Mycorrhiza (2011) 21:1–16
DOI 10.1007/s00572-010-0337-z

REVIEW

Methods for large-scale production of AM fungi: past, present, and future

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Received: 24 March 2010 / Accepted: 5 August 2010 / Published online: 28 August 2010

Abstract Many different cultivation techniques and inoculum products of the plant-beneficial arbuscular mycorrhizal (AM) fungi have been developed in the last decades. Soil- and substrate-based production techniques as well as substrate-free culture techniques (hydroponics and aeroponics) and in vitro cultivation methods have all been attempted for the large-scale production of AM fungi. In this review, we describe the principal in vivo and in vitro production methods that have been developed so far. We present the parameters that are critical for optimal production, discuss the advantages and disadvantages of the methods, and highlight their most probable sectors of application.

Keywords Inoculum production · In vitro cultivation · ROC · Hydroponics · Aeroponics · NFT

Part of the Belgian Coordinated Collections of Microorganisms (BCCM)

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Introduction

Arbuscular mycorrhizal (AM) fungi are worldwide-distributed soil fungi, forming symbiosis with most plant families. Their importance in natural and seminatural ecosystems is commonly accepted and materialized by improved plant productivity and diversity as well as increased plant resistance against biotic and abiotic stresses (Smith and Read 2008). Nowadays, they are increasingly considered in agriculture, horticulture, and forestry programs, as well as for environmental reclamation, to increase crop yield and health and to limit the application of agrochemicals (Gianinazzi et al. 2002; Johansson et al. 2004). Some products are also accessible to the broad public, e.g., for gardening or for specific end users, such as keepers of golf greens. However, the obligate biotrophic nature of AM fungi has complicated the development of cost-efficient large-scale production methods to obtain high-quality AM fungal inoculum. This is one of the reasons why their commercial exploitation is still in its infancy. Other reasons include the sometimes unstable performance of mycorrhizal fungi in plant production systems and the shortage of knowledgeable users. Many different cultivation techniques and products have been developed in the last decades, all having specific advantages and constraints regarding their design, commercialization, and domain of application. Here, we classified the production systems for AM fungi in three main categories. (1) The "classical" sand/soil and more advanced substrate-based production systems. Such systems are widely used and mostly represent a cost-effective way to mass-produce AM fungal inoculum adapted for large-scale applications. (2) The substrate-free cultivation systems ("true" hydroponics and aeroponics) that have been developed to produce relatively clean (sheared) AM fungal inoculum.

However, higher costs associated to these production systems have mostly limited their use to smaller-scale applications and research purposes. (3) The in vitro cultivation systems, are based either on excised roots, the so-called "root organ cultures" (ROC) or on whole autotrophic plants. Despite their current high costs, these systems guarantee the contaminant-free production of pure AM fungi. In vitro cultivation of AM fungi is particularly adapted to the production of high-added-value crops (e.g., crops generated via micropropagation techniques).

In this review, we intend to describe these cultivation techniques for large-scale AM fungal inoculum production, paying particular attention to their advantages and disadvantages, to the parameters that are critical for optimal production, and to the potential sectors of application.

Substrate-based production systems

System description

Classical production of AM fungi is generally performed by the cultivation of plants and associated symbionts in a soil- or sand-based substrate, even though a range of substrate substitutes and amendments are also commonly used (detailed in "Production parameters"). We decided to include nonsterile hydroponic methods that use a physically solid substrate (e.g., sand or perlite) in the present section because the presence of a carrier medium could influence AM fungal propagation.

Large-scale production may be achieved in single pots of various materials (e.g., earthenware or plastic) and sizes (e.g., Millner and Kitt 1992; Sylvia and Schenck 1983) or scaled up to medium-size bags and containers and to large raised or grounded beds (Douds et al. 2005, 2006; Gaur and Adholeya 2002; Fig. 1). The production process is often conducted under controlled or semicontrolled conditions in greenhouses or performed in growth chambers for the easy handling and control of parameters such as humidity and temperature. However, depending on the host plant and climate conditions, large-scale production is sometimes conducted in open air, e.g., for on-farm production (Douds et al. 2005, 2006; Gaur and Adholeya 2002), and infrequently on field plots (e.g., Dodd et al. 1990a, b).

Production parameters

The AM fungi

Mass production in sand/soil or other substrate-based production systems is most often initiated with a single

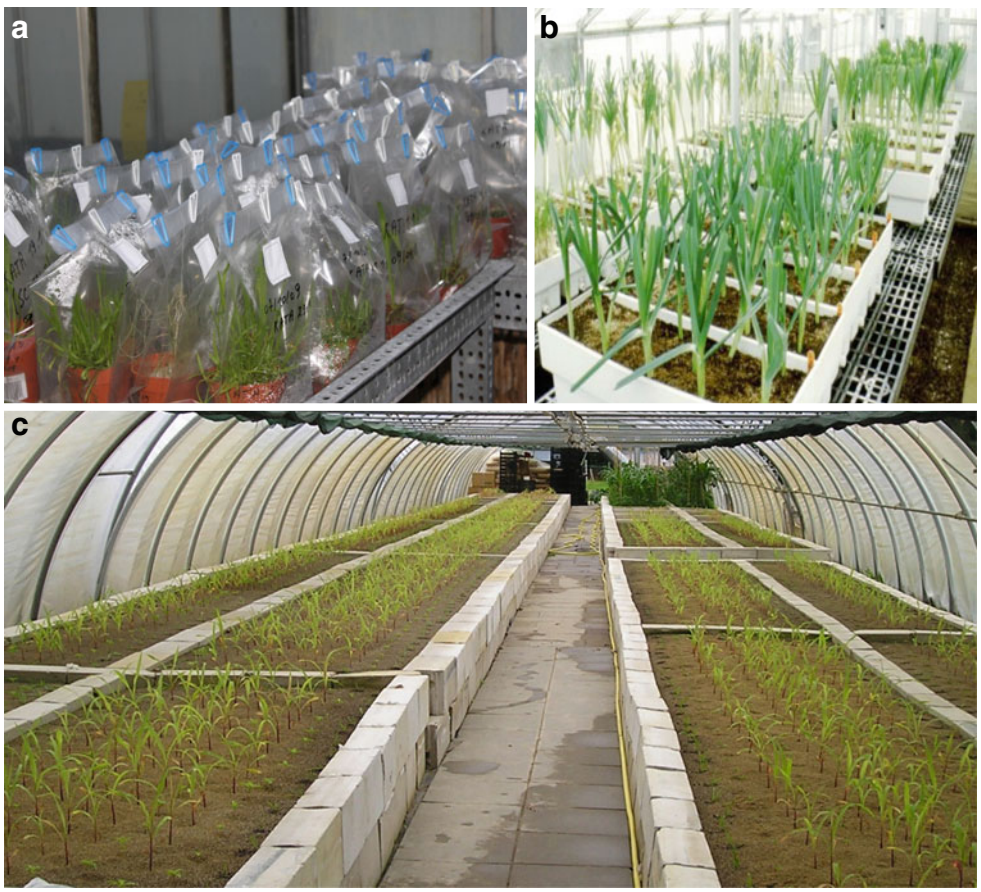

Fig. 1 Substrate-based system for the production of AM fungi. Different scales of production modes: **a** *Plantago lanceolata* pot cultures in sun bags according to the method of Walker and Vestberg (1994). The GINCO, Belgium, is acknowledged for providing the picture; **b** container cultures of *Allium porrum.* Y. Dalpé is thanked for providing the picture with permission from GINCO Canada. **c** Bed cultures of *Zea mays* and *Tagetes erecta* for commercial production of AM fungal inoculum were provided by C. Schneider from the company INOQ who is also acknowledged

identified species or a consortium of selected identified AM fungal species while on-farm production is also sometimes started with species that are indigenous to the site of application and not always identified to the species level (Gaur and Adholeya 2002).

The starter inoculum to initiate production usually consists of isolated spores (e.g., Douds and Schenck 1990a, b) or a mixture of spores and mycorrhizal root pieces (e.g., Gaur and Adholeya 2000). To obtain a mixed inoculum, roots may be dried and chopped into fine pieces while spores are most often obtained by wet sieving and decanting. The soil containing AM fungal hyphae may also be used in a mixed inoculum (Gaur and Adholeya 2000). Mixed inoculum is particularly attractive for those AM fungal species producing intraradical spores and vesicles (Biermann and Linderman 1983; Klironomos and Hart 2002). Besides, direct inoculation of plants with isolated spores or mixed inoculum, plantlets can also be precolonized before their transplantation into beds (e.g., Douds et al. 2005, 2006) or containers.

Spore starter inoculum usually consists of well-identified multiple individuals (e.g., Douds and Schenck 1990a, b) or mixed spore-root inoculum (e.g., Gaur and Adholeya 2000). International culture collections such as INVAM, BEG and GINCO can in most cases guarantee the delivery of well-identified monospecies and offer a clear traceability of the organism via a repository identification code. Culture collections may thus provide material under clear rules (material transfer agreement) or act as repository for inoculum deposited by companies. The traceability of the organism is mainly based on spore vouchers and assures the origin and identification of the isolate. Methods to confirm that intraradical colonization within the field is strictly related to the inoculated isolate are still under development. In the recent years, Krüger et al. (2009) and Stockinger et al. (2010) opened the way to the development of a DNA barcoding of AM fungi, giving the baseline of AM fungal traceability within the field. When the site of application of inoculum is known, indigenous AM fungal species can also be obtained with trap cultures. Monosporal pot cultures to produce pure isolates can be kept isolated, e.g., placed in sun bags (Walker and Vestberg 1994), to avoid cross-contamination by other AM fungal species (Fig. 1a).

The AM host plants

Plants such as onion and leek (*Allium* spp.), maize (*Zea mays* L.), and Bahia grass (*Paspalum notatum* Flugge) are commonly used for the large-scale production of AM fungi (see Table 1 and Fig. 1a, b). These plants offer several advantages, among which a short life cycle, adequate root system development, a good colonization level by a large range of AM fungi, and tolerance to relatively low levels of phosphorus (P). Other relevant characteristics are the low susceptibility to pathogens, the yellow appearance of colonized roots versus white uncolonized roots (e.g., leek and maize), and a wide range of temperature tolerance (Millner and Kitt 1992). Douds et al. (2005, 2006) utilized the C4 Bahia grass and stressed that this tropical plant is frost-killed during the winter in temperate regions, which would favor sporulation. These authors also stated that the use of a plant species, in their case Bahia grass, that is unrelated to most crop species is unlikely to transmit harmful crop pathogens during inoculum application.

Host-dependent sporulation of AM fungal species (e.g., Dodd et al. 1990a; Struble and Skipper 1988) is an important determinant for inoculum production. Gaur and Adholeya (2002) inoculated five different fodder crops with a consortia of indigenous AM fungal species and observed that the level of production of infective propagules was dependent on the host plant species. The type of AM fungal inoculum (e.g., spore or mixed inoculum) that producers aim to process or use partly determines the host plant/fungus association chosen. For example, high intraradical colonization levels are important for the production of mixed spore-root inoculum, while this might not always be needed for the achievement of a spore inoculum. Although correlation between intraradical colonization and extraradical sporulation has sometimes been reported (e.g., Douds 1994), this relation was not found in all cases and is dependent on the plant/fungus association and particular culture conditions (Douds 1994; Douds and Schenck 1990b; Hart and Reader 2002).

The INVAM website (http://invam.caf.wvu.edu) reports that spore numbers in some of the pot cultures in their collection decrease after successive propagation cycles. They suggested to alternate the hosts when this problem occurs. They proposed to shift from C4 Sudan grass (*Sorghum sudanese*), a host commonly used by INVAM, to C3 legume red clover (*Trifolium pratense*), a species that is unrelated to the former host. Moreover, Egerton-Warburton et al. (2007) demonstrated that certain *Glomus* species (rapid colonizers producing small spores) increased their spore production after N fertilization when associated to a C4 host, while in contrast, hyphal growth of *Gigasporaceae* spp. increased after N fertilization when associated with a C3 host (Egerton-Warburton et al. 2007). Burrows and Pfleger (2002) already demonstrated that AM fungal species producing large spores increase sporulation with increased plant diversity, while spore production of species producing small spores varied depending on the hosts. When the AM fungi are connected to diverse host plant species, e.g. in production fields or beds, such decreases in spore production might not occur. Moreover, the number of plants species and individuals,

Table 1 Average production of AM fungi in substrate-based cultivation systems as given in the literature (colonization rates are not included)

Authors	Plant host	AM fungus	Main substrate[a]	Method	Production
Gaur and Adholeya 2002	*Zea mays*	Consortium of indigenous *Glomus*, *Gigaspora*, and *Scutellospora* spp. (inoculum used on all plant species)	Sandy loam/compost	Raised beds	100 IP/g/substrate
	Medicago sativa				105 IP/g/substrate
	Trifolium alexandrium				80 IP/g/substrate
	Avena sativa				70 IP/g/substrate
	Sorghum vulgare				110 IP/g/substrate
Gaur and Adholeya 2000	*Zea mays*	*Glomus intraradices* (DAOM181602)	Perlite	Pots	600 IP/100 ml substrate
			River sand		880 IP/100 ml substrate
			Charcoal		550 IP/100 ml substrate
			Coal marl		400 IP/100 ml substrate
			Clay-brick granules		880 IP/100 ml substrate
Douds et al. 2005	*Paspalum notatum*	Noninoculated	Vermiculite/compost	Raised beds	830 propagules/cm^{-3}
		Glomus mosseae			707 propagules/cm^{-3}
		Glomus etunicatum			465 propagules/cm^{-3}
		Glomus claroideum			365 propagules/cm^{-3}
		Glomus geosporum			2,150 propagules/cm^{-3}
		Glomus intraradices			950 propagules/cm^{-3}
		Gigaspora gigantea			465 propagules/cm^{-3}
		Gigaspora rosea			21 spores/cm^{-3}
Douds and Schenck 1990b	*Paspalum notatum*	*Acaulospora longula* (INVAM316)	Sandy soil	Pots	1,000 spores/cm^{-3} soil
		Scutellospora heterogama (INVAM117)			14 spores/cm^{-3} soil
		Glomus intraradices (INVAM208)			80 spores/cm^{-3} soil
		Gigaspora margarita (INVAM105)			49 spores//cm^{-3} soil
		Gigaspora margarita (INVAM185)			25 spores/cm^{-3} soil
		Gigaspora margarita (INVAM597)			6 spores/cm^{-3} soil
		Gigaspora margarita (INVAM680)			7 spores/cm^{-3} soil
Gryndler et al. 2003	*Allium ampeloprasum*	*Glomus claroideum* (BEG23)	Sand/Cambisol	Pots	2,010 spores/plant
	Plantago lanceolata	Mix of 3 *Glomus* spp	Sand/Cambisol		163 spores/g soil
	Lactuca sativa	*Glomus mosseae* (BEG25)	Sand/vermiculite		1,447 spores/plant
Millner and Kitt 1992	*Zea mays*	*Glomus etunicatum*	Sand	Pots/drip irrigation	341 000/spores per pot
		Glomus mosseae (INVAM156)			210 000/spores per pot
		Gigaspora margarita			64 800/spores per pot
Sylvia and Schenck 1983	*Paspalum notatum*	*Glomus clarum*	Limed loamy sand	Pots	55 spores/g soil
		Glomus mosseae			15 spores/g soil
		Glomus etunicatum			14 spores/g soil
		Glomus macrocarpum			31 spores/g soil
		Gigaspora margarita			20 spores/g soil
		Gigaspora heterogama			700 spores/kg soil
		Gigaspora gigantea			120 spores/kg soil

IP infectious propagules

[a] Substrate amendments and watering/fertilization regimes are not detailed in this table

plant health, and developmental status could impact the performance of the associated AM fungi.

Substrates and amendments

Various substrates either pure or mixed have been used to propagate and large-scale-produce AM fungi (Table 1). Soil, often sandy, has been commonly reported (e.g., Douds and Schenck 1990a, b; Sylvia and Schenck 1983) as well as pure sand (e.g., Millner and Kitt 1992) and to a lesser extent substitutes such as peat (e.g., Ma et al. 2007), glass beads (e.g., Lee and George 2005; Neumann and George 2005), vermiculite (e.g., Douds et al. 2006), perlite (e.g., Lee and George 2005), compost (e.g., Douds et al. 2005, 2006), and calcinated clay (Plenchette et al. 1982).

Substitutes for soil, sand, and substrate amendments have been considered for various purposes. For instance, relatively inert substrates (e.g., vermiculite and perlite) have been used to dilute nutrient-rich soil and compost (Douds et al. 2005, 2006). Conversely, compost or other organic substrates such as peat can be added to nutrient-deficient soils (Gaur and Adholeya 2002; Ma et al. 2007). Many different organic amendments have been reported to influence AM fungal root colonization. For example, chitin (Gryndler et al. 2003) and humic substances (Gryndler et al. 2005) enhanced colonization levels, whereas cellulose reduced colonization by the AM fungus (Avio and Giovannetti 1988; Gryndler et al. 2003). Gryndler et al. (2003) also observed neutral to positive effects of chitin amendment on the spore production of various *Glomus* species. As the effect was possibly due to an increase in actinomycetes, the authors suggest further risk assessment of chitin addition for inoculum production. Inert substrates have also been used as carrier medium to support roots and fungal growth under conditions where plant feeding was mainly provided by a nutrient solution (e.g., Lee and George 2005). Glass beads or (coarse) river sand additions can further simplify the harvesting procedure and clean up the fungal material from any debris (Chen et al. 2001; Neumann and George 2005).

The particle size of the substrate is important for adequate drainage, humidity, and aeration. These parameters have been shown to influence sporulation of AM fungi (Gaur and Adholeya 2000; Millner and Kitt 1992; Saif 1983). In soil-free cultures, nourished with a nutrient solution, sand particle sizes of approximately 250–850 μm have been reported adequate for the cultivation of several *Glomus* species with *Z. mays* as a host (Gaur and Adholeya 2000; Millner and Kitt 1992).

The cultivation substrate is usually pretreated to circumvent problems of contamination by undesirable soil microorganisms (e.g., plant pathogens). The substrate used in pots, containers, and bags can be treated by steam or heat sterilization or by irradiation. The substrates in raised beds can be either fumigated or left untreated (Douds et al. 2005, 2006; Gaur and Adholeya 2002).

Nutrition

Manipulation of nutrient regimes has been demonstrated to impact AM fungal propagule production (Douds 1994; Douds et al. 2006; Douds and Schenck 1990a, b; Millner and Kitt 1992). The nutrient content of the substrate as well as the addition of macronutrients and micronutrients may influence the AM fungi directly but also indirectly by the plant responses to nutrient availability, e.g., by altered root growth or photosynthesis. Although it remains largely unclear as to which extent the used plant/AM fungi associations differ in their nutrient requirements, optimal nutrient regimes should support initial colonization, promote adequate plant (root) growth, and optimize the AM fungal propagule production.

In general, AM fungal colonization is favored under low-nutrient (mainly P) conditions (Amijee et al. 1993; Smith and Read 2008). Nutrient solutions without or with low levels of P have often been reported as beneficial for the AM fungal root colonization and spore production (e.g., Gaur and Adholeya 2000; Millner and Kitt 1992). For instance, Millner and Kitt (1992) cultivated *Glomus mosseae*, *Glomus etunicatum*, and *Gigaspora margarita* on maize plants in a sand-based system and reported a concentration of 20 μMP as optimal. Within their experimental setup, P concentrations below 2 μM resulted in poor plant development and above 100 μM in weak sporulation. Gaur and Adholeya (2000) reported higher propagule production of *Glomus intraradices* grown on maize in sand culture, fertilized with a nutrient solution without P. In contrast, other studies reported that AM fungal strains of *G. intraradices* were tolerant to high P levels (Douds and Schenck 1990a; Sylvia and Schenck 1983) and even nutrient solutions containing over 80 μMP were used to culture *G. mosseae* on lettuce (*Lactuca sativa*) in a nutrient film technique (NFT) system with perlite as carrier substrate (Lee and George 2005). The form of P used (e.g., rock phosphate, superphosphate, organic phosphate) may also impact propagule production. For example, in a study by Sylvia and Schenck (1983), *Gi. margarita*, *Glomus clarum*, *G. mosseae*, and *Gigaspora heterogama* responded positively to superphosphate, while *G. etunicatum*, *Glomus macrocarpum*, and *Gigaspora gigantea* were negatively influenced. Millner and Kitt (1992) did not observe any differences between rock phosphate and solution P. In addition, ratios of N/P in both the substrate and the plant tissues may be of importance in determining colonization and sporulation levels (Blanke et al. 2005; Douds and Schenck 1990a, b).

Although less is known on nitrogen (N) nutrition, NO_3 is the usual form of N addition, since NH_4 can alter the pH and is less readily available to the plants. AM fungi can be nourished with solutions containing both forms of N (e.g., Gryndler et al. 2005; Lee and George 2005), but more frequently Hoagland nutrient solution (Hoagland and Arnon 1950) is used which normally contains only NO_3 as N source. Both full-strength and half-strength Hoagland solutions were often used, sometimes with modified N and P concentrations (e.g., Douds et al. 2006; Millner and Kitt 1992; White and Charvat 1999). Furthermore, the timing of nutrient addition might influence colonization levels and propagule production, as nutrient requirements to culture AM fungi might differ throughout time. Whereas high P availability often suppresses colonization, the addition of P in later stage might enhance AM fungal growth and sporulation. For example, Neumann and George (2005) allowed *G. intraradices* to forage in a compartment differing in P and extracted more hyphae from a high-P compartment than from a low-P compartment.

Additional factors

Many other factors may influence propagule production. Among these, the factors that influence the plant photosynthesis (e.g., light intensity—Furlan and Fortin 1977) and C allocation to the roots may indirectly impact the AM fungi colonization and spore production. Soil characteristics such as pH, cation exchange capacity (CEC), temperature (T), and water content are closely related to characteristics of the substrate. A change in substrate may cause an alteration in more then one aspect, and in the light of this paper it was not possible to elucidate all separate factors.

To avoid fluctuations in pH, Lee and George (2005) and Millner and Kitt (1992) proposed to buffer their nutrient solution with four-morpholine ethanesulfonic acid (MES). Millner and Kitt (1992) further classified their *Gi. margarita* and *G. etunicatum* isolates as acidophilic and six isolates of *G. mosseae* as basophilic, confirming that their isolates had pH optima. Another study by Medeiros et al. (1994) on intraradical colonization levels only also revealed pH optima of several *Glomus* species and isolates.

Host plants and AM fungi should have access to sufficient water, at the same time avoiding water excess and oxygen deprivation. In substrate-based hydroponics systems, the side effects of water may be counterbalanced by an adequate aeration of the medium (White and Charvat 1999). Drought has also been considered as a potential factor impacting spore production. While Sylvia and Schenck (1983) did not observe any effect of drought on sporulation, Gaur and Adholeya (2000) noted a reduced production of propagules when the particle size of the substrate was high, i.e., 1.7–0.78 mm, and argued that this was due to lower water retention capacity of the substrate.

Advantages, disadvantages, and sectors of application

Substrate-based cultivation of AM fungi in pots, bags, or beds is the most widely adopted technique for AM fungal inoculum production because relatively low technical support is needed and consumables are cheap. Substrate-based production systems are the least artificial and support the production of a large set of AM fungal species, either alone or in consortia of several species. In general, they are considered as a convenient system for large-scale production that is able to reach inoculum densities set for mass production of 80–100 propagules per cubic centimeter (Feldmann and Grotkass 2002). When inert carrier media are used, the nutrient supplies to the AM fungus and plant can be monitored and regulated (Lee and George 2005). More controlled culture conditions are an advantage as this can lead to insights on factors to optimize propagule production.

A disadvantage of substrate-based cultivations systems is that, in most cases, they cannot formally guarantee the absence of unwanted contaminants, even if strict quality control systems could be applied. Besides, these methods are often space consuming and need pest control. Harvesting is usually performed by wet sieving and decanting, which can be followed by centrifugation. When the substrate is not used as a carrier, the final inoculum can be difficult to prepare due to the attachment of clay particles and organic debris (Millner and Kitt 1992). Technical adaptations such as the addition of glass beads, river sand, or vermiculite seem to have limited this problem and facilitate harvesting of relatively clean AM fungal spores and roots that can be chopped into pieces. The presence of a substrate, however, provides an inoculum which is not directly suitable for mechanical application, as is the case for substrate-free production methods (Mohammad et al. 2004).

Substrate-free cultivation systems

System description

A wide variety of substrate-free cultivation techniques, also termed “solution culture techniques,” exists. They mainly differ in the mode of aeration and application of the nutrient solution. In static systems (i.e. in which the solution is not flowing), the nutrient solution needs to be aerated via an aeration pump to prevent roots to suffer from oxygen deprivation. However, strong movement of the nutrient solution and bursting of air bubbles might damage the development of the delicate extraradical hyphae. To prevent

this problem, pumps may be switched on only periodically to minimize the impact on AM fungal growth and development (Dugassa et al. 1995; Hawkins and George 1997).

An alternative to the systems above is the NFT (or nutrient flow technique; Fig. 2), in which a thin nutrient solution (i.e., film) flows into the often inclined channels (also called gulls) where the plant roots and AM fungus develop. The use of a nutrient film that covers the roots increases the relative area for gas exchange and overcomes problems due to insufficient aeration. This technique was used to culture AM fungi in the early 1980s (Elmes and Mosse 1984) and was patented by Mosse and Thompson in 1981 (US Pat. No. 4294037). More recently, it was used by Lee and George (2005) as a substrate-based system (see "Substrate-based production systems").

Aeroponics is a form of hydroponics in which the roots (and AM fungus) are bathed in a nutrient solution mist (Zobel et al. 1976). Spraying of microdroplets increases the aeration of the culture medium, and in addition, the liquid film surrounding the roots allows gas exchange. This mist can be applied by various techniques that differ mainly in the size of the fine droplets produced. Comparisons to test the suitability of different aeroponic techniques have been performed by Jarstfer and Sylvia (1995) and Mohammad et al. (2000). In the first study, Jarstfer and Sylvia (1995) tested three types of aeroponic devises, atomizing disk, pressurized spray through a microirrigated nozzle, and an ultrasonically generated fog of nutrient solution with droplets of 3–10 μm diameter. They reported that pump and nozzle spray systems were the most adapted systems for AM fungi cultivation. In the study of Mohammad et al. (2000), atomizing disk was compared with the latest ultrasonic nebulizer technology (resulting into microdroplets of 1 μm in diameter). These authors reported the ultrasonic nebulizer as the most successful aeroponic method for the cultivation of *G. intraradices* associated to Sudan grass (*S. sudanese* Staph.).

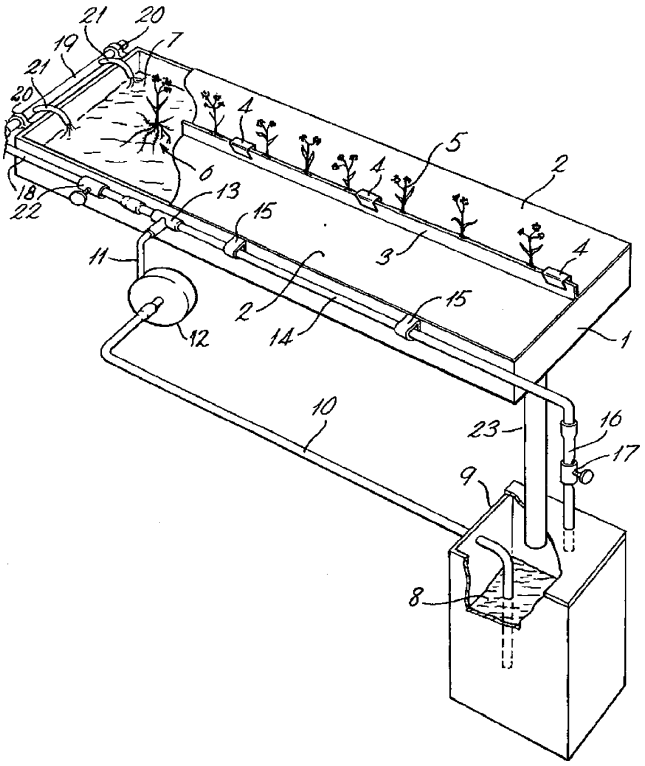

Fig. 2 Substrate-free system for the production of AM fungi. NFT (Mosse and Thompson 1981, US Pat. No. 4294037), as example of a "solution culture technique." Within this system, the mycorrhized roots of the plants and the AM fungal extraradical parts develop in a nutrient solution

In substrate-free production systems (i.e., hydroponics and aeroponics) precolonized plants are produced prior to their introduction into the systems. For preinoculation, plant seedlings and AM fungal propagules (both preferably surface-sterilized) are usually precultured in pots containing a substrate (e.g., mixture of sand and perlite) for several weeks. The container in which the roots (and AM fungus) develop is usually protected from light to prevent the development of algae (Jarstfer and Sylvia 1995).

Production parameters

The AM fungi

Several *Glomus* species (e.g., *G. intraradices*, *G. mosseae*, *G. fasciculatum*, and *G. caledonium*) have been successfully used to set up substrate-free inoculum production (Dugassa et al. 1995; Elmes and Mosse 1984; Hawkins and George 1997; Tajini et al. 2009; Table 2). *Acaulospora laevis* associated to bean plants was reported in NFT (Elmes and Mosse 1984), and *Entrophospora kentinensis* was grown in aeroponics (Wu et al. 1995). *G. rosea* failed to grow in a so-called tripartite hydroaeroponic system with bean and rhizobia in a study performed by Tajini et al. (2009), while *G. intraradices* was successfully grown in this system. However, no clear reasons have been proposed to explain the failure to grow and propagate AM fungi in a moist environment when adequate aeration was supplied. Recently, AM fungi associated with aquatic plants were cultured in substrate-based hydroponics (White and Charvat 1999), and it can be hypothesized that isolates from such habitats might be better adapted to this cultivation technique.

The AM host plants

Substrate-free cultivation techniques have been used with several plant species that are similar to those used in substrate-based production systems (Table 2). Jarstfer and

Table 2 AM fungal propagule production in substrate-free cultivation systems as given in the literature (colonization rates are not shown)

Authors	Plant host	AM fungus (code)	Method	Production
Elmes and Mosse 1984	*Zea mays*	*Glomus mosseae* (YV)	NFT	Not available
Hawkins and George 1997	*Triticum aestivum*	*Glomus mosseae*	Hydroponics	Not available
	Sorghum bicolor			
	Linum usitatissimum			
Hung and Sylvia 1988	*Ipomoea batatas*	*Glomus deserticola* (S306)	Aeroponic	7.8 spores/cm colonized root
		Glomus etunicatum (S329)		3.6 spores/cm colonized root
		Glomus intraradices (S303)		50.7 spores/cm colonized root
	Paspalum notatum	*Glomus etunicatum* (S329)		6.5 spores/cm colonized root
		Glomus intraradices (S303)		13.4 spores/cm colonized root
Dugassa et al. 1995	*Linum usitatissimum*	*Glomus intraradices*	Hydroponics	Not available
	Paspalum notatum			
Jarstfer et al. 1988	*Allium cepa*	*Glomus* sp. (INVAM-FL329)	Hydroponics	3,738 spores/plant
Tajini et al. 2009	*Phaseolus vulgaris*	*Glomus intraradices* (BEG157)	Tripartite hydroponics[a]	Not available
Mohammad et al. 2000	*Sorghum sudanese*	*Glomus intraradices*	Aeroponics	175,000 propagules/g dw inoculum

NFT nutrient film technique

[a] Consisted of bean, rhizobia, and AM fungus

Sylvia (1997) mentioned that at least 21 genera of host plants have been cultured in solution culture techniques. Among the plants suggested for static hydroponics are wheat (*Triticum aestivum* L.) and linseed (*Linum usitatissimum* L.) (Dugassa et al. 1995; Hawkins and George 1997). Elmes and Mosse (1984) tested several host/AM fungus combination and grew especially maize (*Z. mays*) plants associated to AM fungi successfully in the NFT System. Bahia grass (*P. notatum* Flügge), Sudan grass (*S. sudanese* Staph.), and sweet potato (*Ipomoea batatas* L.) have also been reported as adequate for aeroponic production of AM fungi (Hung and Sylvia 1988; Jarstfer and Sylvia 1995; Mohammad et al. 2000; Wu et al. 1995). The choice of the host plant may influence the colonization levels obtained with some AM fungal species (Elmes and Mosse 1984; Hawkins and George 1997) and possibly also impact sporulation. In addition, it should be taken into account that nutrient solution requirements might differ among host species.

Nutrition

Most, if not all, studies use diluted and modified (e.g., solution with low P content) versions of existing nutrient solutions (e.g., Knop's, Hoagland's, Long Ashton). Concentrations of P appeared crucial (Elmes and Mosse 1984; Hawkins and George 1997; Jarstfer and Sylvia 1995), and levels that are recommended for substrate-free cultivation of AM fungi as mentioned by Hawkins and George (1997) are within the range of P concentrations found in natural soil solutions, i.e., 1–50 μM. Otherwise, concentrations are as within the most common AM fungal habitats where low concentrations (0.5–10 μM) of available P are found in the soil solution (Smith and Read 2008). According to Jarstfer and Sylvia (1997), most often, the nutrient solutions for solution culture techniques range from <1 to 24 μMP, and these authors have successfully used 0.3 μM to culture AM fungi in aeroponics (Jarstfer and Sylvia 1995; Jarstfer et al. 1988). Hawkins and George (1997) reported that the solution containing 10 μM (Long Ashton) resulted in higher colonization of wheat plants by *G. mosseae* compared to the solution containing 0.9 mM (Knop and Hoagland). *G. intraradices* (BEG157) however colonized rhizobial bean plants even when 75 and 250 μM P was supplied (Tajini et al. 2009). The addition of iron (Fe) in the form of iron chelate ((Na)FeEDTA) has been reported (e.g., Dugassa et al. 1995; Hawkins and George 1997) to prevent plants from suffering from chlorosis (Elmes and Mosse 1984). The addition of molybdenum (Mo) was reported (e.g., Elmes and Mosse 1984; Jarstfer et al. 1988; Tajini et al. 2009) as an element required for fungal growth (Hawkins and George 1997).

Within hydroponic and aeroponic cultivation systems, the nutrient solution is regularly renewed, e.g., weekly (Dugassa et al. 1995) or after nutrient levels drop under a fixed threshold (Hawkins and George 1997). Besides prevention of mineral depletion, Jarstfer and Sylvia (1995) further mentioned that periodic changes in the nutrient solution reduced the problems that occur by accumulation of undesirable toxins (e.g., accumulated exudates) and pathogens in the medium.

Similar to substrate-based production systems, optimal nutrient supply to the AM fungus and the plant is expected to be dependent on the specific host plant/AM fungal isolate combination. For the optimization of AM fungal propagule production, it is, however, important to realize that nutrients in the culture solution are directly available to the plant, and the AM fungi might not be needed from the plant's perspective.

Additional factors

The reported pH of the culture medium most often varied between 6.5 and 7.2 and was sometimes adjusted to the known pH requirements of AM fungal isolates (Elmes and Mosse 1984). Hawkins and George (1997) used a MES-KOH buffer along with the Long Ashton medium that was low in P concentrations. Jarstfer and Sylvia (1995) mentioned that both pH and °T needed to be controlled, i.e., °T set between 15°C and 35°C. Plants were often exposed to complementary superficial illumination during the cultivation (Hawkins and George 1997; Jarstfer and Sylvia 1995; Jarstfer et al. 1988; Mohammad et al. 2000; Tajini et al. 2009), especially during the winter. Cultivation has also been conducted in nonshaded greenhouses without supplementary light (Hung and Sylvia 1988; Wu et al. 1995). Jarstfer and Sylvia (1997) proposed to use adequate light wavelength (λ=400–700 nm) and high photosynthetic photon flux density (>500 $\mu mol\,m^{-2}\,s^{-1}$). When placed in a controlled growth chamber, relative humidity was 60% Hawkins and George (1997).

Advantages, disadvantages, and sectors of application

The main advantage of the substrate-free cultivation system is the production of inoculum, free from attached substrate particles. Sheared-root inoculum (roots chopped up in a food processor and washed over sieves) with high propagule density can directly be used for application or can be processed for storage (Sylvia and Jarstfer 1992; Jarstfer and Sylvia 1995). Spores can be easily separated from the roots in absence of debris on the root material (Millner and Kitt 1992). Samples of clean roots can also be harvested and analyzed without roughly interrupting the cultivation of the AM fungus. Moreover, the risk of cross-contamination by other AM fungi is low in such systems. When the container is covered, cross-contamination may only depend on the preinoculation phase. Jarstfer and Sylvia (1995) therefore found this method suitable for the multiplication of pure AM fungal strains. In addition, nutrient supply and pH can be monitored and/or manipulated in substrate-free cultivation systems to optimize cultivation settings for a particular host/AM fungus association.

As a disadvantage, liquid nutrient solutions are prone to the multiplication and dissemination of microbial contaminants as well as the development of algae (Elmes and Mosse 1984). Covering channels, addition of clean P sources, and the utilization of soil-free substrate in the preinoculation phase will solve part of the problems. Dugassa et al. (1995) for example precultured plants in a sand substrate before transferring them into the common nutrient solution, while Voets et al. (2009) successfully transplanted autotrophic in vitro produced mycorrhizal plants.

The lack of a carrier substrate could affect spore production rates, even though to our knowledge evidence for this is only supported by *in vitro* studies (Jolicoeur 1998). Rapid root growth of plants in solution culture can cause low AM fungal colonization rates during early periods of the cultivation (Dugassa et al. 1995). Furthermore, Hung and Sylvia (1988) reported lower germination rates of *G. etunicatum* grown in aeroponic culture as compared to spores obtained from soil, but without affecting the inoculum infectivity potential.

Hydroponics, aeroponics, and NFT have all been successfully used to mass-propagate AM fungi. Aeroponics appeared to be suitable for the propagation of pure strains; NFT systems have been used to preinoculate plants, while static hydroponics has so far mainly been used in research studies. Particularly, aeroponic cultivation and possibly NFT have potential for large-scale production of AM fungi. Mohammad et al. (2000) reported a high number of viable AM fungal propagules obtained by aeroponic culture, and such inoculum was used in a field experiment (Mohammad et al. 2004). Elmes and Mosse (1984) reported and visualized the production of many sporocarps by *G. mosseae* in NFT. Precolonized plants that were grown in an adapted NFT system, with improved aeration and glass beads as solid support, resulted in the development of extensive hyphal mats within 4 weeks (Lee and George 2005). Both aeroponics and NFT may thus appear to be suitable systems for the large-scale production of AM fungi.

In vitro production systems

System description

The first attempts to culture AM fungi in vitro date back the late 1950s (Mosse 1959); shortly thereafter, Mosse (1962)

reported the first association of an *Endogone* species with a plant. Since then, several progresses have paved the way to mass-produce AM fungi. In the mid-1970s, Mosse and Hepper (1975) successfully established a culture of an AM fungus associated with excised roots of tomato (*Lycopersicum esculentum Mill.)* and red clover (*T. pratense* L.) on a gelled medium. Ten years later, Mugnier and Mosse (1987) and Bécard and Fortin (1988) used Ri T-DNA transformed carrot roots as host in the so-called ROC system. To facilitate the access to the AM fungus and increase the production of propagules, St-Arnaud et al. (1996) used a split-plate method, i.e., a bicompartmental ROC, separating a proximal compartment containing the root and AM fungus from a distal compartment in which only the AM fungus developed. Using this split-plate method, Douds (2002) demonstrated that the AM fungus continued sporulation after medium from the distal compartment was partially replaced, and glucose was provided to the proximal compartment, which resulted in repeated harvests of the same Petri plate culture.

Different production systems have been derived from the basic ROC in Petri plates. For example, Tiwari and Adholeya (2003) cultured root organs and AM fungi in small containers, by which large-scale production was obtained (Adholeya et al. 2005). 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 a substrate (Jolicoeur 1998), and in a bioreactor containing solid (i.e., gelled medium) support elements (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 a 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 of Voets et al. (2005), the shoot developed outside the Petri plate while the roots and AM fungus were associated inside the Petri plates filled with a suitable gelled medium. In the system of 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 cultivation systems are then placed in growth chambers to provide controlled environmental conditions adequate for plant growth, while the Petri plates are protected from light exposure (Fig. 3a, b). Of these two methods, the one of Voets et al. (2005) is more laborious and prone to contamination but also appears more suitable for the production of AM fungal spores. In the study of Dupré de Boulois et al. (2006), ~1,600 spores were obtained in a period of 12 weeks in the root compartment of a bicompartmented Petri plate (half the size of the Petri plates used by Voets et al. 2005), while Voets et al. (2005) obtained on average 4,500 spores within the same period and more than 12,000 spores per Petri plate after 22 weeks of cultivation.

A derived plant in vitro production system has recently been detailed in a patent proposal (Declerck et al. 2009; Fig. 3c). In this system, each preinoculated 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 on the mycorrhizal roots.

Cultivation parameters

The AM fungi

Many different species and strains of AM fungi have been cultured in the ROC system. From the literature and culture collections, it is estimated that over 100 (S. Cranenbrouck, personal communication) different strains are maintained in vitro. For instance, the GINCO maintains at least 20 species and 30 strains, among which are representatives of the Glomeraceae and Gigasporaceae. However, only a few species are fast growers and colonizers that are able to produce many thousands in vitro propagules in a few months and thus nowadays represent a potential for large-scale production. Species from the *G. intraradices* clade/species complex (Table 3) are among the most productive so far. This species sensu lato is without any doubt the most frequently cultured AM fungus in vitro. Thus, we choose to compare the productivity of different in vitro cultivation systems based on *G. intraradices*. At this point, it is important to notice that a number of strains have been determined as *G. intraradices* in the past, but recent work has shown that most of them belong to a different clade in the *Glomus* GlGrAb grouping of Schwarzott et al. (2001) and are now known to be phylogenetically in the same clade as *Glomus irregulare* (Stockinger et al. 2009). The relationships among AM fungi in this group are not yet clear, and consequently, we continue to use the designation used in the literature.

The AM hosts

The in vitro large-scale production of *G. intraradices* spores was first attempted on ROC (e.g., Chabot et al. 1992; Declerck et al. 2001; Douds 2002; St-Arnaud et al. 1996) and later extended to plant systems (Voets et al. 2005, 2009). ROC is most often initiated on carrot (*Daucus carota* L.) roots. However, in the recent years, different excised roots, among which chicory (*Cichorium intybus* L.)

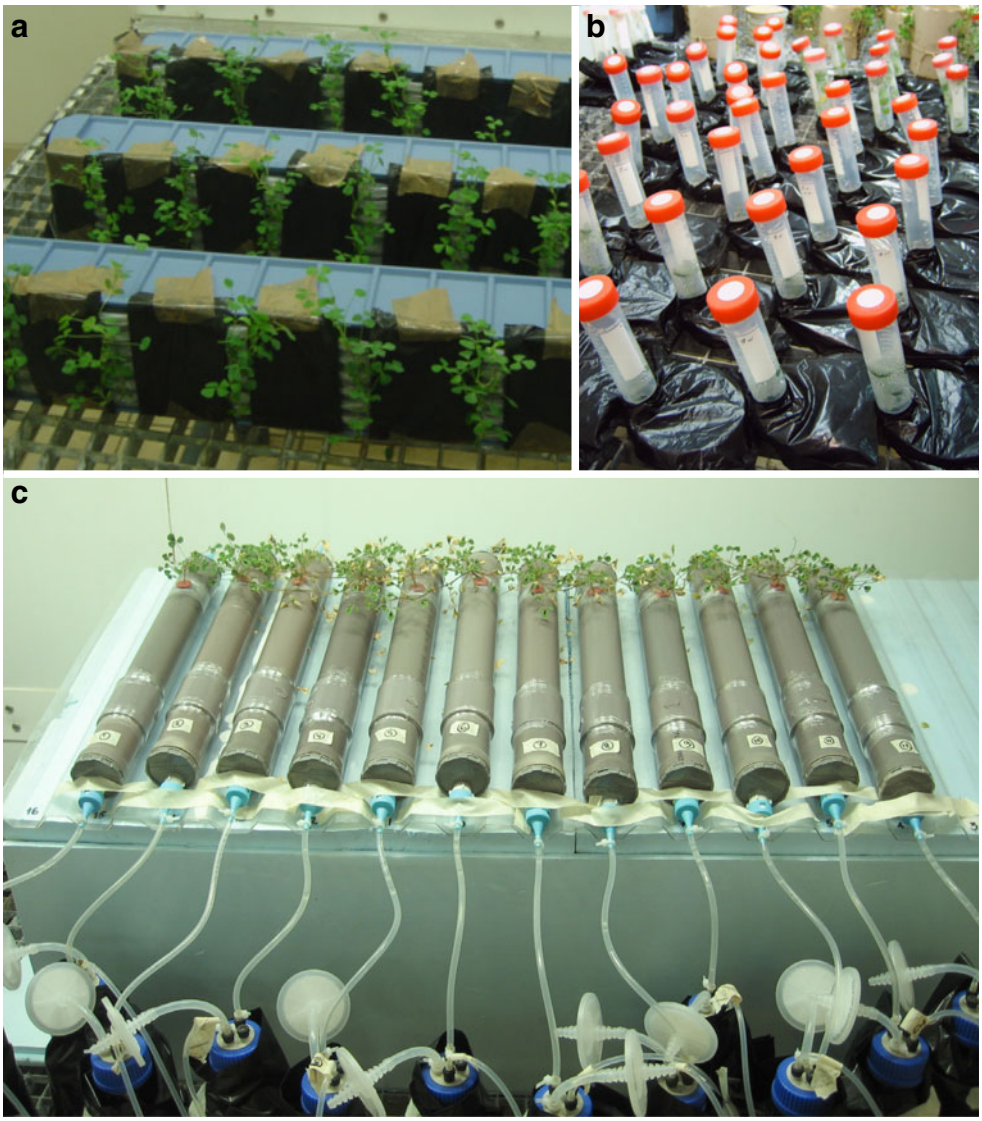

Fig. 3 In vitro plant systems for the production of AM fungi. AM fungi cultured on *Medicago truncatula* in a controlled growth chamber in various systems: **a** plant system as described in Voets et al. (2005); **b** plant system as described in Dupré de Boulois et al. (2006), who are kindly acknowledged for providing the picture; **c** hydroponics as described in Declerck et al. 2009 (WO/2009/090220). Thanks to H. Rouhier for providing the picture

Table 3 Average production of *G. intraradices* strains under in vitro cultivation systems

Authors	Host type	Host species	Fungal Code	Culture (weeks)	Method	Approximate production/unit
St-Arnaud et al. 1996	ROC	*Daucus carota*	DAOM 181602	16	Petri plate (split)	15,000/Petri plate
Jolicoeur et al. 1999	ROC	*Daucus carota*	Not coded	12	Airlift bioreactor	12,400 spores/bioreactor
Declerck et al. 2001	ROC	*Daucus carota*	MUCL 41833	15	Petri plate	8,400 spores/Petri plate
Douds 2002	ROC	*Daucus carota* (DC1)	DAOM 181602	28	Petri plate (split)[a]	65,000 spores/initial culture
Elsen et al. 2003	ROC	*Daucus carota*	MUCL 41833	18	Petri plate	16,800 spores/Petri plate
Gadkar et al. 2006	ROC	*Daucus carota* (DC2)	DAOM 181602	8	Container	No data on sporulation
Voets et al. 2009	Plant	*Medicago truncatula*	MUCL 41833	4	Petri plate[b]	7,300 spores/Petri plate
Voets et al. 2005	Plant	*Solanum tuberosum*	MUCL 43194	22	Petri plate	12,250 spores/Petri plate
IJdo et al. 2010	Plant	*Medicago truncatula*	MUCL 49410	12	Petri plate	7,200 spores/Petri plate

DC1, *2 Daucus carota* transformed roots, clone 1 and clone 2

[a] Medium was regularly renewed

[b] Plants were preinoculated with AM fungus

and barrel medic (*Medicago truncatula* Gaertn.), 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 could impact AM fungal spore production (Tiwari and Adholeya 2003).

Voets et al. (2005) used a potato plant (*Solanum tuberosum* L.) and obtained a production of ~12,000 spores in 12 weeks of cultivation. Recently, a spore production of ~50,000 per Petri plate (I.M. van Aarle, personal communication) was yielded with a bicompartmented plant system in which *G. intraradices* (MUCL 41833) associated to *Medicago truncatula* was grown during 14 weeks. Other hosts, such as banana (Koffi et al. 2009) and grapevine (Nogales, personal communication), were found suitable for association but less effective for the large-scale production of spores.

Cultivation media

Two culture media are frequently used to culture AM fungi on ROC: the minimal medium (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 micronutrients and macronutrients as well as vitamins and sucrose (see for description Cranenbrouck et al. 2005). Both media are solidified with a gelling agent such as PhytaGel (Sigma) and GelGro (MP Biomedicals). ROC systems in bioreactors (Jolicoeur et al. 1999) and containers (Gadkar et al. 2006) were performed with liquid M medium. In the compartmented culture system of 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).

The whole-plant in vitro culture systems were conducted on the MSR medium that lack sucrose and vitamins (Voets et al. 2005; Dupré de Boulois et al. 2006) and were similarly solidified with either Phytagel or Gelgro. The addition of vitamins and sucrose is not necessary in whole-plant in vitro culture systems as the autotrophic plant provides sugars obtained by photosynthesis and metabolizes vitamins that are needed for plant growth. The hydroponic in vitro plant-based system of Declerck et al. (2009, WO/2009/090220) also utilizes MSR without sugars and vitamins. Similar to in vivo NFT systems, a thin layer of liquid prevents aeration problems in this cultivation system.

Additional factors

Spore production of AM fungal species can be obtained in monocompartmented as well as bicompartmented Petri plates of various sizes. Optimal spore production of different AM fungal species and strains can vary in such systems. For example, spore production of *G. intraradices* was increased by the introduction of a sugar-free compartment (St-Arnaud et al. 1996) and *Gigasporaceae* species were often cultured in large (9×9 cm) square Petri plates (e.g., Diop et al. 1992).

Voets et al. (2009) described a method by which plantlet can be precolonized by an AM fungus. This precolonization step have resulted in higher sporulation of the AM fungus after the plantlet was transferred to a Petri plate containing fresh culture medium (Voets et al. 2009).

Advantages, disadvantages, and sectors of application

The most obvious advantage shared by all in vitro cultivation systems is the absence of undesirable microorganisms, which makes them more suitable for large-scale production of high-quality inoculum. While cross-contaminations by other AM fungi are evidently excluded (if the starter inoculum is monospecific), the 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. The possibility to follow 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) in vitro cultures.

As a disadvantage, the diversity (in terms of genera) of AM fungi that have been grown in vitro is lower than under pot cultivation systems. Another disadvantage of in vitro production is the costs associated with the production systems, requiring skilled technicians and laboratory equipments such as sterile work flows, controlled incubators for ROC, and growth chambers for plant systems.

An advantage restricted to hydroponic in vitro cultivation for both ROC and plant systems is the possibility of monitoring and regulating the culture medium. Other advantages of the ROC systems are the low requirements in the follow-up of the cultures. Once successfully initiated, the cultures may be maintained for periods exceeding 6 to 12 months without intervention. Cultures that use genetically modified root organs could however present a drawback for field application and thus for commercial mass production. The harvesting method of solid in vitro cultures involves a solubilization of the medium, while

roots from in vitro hydroponics can be cut and processed into a sheared-root inoculum.

The high production levels observed under in vitro plant cultivation, which might be a result of continuous C flow from the plant to AM fungus, is a great advantage for commercial large-scale production of AM fungal inoculum. As a disadvantage, in vitro plant cultures need regular additions of fresh culture medium to compensate nutrient and water loss, especially in the system of Voets et al. (2005), where water loss by transpiration from the plant leaves occurs. On the one hand, such additions increase the risks of contamination by manipulation, while on the other hand increased periods of access to water and nutrients to the plant–AM fungus association could favor sporulation.

The application of sterile produced 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 reintroduced endangered plant species. It could also be used to enhance the production of secondary metabolites used in the pharmaceutical industry (Kapoor et al. 2008).

Although in vitro cultivation methods are currently still costly, it seems likely that the criteria for quality control of AM fungal inoculum will result in the utilization of techniques that are able to reduce contamination risks. Cultivation by in vitro methods may then become an important method to meet future quality standards for commercial mass production.

Patents

Over 40 patents involving AM fungi have been deposited in the last decades. Many of them concerned the beneficial properties of AM fungi. Other patents focused on inoculum preparation (Sylvia and Jarstfer 1992), on formulation and applications (Cano and Bago 2007; Fernandez et al. 2006), and on cultivation methods either in substrate-free (Mosse and Thompson 1981) or under in vitro culture conditions (Declerck et al. 2009, WO/2009/090220; Fortin et al. 1996; Mugnier et al. 1986; Wang 2003).

The most recent patents on production methods involved in vitro cultures. Two patents are based on ROC (Fortin et al. 1996, US Pat. No. 5554530; Wang 2003, US Pat. No. 6759232), while another patent is based on autotrophic in vitro mycorrhizal plants (Declerck et al. 2009, WO/2009/090220). Fortin et al. (1996, US Pat. No. 5554530) cultivated the AM fungi in a compartmented bioreactor. Wang (2003, US Pat. No. 6759232) utilized a container in which the AM fungus was temporarily exposed to a nutrient solution. In the third patent on in vitro cultivation, deposited recently by Declerck et al. (2009, WO/2009/090220), premycorrhized in vitro produced plants (Voets et al. 2009) grew in a slightly inclined growth tube in which the nutrient solution was continuously flowing on the mycorrhized root system.

Conclusion and future directions

Numerous methods have been developed for decades for the large-scale production of AM fungi. It is tempting to extrapolate by saying that there are almost as many methods as there are laboratories working with AM fungi, since production is a prerequisite to fundamental research as well as for application purposes. The sectors of utilization widely vary from lab scale to large field, with production methods (and thus costs) and factors (e.g., host plant, AM fungi, substrate, nutrition) specifically custom-made.

Nowadays, large-scale production of AM fungi is not possible in the absence of a suitable host, and species cannot be identified in their active live stages (growing mycelium). As a consequence, quality control is often a problem, and tracing the organisms into the field to strictly relate positive effects to the inoculated AM fungus is nearly impossible. In addition, no clear criteria have been set for the quality control of commercial inoculum, but most likely, the legislation dealing with the application of beneficial microorganisms will become more drastic in the coming decades. Pringle et al. (2009) have already indicated the risks associated with the transport of AM fungi around the world and have detailed the problem that can arise with the introduction of exotic material. Furthermore, unwanted microorganisms associated with the inoculum might be introduced unnoticed (Schwartz et al. 2006; Pringle et al. 2009). While until now no invasive strains of AM fungi have been detected, the introduction of exotic species can be constrained by the use of indigenous AM fungal strains. The introduction of undesirable microorganisms can however only been avoided when the inoculum is produced under strict in vitro conditions.

It is expected that in the future new cultivation techniques will emerge, taking into consideration several of these aspects. As an example, the production of AM fungi on plants under in vitro conditions has been recently proposed (Voets et al. 2005) and extended to hydroponic systems (Declerck et al. 2009, WO/2009/090220). Following the preinoculation of a suitable autotrophic host plant in the system of Voets et al. (2009), a culture is transferred in a hydroponic cultivation system favoring the production of large quantities of propagules. Other in vitro methods might come up, which could involve spore production on callus or sporulation in sterile alginate beads or fully closed

hydroponic plant cultivation suitable for the production of AM fungi (upscaling the system of Dupré de Boulois et al. 2006). However, other relatively clean methods (e.g., in aeroponics) also have a strong developmental potential and could be further developed in the future. Biermann and Linderman (1983) already discussed that techniques such as sonication and gradient flotation as well as enzymatic methods could be developed to separate intraradical spores and vesicles from roots. With an AM fungus as the only endophyte, such intraradical propagules can serve as a high-quality inoculum.

We are at the beginning of an era where the utilization of beneficial microbes among which AM fungi will take more and more importance. The continued development of high-quality and low-cost inoculum methods can therefore be expected, which could lead to more new and advanced methods for AM fungal large-scale inoculum production to emerge in the close future.

Acknowledgements This research was funded by a Marie Curie Early-Stage Research Training Fellowship of the European Community's Sixth Framework Program, under contract number MEST CT-2005-021016. S. C. acknowledges the financial support from the Belgian Federal Office for Scientific, Technical, and Cultural affairs under contract number C4/00/001.

References

Adholeya A, Tiwari P, Singh R (2005) Large-scale production of arbuscular mycorrhizal fungi on root organs and inoculation strategies. In: Declerck S, Strullu DG, Fortin JA (eds) In vitro culture of mycorrhizas. Springer, Heidelberg, pp 315–338

Amijee F, Stribley DP, Tinker PB (1993) The development of endomycorrhizal root systems. VIII effects of soil phosphorus and fungal colonization on the concentration of soluble carbohydrates in roots. New Phytol 123:297–306

Avio L, Giovannetti M (1988) Vesicular–arbuscular mycorrhizal infection of lucerne roots in a cellulose-amended soil. Plant Soil 112:99–104

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

Biermann B, Linderman RG (1983) Use of vesicular–arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. New Phytol 95:97–105

Blanke V, Renker C, Wagner M, Füllner K, Held M, Kuhn AJ, Buscot F (2005) Nitrogen supply affects arbuscular mycorrhizal colonization of *Artemisia vulgaris* in a phosphate-polluted field site. New Phytol 166:981–992. doi:10.1111/j.1469-8137.2005.01374.x

Boisson-Dernier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker DG (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol Plant Microb Interact 14:695–700. doi:10.1094/MPMI.2001.14.6.695

Burrows RL, Pfleger FL (2002) Arbuscular mycorrhizal fungi respond to increasing plant diversity. Can J Bot 80:120–130

Cano CP, Bago A (2007) Aseptic mycorrhization inoculant and in vitro and ex vitro application methods. WO/2007/014974

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

Chen B, Christie P, Li X (2001) A modified glass bead compartment cultivation system for studies on nutrient and trace metal uptake by arbuscular mycorrhiza. Chemosphere 42:185–192. doi:10.1016/S0045-6535(00)00124-7

Cranenbrouck S, Voets L, Bivort C, Renard L, Strullu DG, Declerck D (2005) Methodologies for in vitro cultivation of arbuscular mycorrhizal fungi with root organs. In: Declerck S, Strullu DG, Fortin JA (eds) In vitro culture of mycorrhizas. Springer, Heidelberg, pp 341–375

Declerck S, Stullu 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

Declerck S, D'Or D, Cranenbrouck S, Le Boulengé E (2001) Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. Mycorrhiza 11:225–230

Declerck S, IJdo M, Fernandez K, Voets L, de la Providencia I (2009) Method and system for in vitro mass production of arbuscular mycorrhizal fungi. WO/2009/090220

Diop TA, Bécard G, Piche Y (1992) Long-term *in vitro* culture of an endomycorrhizal fungus *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. Symbiosis 12:249–259

Dodd JC, Arias I, Koomen I, Hayman DS (1990a) The management of populations of vesicular–arbuscular mycorrhizal fungi in acid-infertile soils of savanna ecosystem. I. The effect of pre-cropping and inoculation with VAM-fungi on plant growth and nutrition in the field. Plant Soil 122:229–240

Dodd JC, Arias I, Koomen I, Hayman DS (1990b) The management of populations of vesicular–arbuscular mycorrhizal fungi in acid-infertile soils of savanna ecosystem. II The effects of pre-crops on the spore populations of native and introduced VAM-fungi. Plant Soil 122:241–247

Douds DD Jr (1994) Relationship between hyphal and arbuscular colonization and sporulation in a mycorrhiza of *Paspalum notatum* Flugge. New Phytol 126:233–237. doi:10.1111/j.1469-8137.1994.tb03941.x

Douds DD Jr (2002) Increased spore production by *Glomus intraradices* in the split-plate monoxenic culture system by repeated harvest, gel replacement, and resupply of glucose to the mycorrhiza. Mycorrhiza 12:163–167. doi:10.1007/s00572-002-0174-9

Douds DD Jr, Schenck NC (1990a) Increased sporulation of vesicular–arbuscular mycorrhizal fungi by manipulation of nutrient regimens. Appl Environ Microbiol 56:413–418

Douds DD Jr, Schenck NC (1990b) Relationship of colonization and sporulation by VA mycorrhizal fungi to plant nutrient and carbohydrate contents. New Phytol 116:621–627

Douds DD Jr, Nagahashi G, Pfeffer PE, Kayser WM, Reider C (2005) On-farm production and utilization of arbuscular mycorrhizal fungus inoculum. Can J Plant Sci 85:15–21. doi:10.4141/P03-168

Douds DD Jr, Nagahashi G, Pfeffer PE, Reider C, Kayser WM (2006) On-farm production of AM fungus inoculum in mixtures of compost and vermiculite. Biores Tech 97:809–818. doi:10.1016/j.biortech.2005.04.015

Dugassa DG, Grunewaldt-Stöcker G, Schönbeck F (1995) Growth of *Glomus intraradices* and its effect on linseed (*Linum usitatissimum* L.) in hydroponic culture. Mycorrhiza 5:279–282. doi:10.1007/BF00204962

Dupré de Boulois H, Voets L, Delvaux B, Jakobsen I, Declerck S (2006) Transport of radiocaesium by arbuscular mycorrhizal

fungi to *Medicago truncatula* under in vitro conditions. Environ Microbiol 8:1926–1934. doi:10.1111/j.1462-2920.2006.01070.x

Egerton-Warburton LM, Johnson NC, Allen EB (2007) Mycorrhizal community dynamics following nitrogen fertilization: a cross-site test in five grasslands. Ecol Monogr 77:527–544

Elmes RP, Mosse B (1984) Vesicular-arbuscular endomycorrhizal inoculum production II Experiments with maize (*Zea mays*) and other hosts in nutrient flow culture. Can J Bot 62:1531–1536

Elsen A, Declerck S, De Waele D (2003) Use of root organ cultures to investigate the interaction between *Glomus intraradices* and *Pratylenchus coffeae*. Appl Environ Microbiol 69:4308–4311. doi:10.1128/AEM.69.7.4308-4311.2003

Feldmann F, Grotkass C (2002) Directed inoculum production—shall we be able to design AMF populations to achieve predictable symbiotic effectiveness? In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture: from genes to bioproducts. Birkhauser, Basel, pp 261–279

Fernandez F, Dellamico JM, Perez Y (2006) Inoculum mycorhizogene liquide. WO/2006/060968

Fontaine J, Grandmougin-Ferjani A, Glorian V, Durand R (2004) 24-Methyl:methylene sterols increase in monoxenic roots after colonization by arbuscular mycorrhizal fungi. New Phytol 163:159–167. doi:10.1111/j.1469-8137.2004.01075.x

Fortin JA, St-Arnaud M, Hamel C, Chaverie C, Jolicoeur M (1996) Aseptic in vitro endomycorrhizal spore mass production. US Pat. No. 5554530

Furlan V, Fortin JA (1977) Effects of light intensity on the formation of vesicular–arbuscular endomycorrhizas on *Allium cepa* by *Gigaspora calospora*. New Phytol 79:335–340

Gadkar V, Driver JD, Rillig MC (2006) A novel in vitro cultivation system to produce and isolate soluble factors released from hyphae of arbuscular mycorrhizal fungi. Biotechnol Lett 28:1071–1076. doi:10.1007/s10529-006-9055-1

Gaur A, Adholeya A (2000) Effects of the particle size of soil-less substrates upon AM fungus inoculum production. Mycorrhiza 10:43–48. doi:10.1007/s005720050286

Gaur A, Adholeya A (2002) Arbuscular-mycorrhizal inoculation of five tropical fodder crops and inoculum production in marginal soil amended with organic matter. Biol Fertil Soils 35:214–218. doi:10.1007/s00374-002-0457-5

Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (2002) Mycorrhizal technology in agriculture: from genes to bioproducts. Birkhauser, Basel

Gryndler M, Jansa J, Hršelová H, Chvátalové I, Vosátka M (2003) Chitin stimulates development and sporulation of arbuscular mycorrhizal fungi. Appl Soil Ecol 22:283–287. doi:10.1016/S0929-1393(02)00154-3

Gryndler M, Hršelová H, Sudová R, Gryndlerová H, Řezáčová V, Merhautová V (2005) Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimulated by humic substances. Mycorrhiza 15:483–488. doi:10.1007/s00572-005-0352-7

Hart MM, Reader RJ (2002) Does percent root length colonization and soil hyphal length reflect the extent of colonization for all AMF? Mycorrhiza 12:297–301. doi:10.1007/s00572-002-0186-5

Hawkins HJ, George E (1997) Hydroponic culture of the mycorrhizal fungus *Glomus mosseae* with *Linum usitatissimum* L., *Sorghum bicolor* L. and *Triticum aestivum* L. Plant Soil 196:143–149. doi:10.1023/A:1004271417469

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Stat Circ 347:1–32

Hung LLL, Sylvia DM (1988) Production of vesicular–arbuscular mycorrhizal fungus inoculum in aeroponic culture. Appl Environ Microbiol 54:353–357

IJdo M, SchtickzelleN, Cranenbrouck S, Declerck S (2010) Do arbuscular mycorrhizal fungi with contrasting life history strategies differ in their responses to repeated defoliation? FEMS Microbiol Ecol 72:114-122. doi:10.1111/j.1574-6941.2009.00829.x

Jarstfer AG, Sylvia DM (1995) Aeroponic culture of VAM fungi. In: Varma A, Hock B (eds) Mycorrhiza. Springer, Heidelberg, pp 427–441

Jarstfer AG, Sylvia DM (1997) Isolation, culture and detection of arbuscular mycorrhizal fungi. In: Hurst CJ (ed) Manual of environmental microbiology. American Society of Microbiology, Washington, pp 406–412

Jarstfer AG, Farmer-Koppenol SDM, Sylvia DM (1988) Tissue magnesium and calcium affect arbuscular mycorrhiza development and fungal reproduction. Mycorrhiza 7:237–342. doi:10.1007/s005720050186

Johansson JF, Paul LR, Finlay RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol Ecol 48:1–13. doi:10.1016/j.femsec.2003.11.012

Jolicoeur M (1998) Optimisation d'un procédé de production de champignons endomycorhiziens en bioréacteur. Dissertation, École Polytechnique de Montréal

Jolicoeur M, Williams RD, Chavarie C, Fortin JA, Archambault J (1999) Production of *Glomus intraradices* propagules, an arbuscular mycorrhizal fungus, in an airlift bioreactor. Biotechnol Bioeng 63:224–232

Kapoor R, Sharma D, Bhatnagar AK (2008) Arbuscular mycorrhizae in micropropagation systems and their potential applications. Sci Hortic 116:227–239. doi:10.1016/j.scienta.2008.02.002

Klironomos JM, Hart NN (2002) Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza 12:181–184. doi:10.1007/s00572-002-0169-6

Koffi MC, de la Providencia IE, Elsen A, Declerck S (2009) Development of an in vitro culture system adapted to banana mycorrhization. Afr J Biotechnol 8:2750–2756

Krüger M, Stockinger H, Krüger C, Schüßler A (2009) DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183:212–223

Lee YJ, George E (2005) Development of a nutrient film technique culture system for arbuscular mycorrhizal plants. HortScience 40:378–380

Ma N, Yokoyama K, Marumoto T (2007) Effect of peat on mycorrhizal colonization and effectiveness of the arbuscular mycorrhizal fungus *Gigaspora margarita*. Soil Sci Plant Nutr 53:744–752. doi:10.1111/j.1747-0765.2007.00204.x

Medeiros CAB, Clark RB, Ellis JR (1994) Growth and nutrient uptake of sorghum cultivated with vesicular–arbuscular mycorrhiza isolates at varying pH. Mycorrhiza 4:185–191. doi:10.1007/BF00206778

Millner PD, Kitt DG (1992) The Beltsville method for soilless production of vesicular–arbuscular mycorrhizal fungi. Mycorrhiza 2:9–15. doi:10.1007/BF00206278

Mohammad A, Khan AG, Kuek C (2000) Improved aeroponic culture of inocula of arbuscular mycorrhizal fungi. Mycorrhiza 9:337–339. doi:10.1007/s005720050278

Mohammad A, Mirta B, Khan AG (2004) Effects of sheared-root inoculum of *Glomus intraradices* on wheat grown at different phosphorus levels in the field. Agric Ecosyst Environ 103:245–249. doi:10.1016/j.agee.2003.09.017

Mosse B (1959) The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular–arbuscular mycorrhiza. Trans Br Mycol Soc 42:273–286

Mosse B (1962) The establishment of vesicular–arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mosse B, Hepper CM (1975) Vesicular-arbuscular infections in root–organ cultures. Physiol Plant Pathol 5:215–233

Mosse M, Thompson JP (1981) Production of mycorrhizal fungi. US Pat. No 4294037

Mugnier J, Mosse B (1987) Vesicular–arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Mugnier J, Jung G, Prioul J-L (1986) Method of producing endomycorrhizian fungi with arbuscules and vesicles in vitro. US Pat. No 4599312

Neumann E, George E (2005) Extraction of extraradical arbuscular mycorrhizal mycelium from compartments filled with soil and glass beads. Mycorrhiza 15:533–537. doi:10.1007/s00572-005-0361-6

Plenchette C, Furlan V, Fortin JA (1982) Effects of different endomycorrhizal fungi on 5 host plants grown on calcined montmorillonite clay. J Am Soc Hortic Sci 107:535–538

Pringle A, Bever JD, Gardes M, Parrent JL, Rillig MC, Klironomos JN (2009) Mycorrhizal symbioses and plant invasions. Annu Rev Ecol Evol Syst 40:699–715. doi:10.1146/annurev.ecolsys.39.110707.173454

Saif SR (1983) The influence of soil aeration on the efficiency of vesicular-arbuscular mycorrhizas. II Effect of soil oxygen on growth and mineral uptake in *Eupatorium odoratum* L., *Sorghum bicolor* (L.) Moench and *Guizotia abyssinica* (L.f.) Cass. inoculated with vesicular–arbuscular mycorrhizal fungi. New Phytol 95:405–417

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515. doi:10.1111/j.1461-0248.2006.009.10.x

Schwarzott D, Walker C, Schüßler A (2001) *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. Mol Phylogenet Evol 21:190–197. doi:10.1006/mpev.2001.1007

Smith SE, Read DJ (eds) (2008) Mycorrhizal symbiosis, 3rd edn. Academic, 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

Stockinger H, Walker C, Schüßler A (2009) '*Glomus intraradices* DAOM197198', a model fungus in arbuscular mycorrhizal research, is not *Glomus intraradices*. New Phytol 183:1176–1187. doi:10.1111/j.1469-8137.2009.02874.x

Stockinger H, Krüger M, Schüßler A (2010) DNA barcoding of arbuscular mycorrhizal fungi. New Phytol 187:461–474

Struble JE, Skipper HD (1988) Vesicular–arbuscular mycorrhizal fungal spore production as influenced by plant species. Plant Soil 109:277–280

Strullu DG, Romand C (1986) Méthode d'obtention d'endomycorhizes a vesicules et arbuscules en condition axeniques. C R de 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

Sylvia DM, Jarstfer AG (1992) Sheared roots as a VA-mycorrhizal inoculum and methods for enhancing growth. US Pat. No 5096481

Tajini F, Suriyakup P, Vailhe H, Jansa J, Drevon JJ (2009) Assess suitability of hydroaeroponic culture to establish tripartite symbiosis between different AMF species, beans, and rhizobia. BMC Plant Biol 9:73. doi:10.1186/1471-2229-9-73

Tiwari P, Adholeya A (2003) Host dependent differential spread of *Glomus intraradices* on various Ri T-DNA transformed root in vitro. Mycol Prog 2:171–177. doi:10.1007/s11557-006-0055-2

Voets L, Dupré de Boulois H, Renard L, Strullu DG, Declerck S (2005) Development of an autotrophic culture system for the in vitro mycorrhization of potato plantlets. FEMS Microbiol Lett 248:111–118. doi:10.1016/j.femsle.2005.05.025

Voets L, de la Providencia IE, Fernandez K, IJdo M, Cranenbrouck S, Declerck S (2009) Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under in vitro conditions. Mycorrhiza 19:347–356. doi:10.1007/s00572-009-0233-6

Walker C, Vestberg M (1994) A simple and inexpensive method for producing and maintaining closed pot cultures of arbuscular mycorrhizal fungi. Agri Sci Finland 3:233–240

Wang WK (2003) Method of facilitating mass production and sporulation of arbuscular mycorrhizal fungi aseptic. US Pat. No. 6759232

White JA, Charvat I (1999) The mycorrhizal status of an emergent aquatic, *Lythrum salicaria* L., at different levels of phosphorus availability. Mycorrhiza 9:191–197. doi:10.1007/s005720050266

Wu CG, Liu YS, Hung LL (1995) Spore development of *Entrophospora kentinensis* in an aeroponic system. Mycologia 87:582–587

Zobel RW, Del Tredici P, Torrey JG (1976) Method for growing plants aeroponically. Plant Physiol 57:344–346