extracted from roots by enzymatic maceration were able to regenerate vegetative mycelium in *Glomus* species. When associated with tomato roots, the mycelium formed new and typical mycorrhizae. This system was successfully used by Diop et al (1994a) for dual axenic culture of mycorrhizal root-segments containing *G. versiforme* or *R. irregularis* associated with non-transformed tomato roots. The cultivation system was further improved by using transformed carrot roots as host with several *Glomus* species (Diop 1995). Diop et al (1994a) obtained approximately 2000 spores per Petri dish over a period of three months using a dual culture of leek (*Allium* sp.) root-segments colonized by *G. versiforme* associated with tomato roots. They further demonstrated that the fungus both in tomato root segments and as spores, produced in sterile conditions, germinated well and was able to complete its life cycle in association with isolated

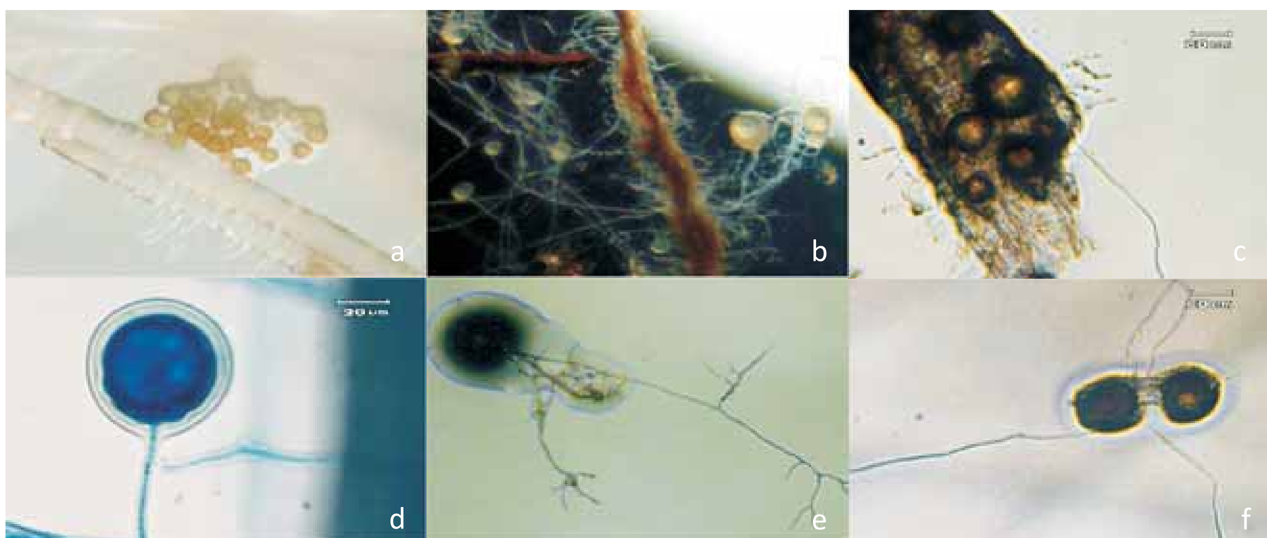

Figure 1. a. Inoculation of Ri T-DNA transformed roots of *Cichorium intybus* L. using AM spores; b. Healthy spores from pure culture (pot culture); c. *In vitro* hyphal growth in colonized root fragment; d. Intra-radical vesicle as *in vitro* propagule; e. Spore germination in *Glomus* sp.; f. Spore germination in *Acaulospora* sp.

tomato roots. Inoculation of *Faidherbia albida* (Delile) A. Chev. plantlets with the newly produced spores and mycorrhizal root-segments were also successfully demonstrated (Diop et al 1994a). Species from *R. irregularis* clade/species complex are found among the most productive so far. This species *sensu lato* is the most frequently cultured AM fungus *in vitro*.

Strullu and Plenchette (1990a, b) demonstrated the ability of entrapped, disinfected mycorrhizal root fragments to form new mycorrhizae, even after storage for 1 month at 4C. Intra-radical vesicles separated from roots and encapsulated were also shown to retain their inoculum potential (Plenchette and Strullu 2003) and hence represent another practical source of inoculum. In a study by Declerck et al (1996a), *in vitro* produced spores of *G. versiforme* were entrapped in alginate beads and their inoculum potential was evaluated by a biological assay. The results showed that the encapsulated spores were able to germinate and the regenerated mycelium retained its ability to colonize roots under controlled conditions. Declerck and Angelo-van Coppenolle (2000) developed a cryopreservation technique based on the entrapment of monoxenically produced spores of *R. irregularis* in alginate beads. These studies indicate the feasibility of encapsulation of *in vitro* produced spores and therefore represent a new kind of high quality inoculum, free of pathogens.

For all AM propagules, appropriate selection and efficiency of sterilization process are keys to 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's (1994a, b) method. Vesicles are then easily isolated by enzymatic digestion of the heavily colonized roots. Surface sterilization involves baths in chloramine T (2%) solution with traces of a surfactant (Tween 20/80) and antibiotics, such as streptomycin or gentamycin. To maintain spore dormancy, all steps from spore isolation to rinsing should be done on ice. If spores are not to be used immediately, they should be stored at 4C, either in distilled water, on water agar, or on 0.1% $MgSO_4 \cdot 7H_2O$ solidified with 0.4% gellan gum. To limit the risk of contamination by bacteria or fungi that were not eliminated during the sterilization process, spore number should be limited in each Petri plate.

Generally AM fungal spores do not need specific conditions or the presence of a host root to germinate. However, root exudates and 2% CO_2 can stimulate germination and/or post germination hyphal growth (Buée 2000). Recalcitrant spores can be placed alongside a growing root. If spores fail to germinate within 20 days, either the sterilization treatment is possibly strong or the spores are immature, dormant, or dead. It is well known that spores of some AM fungal species require cold stratification (4C) prior to germination (Smith and Read 2008). This requirement can vary within a genus. *G. gigantea* (Koske 1981) and *G. margarita* require cold treatment, whereas *G. rosea* did not (Bécard and Fortin 1988). The cold treatment (14–21 days) is best applied prior to spore isolation, when the spores are still attached to the extra-radical mycelium (Fortin et al 2002).

Continuous Cultures

The first continuous culture was achieved by Strullu and Romand (1986) and is now commonly used for a wide range of *Glomus species* (Declerck et al 1998). Continuous culture is obtained by transferring mycorrhizal roots to fresh medium either with or without spores (St-Arnaud et al 1996; Declerck et al 1996a). Following this transfer, the pre-existing root-fungus association continues to proliferate. While using older mycorrhizal roots, it is preferable to transfer them to a Petri plate containing an actively growing root (Declerck et al 1998). In the method by St-Arnaud et al (1996), apical segments of actively growing mycorrhizal roots with or without extra-radical mycelium that are supporting the spores are transferred to a fresh medium. The root and associated fungus continues to grow across successive transfers onto fresh medium. This procedure requires the use of young, actively growing cultures, to allow continuous growth of the host root. The method is effective for *Glomus* species having a well-developed intra-radical phase, such as *R. irregularis*. For AM fungal species that do not produce intra-radical vesicles (*Gigaspora* and *Scutellospora* species), direct sub-culturing is possible but it is more difficult to achieve (Fortin et al 2002). Alternatively, with older cultures, *in vitro* produced spores can be used to inoculate new roots (Bécard and Fortin 1988).

Diop (1995) established a bank of germplasm of AM fungi monoxenically cultivated in association with isolated tomato or transformed carrot roots. The propagules produced (spores, hyphae, colonized roots) germinated and re-colonized new plants efficiently. Encapsulation stabilizes biological properties of mycorrhizal roots and the isolated vesicles or spores (Declerk et al 1996b). This also preserves infectivity of AM propagules under *in vitro* or *in vivo* assays.

Fungal Morphological Features in ROC System

The use of ROC of AM enables the aseptic production of spores of various AM fungal species. Germination of the AM fungal propagules usually proceeds from the

pushing of the inner spore wall through the lumen of the subtending hyphae (De-Souza and Berbara 1999), directly through the spore wall (Tommerup and Kidby 1980), or the subtending hyphal wall (Giovannetti et al 1991). Spore germination gives rise either to straight, thick-walled hyphae (De-Souza and Berbara 1999) or to stunted hyphae, depending on the physiological status of spore (Juge et al 2002) (Fig. 1e, f & 2a). The germination of isolated intra-radical vesicles was clearly demonstrated by Diop et al (1994a, b). Germination occurred through the lumen of the subtending hyphal attachment (Declerck et al 1998), the germ tubes generating runner and ramified hyphae similar to those of AM fungi spores. To date, no systematic investigation has been conducted on factors influencing vesicle germination. Among other fungal structures capable of re-growth are hyphae from the peridium of *F. mosseae* sporocarps which have the capability to elongate and differentiate into vesicle-like structures (Budi et al 1999). Furthermore, the "germination" of colonized root segments is currently used to replicate AM fungi monoxenic cultures (Strullu et al 1991). The root vesicles and eventually intra-radical spores are certainly the fungal propagules involved in root segment "germination" as colonized root segments, where vesicles and spores were absent, remained unsuccessful in propagation. Germ tube growth is dependent on the availability of spore reserves (Sancholle et al 2001), and the protoplasm contains all the organelles required to ensure development (Meier and Charvat 1992). This consists of a straight-growing hypha (runner hyphae, RH) exploring the media by successive branching into thinner-diameter filaments (Declerck et al 2000) (Fig. 2b). In the case of no hyphal root contact or host signal detection, germ tube growth stops within a few days (Bécard and Piché 1989b). The protoplasm shrinks back from the hyphal apex, and is sequestered from the empty hyphae by repeated septation (Logi et al 1998). These germination attempts resemble a well-orchestrated survival scenario, providing repeated chances for the fungus to establish symbiosis. Spore germination does not generally require the presence of a host root (the non-symbiotic stage). However, for further growth and development, the AM fungus becomes dependent upon the presence of, but not necessarily physical contact with an adequate host (Giovannetti et al 1996). Using *G. rosea,* it was shown that this activated physiological stage (the pre-symbiotic stage) requires the simultaneous presence of root exudates and CO_2 (Poulin et al 1993). Bécard and Piché (1989*a*) suggested that *G. rosea* was capable of fixing CO_2 as a mineral source of carbon. *In vitro* labeling with $^{13}CO_2$ and NMR spectroscopic analysis has confirmed that substantial dark fixation of CO_2 occurs in *R. irregularis* during spore germination (Bago et al 1999a).

In monoxenic cultures, root colonization levels vary according to the host plant species and fungal isolates (Elsen et al 2003). Acidification of the media directly influences AM fungal development. The pH 5.5 value of standard monoxenic culture systems might limit the growth of some isolates, but an increase in pH of the nutritive media may alter the solubility and balance of the media components. Buffered media may counteract such imbalances. Most monoxenic culture plants support the *Arum*-type colonization (Glorian 2002). A contrary situation occurs with carrot root culture, an Apiaceae

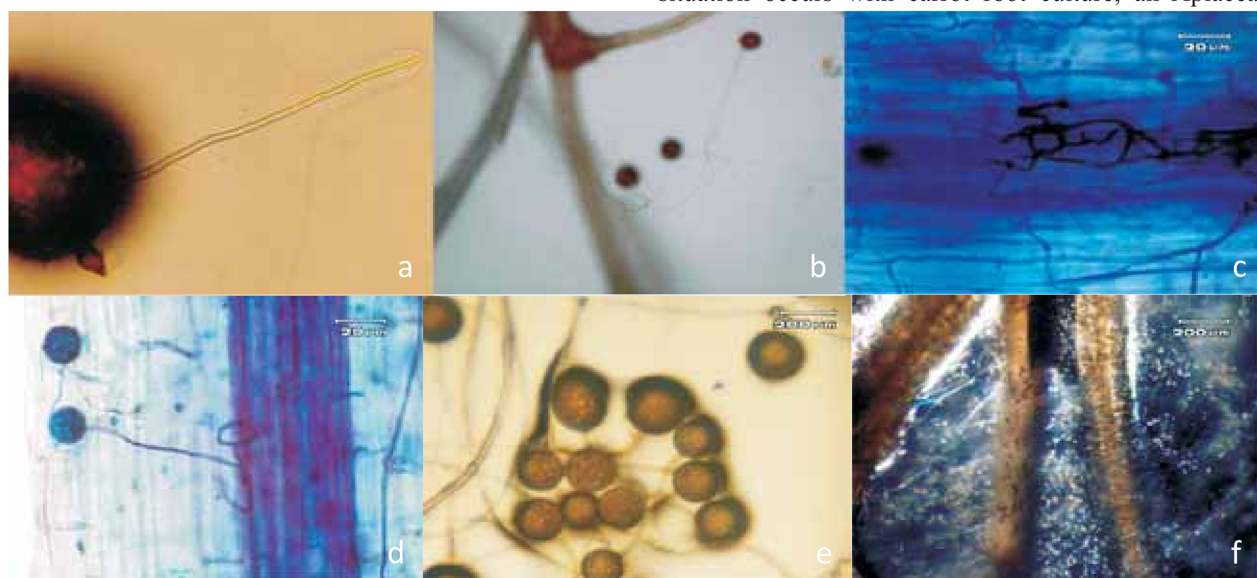

Figure 2. a. Spore germination in *Scutellospora* sp.; b. Hyphal growth in *in vitro*; c. Hyphal colonization in transformed root; d. VLS in transformed root; e. Sporulation in *Rhizophagus irregularis*; f. Sporulation in *Glomus clarum*

(Umbelliferae) recognized as supporting both *Paris*- and *Arum*-type colonization (Smith and Smith 1997), whereby *Arum*-type colonization is differentiated more. Only one *F. caledonius* isolate has differentiated *Paris*-type colonization with a carrot root culture (Karandashov et al 2000), and one *G. etunicatum* isolate had mixed types, differentiating hyphal coils in the first layer of cortical cells (Pawlowska et al 1999). *Paris* and *Arum* morphotypes were long considered to be determined by the plant genome (Smith and Smith 1997), but the typical *Paris* anatomical type observed in carrot root culture colonized by *F. caledonius* emphasizes the impact of the fungal genome on the regulation of fungal morphology (Cavagnaro et al 2001). Budi et al (1999) reported that the hyphae from the peridium of *F. mosseae* sporocarps have the capability to elongate and differentiate into vesicle-like structures (VLS). When differentiated, VLS occurred within 2–8 days after root contact and their size ranged between 20 and 100 µm, depending on the species (Declerck et al 2005). VLS are small, hyaline thin-walled swellings resembling miniature spores (Strullu and Romand 1987). Hypotheses about their role range from a survival process during the pre-symbiotic stage to an aborted sporulation tentative (Declerck et al 2005) (Fig. 2 c & d).

The basic structure of the mycelium is composed of large, straight growing relatively un-branched thick walled runner hyphae (RH) (Friese and Allen 1991), small-diameter thin walled branched hyphae called arbuscule-like structures (ALS) (Bago et al 1998a) or fine branching (FB) (Juge et al 2009), and spores. Runner hyphae are similar to pre-symbiotic hyphae in their capacity to extend rapidly, to colonize the substrates, and to establish root contact. Microscopical cellular and subcellular observations allow detection of protoplasmic streaming, nuclei migration and organelle morphology (Bago et al 2001). Hyphae are either single-walled, as with *G. versiforme* (Garriock et al 1989), or double-walled as found through ultrastructure work on *R. fasciculatus* (Bonfante-Fasolo and Grippiolo 1982). The abundance of runner and branched hyphae determines the mycelium architecture. Once a successful symbiosis is established, numerous ALS are differentiated along hyphae (Bago et al 1998a). Ultra-structural investigations revealed that ALS (renamed as branched absorbing structures or BAS; Bago et al 1998d) are very similar to intra-radical arbuscules and, like arbuscules, they are sites of intense metabolic activity. Arbuscules and BAS are also similar in terms of their gross morphology (thinner diameter with increased dichotomous branching). The extent to which these structures are functionally comparable remains to be elucidated. However, prolific branching of the fungus to form BAS results into an important increase in surface area producing a structure better adapted for nutrient uptake. It has also been shown that increased acidification of the medium coincides with a higher production of spore-associated BAS. This change in pH could be a direct consequence of a greater phosphate uptake, to provide storage products for the spores (Bago et al 1998b, 1998c). It also appears that inorganic nitrogen and phosphate absorption by extra-radical mycelium is closely correlated with BAS development (Bago et al 1998*d*). BAS may adopt variable morphologies, the most striking being the large and stunted ramified structures of *F. caledonius* (Karandashov et al 1999).

Spore differentiation occurs either apically or intercalary along lateral branches of RH, often in association with BAS (Bago et al 1998d). The outer evanescent spore wall then originates from the hyphal wall. The spore apical hyphae, even though collapsed, remain attached to the spore during most of the maturation process. Intra-radical spores have sometimes been observed in monoxenic cultures (De-Souza and Barbara 1999). Spore production differs considerably between species and between isolates of a single species, and seems to be related to spore size. With the small to medium size spore species *R. proliferus* and *R. irregularis*, an average of 7,800 and 8,200 spores were differentiated in mono-compartment (Declerck et al 2001) and bi-compartment growth systems (St-Arnaud et al 1996) respectively. Most *Glomus* species exhibit an asynchronous mode of sporulation, *i.e.* with a lag, log and plateau phase (Declerck et al 1996a, 2001) (Fig. 2 e & f).

Research into major differences between AM fungal cultures has dealt primarily with mycelium architecture, hyphal network density, pattern of ramification, spore abundance, and positioning and clustering of spores. Large-spore species usually exhibit a less dense mycelium and fewer anastomoses. Stunted BAS, together with typical *Paris*-type root colonization, characterize *F. caledonius* isolates (Karandashov et al 2000). Spore maturation of monoxenic cultured AM fungi follow similar ontogeny steps as those in pot-culture. Differences reside essentially in the clean, contaminant-free quality of monoxenic cultured spores, with abundant fungal material available at precise age and physiological stages. The comparison between monoxenically cultured species shows ready segregation between large- and small-spore species in terms of apical mode of development, single spore differentiation, and low sporulation levels. By contrast, smaller spore species present a variable growth pattern, mainly with intercalary sympodial spore growth, clustered spores, and high sporulation levels. Spore wall morphology of monoxenically differentiated spores does not differ fundamentally from field-collected ones, apart from the

lower mean spore diameter measured for some AM fungal isolates (Pawlowska et al 1999). With monoxenic cultures, all elements of spore wall architecture remain observable throughout maturation, including the evanescent outer wall, often absent in soil-propagated AM fungal spores, due to abrasion and/or digestion by soil micro-organisms.

Fundamental and Practical Studies

Although *in vitro* culture is an artificial system, it may be a valuable tool to study fundamental and practical aspects of AM symbiosis, complementing experimental approaches. The compartmentalized Petri dish system (St-Arnaud et al 1996) is particularly suitable for the study of nutrient uptake and translocation in AM fungi under strictly controlled conditions. It also allows the differentiation between intra-radical and extra-radical fungal metabolism (Bago et al 2000). The compartmentalized system has been used for example, by Joner et al (2000) to study P transport by the extra-radical hyphae of *R. irregularis*, and in N nutrition, the compartmentalized *in vitro* system was used to show that the extra-radical hyphae of *R. irregularis* facilitate nitrate (Bago et al 1996) and ammonium absorption (Villegas 2001).

The first report of interactions between soil microbes and AM fungi under aseptic *in vitro* conditions was by Mosse (1962), who observed that root colonization could not be established without adding either a suspension of *Pseudomonas* species or various types of bacterial filtrates. Following this pioneering work, a wide range of soil bacteria and fungi has been shown to enhance *in vitro* germination of spores and hyphal growth of *F. mosseae* without direct contact between the organisms. These results suggest involvement of volatile (*e.g.* CO_2) or highly diffusible substances (Azcón, 1989). Simultaneously, spore-associated bacteria have been identified from the genera *Pseudomonas* and *Corynebacterium* (Mayo et al 1986), and that cell-free fractions from rhizosphere bacteria cultures have the same stimulatory effect as complete bacterial cultures (Azcón 1987). AM fungi can contribute to root disease suppression through mechanisms not well understood (Linderman 1994) but the most obvious effect of AM fungi has been attributed to amelioration of nutrient uptake (P and others), resulting in more vigour in growing plants that are better able to ward off or tolerate root disease. St-Arnaud et al (1995a) proposed a compartmentalized *in vitro* system to elucidate interactions between *R. irregularis* and the root pathogen *Fusarium oxysporum* f. sp *chrysanthemi*. Significant negative correlations were found between conidia production and *R. irregularis* hyphae or spore concentrations. McAllister et al (1994) found no *in vitro* interactions between spores of *F. mosseae* and *Trichoderma koningii* Oudem or *Fusarium solani* (Mart.) Sacc. Life cycles of *R. irregularis* and the burrowing nematode, *Radopholus similis* (Cobb) Thorne, were achieved in monoxenic cultures (Elsen et al 2001). The AM fungus reduced the nematode population by 50%. Also, AM root-organ cultures showed a synergistic interaction between the extra-radical mycelium of *R. irregularis* and soil bacteria in a study of rhizosphere nutritional dynamics (Villegas 2001). In this study, species-specific interactions were obtained between *R. irregularis* and *Pseudomonas aeruginosa* (Schröter) Migula, *P. putida* Trevisan and *Serratia plymutica* Lehman and Neumann. Although the inherent ability of the fungus and the bacteria to solubilize a recalcitrant form of calcium phosphate was low, *P. aeruginosa* and *P. putida* interacting with the extra-radical mycelium markedly increased P availability in the growth medium. This increase was dependent on the N source, which allowed a reduction of the pH (Villegas and Fortin 2001). Associations found between some bacterial strains and AM fungal propagules may have a promotional effect on short-term pre-symbiotic mycelium development but little impact on AM propagule germination (Fernández Bidondo et al 2011).

Advantages and Disadvantages of the Root Organ Culture System

Although *in vitro* culture is an artificial system, it may be a valuable tool to study fundamental and practical aspects of AM symbiosis, complementing the experimental approaches. The most evident advantage shared by all *in vitro* cultivation systems is the absence of undesirable microorganisms due to controlled conditions, rendering greater suitability for large-scale production of high-quality inoculum. Contamination by other microorganisms may occur either at the establishment of the cultivation process or at later stages of culture. Therefore, it may be useful to control the cultures visually, by standard plate-counting techniques and by molecular techniques. The cultures may be placed in a growth chamber requiring minimal space for incubation with no light required in the case of ROCs. Following sporulation dynamics during cultivation also provides a means to control the level of spore production and to determine the optimal harvesting time. Factors that influence optimal production (e.g. nutrient availability, presence of contaminants) can be more easily detected and controlled in liquid cultures. As a drawback, the diversity in terms of genera of AM fungi that have been grown *in vitro* is lower than under pot cultivation systems. Once successfully initiated, the cultures may be maintained for periods exceeding 6 to 12 months without intervention. The harvesting method of solid *in vitro* cultures involves solubilization of the medium by citrate buffer i.e. the gelling agent may be

removed from the culture medium so as to stimulate re-growth of the fungus (Doner and Bécard 1991). Monoxenic cultures provide access to abundant and high-quality fungal material suitable for taxonomic and evolutionary studies (Fortin et al 2002). In terms of biodiversity, monoxenic cultures provide a tool for basic comparative analyses of root populations and strain potential, long-term propagation capabilities, and fungal adaptation to environment.

Conclusion

The continued development of high quality and low-cost methods for production of AM fungal spores under *in vitro* systems may lead to new and advanced methods of large-scale inoculum production of AM fungi in the near future. The application of contaminant free inoculum can be of great value for *in vitro* propagation of high-value crops and ornamental plants (Kapoor et al 2008). In addition, *in vitro* propagation in association with AM fungi could reduce mortality rates and the transplantation shock of re-introduced endangered plant species. It could also be used to enhance the production of secondary metabolites used in the pharmaceutical industry (Kapoor et al 2008).

Intra-radical propagules can serve as a source of high-quality inoculum. Techniques such as sonication and gradient flotation as well as enzymatic methods, may be developed to separate intra-radical spores and vesicles from roots (Biermann and Linderman 1983).The encapsulation of AM fungi produced monoxenically in alginate beads offers an opportunity to diversify inoculation process. It might be helpful to incorporate stimulatory compounds such as flavonoids in beads containing AM fungi (Gianinazzi-Pearson et al 1989) or synergic microorganisms (Hildebrant et al 2002).

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

The production of AM fungi on plants under *in vitro* conditions has recently been proposed (Voets et al 2005) and extended to hydroponic systems (Declerck et al 2009). Following the pre-inoculation of a suitable autotrophic host plant in the system developed by Voets et al (2009), a culture is transferred in a hydroponic cultivation system favouring production of large quantities of propagules. However, the effectiveness of the *in vitro* produced propagules under adverse conditions remains uncertain. Some AM fungi lose their colonization potential 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 conserve permanent fungal biodiversity, *in vitro* and *in vivo* collections must be maintained consistently.

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Received: 25 Apr 2013 Accepted: 03 May 2013