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TECHNICAL NOTE

Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under in vitro conditions

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Abstract Actively growing extraradical hyphae extending from mycorrhizal plants are an important source of inoculum in soils which has seldom been considered in vitro to inoculate young plantlets. Seedlings of *Medicago truncatula* were grown in vitro in the extraradical mycelium network extending from mycorrhizal plants. After 3, 6, 9, 12, and 15 days of contact with the mycelium, half of the seedlings were harvested and analyzed for root colonization. The other half was carefully transplanted in vitro on a suitable growth medium and mycelium growth and spore production were evaluated for 4 weeks. Seedlings were readily colonized after 3 days of contact with the mycelium. Starting from 6 days of contact, the newly colonized seedlings were able to reproduce the fungal life cycle, with the production of thousands of spores within 4 weeks. The fast mycorrhization process developed here opens the door to a broad range of in vitro studies for which either homogenous highly colonized seedlings or mass-produced in vitro inoculum is necessary.

Keywords Arbuscular mycorrhizal (AM) fungi · Seedling · Root organ culture · Mycelium network · *Medicago truncatula*

Liesbeth Voets and Ivan Enrique de la Providencia contributed equally to this work.

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Introduction

Root organ cultures (ROC) of Ri T-DNA-transformed carrots associated to arbuscular mycorrhizal (AM) fungi (Bécard and Fortin 1988) have been widely used to explore in vitro most areas of AM fungal biology (as reviewed in Declerck et al. 2005). By permitting nondestructive long-term observations, ROC provide access to detailed information that is difficult to obtain through other means, e.g., the mycelium architecture (Bago et al. 1998; de la Providencia et al. 2005; Voets et al. 2006), the dynamics of fungal development (Declerck et al. 1996, 2001; de Souza and Declerck 2003), and the spore ontogeny (de Souza and Berbara 1999; Pawlowska et al. 1999; Declerck et al. 2000; Dalpé and Declerck 2002; de Souza et al. 2005). The physical partition in two distinct compartments (St-Arnaud et al. 1996) further separates the extraradical mycelium on one side from the excised mycorrhizal root on the other side. This improvement allowed to study, e.g., the lipid metabolism (Bago et al. 2002) and the transport of elements to roots (Rufyikiri et al. 2002; Nielsen et al. 2002; Dupré de Boulois et al. 2005) and to isolate microbial-free AM fungal mycelium and spores for molecular analysis

(Koch et al. 2004; Pawlowska and Taylor 2004). However, the absence of a true sink (i.e., a photosynthetic active shoot) represents a limitation for other studies such as, e.g., transport of elements to and from the shoot (i.e., phosphorus from fungus to plant shoot and carbohydrate from plant shoot to fungus), water stress, and impact of CO_2 elevation on plant–fungus relationship.

Several authors have proposed approaches for the in vitro mycorrhization of autotrophic plants. Pioneer work was conducted by Hepper (1981) with *Trifolium* sp. associated in vitro to *Glomus mosseae* and *Glomus caledonium* using agar, paper, or glass as support. This author was able to follow the early stage of root penetration. Later on, Elmeskaoui et al. (1995) developed a tripartite culture system associating in a single container, mycorrhizal carrot roots with strawberry. This system was used to investigate water stress (Hernandez-Sebastia et al. 1999, 2000) and the effect of CO_2 on the mycorrhizal formation (Louche-Tessandier et al. 1999). However, few other applications derived from this system, possibly because of the presence of three organisms and the complexity of the setup. Recently, Voets et al. (2005) and Dupré de Boulois et al. (2006) developed two new in vitro autotrophic culture systems associating in vitro produced AM fungal spores with *Solanum tuberosum* and *Medicago truncatula*, respectively. Mass production of spores (i.e., approx. 12,000 in 22 weeks) was obtained with potato (Voets et al. 2005), and the transport of C from the shoot of *M. truncatula* to the fungus (Voets et al. 2008) and of P and Cs from a root-free labeled compartment to the shoot via the extraradical mycelium (Dupré de Boulois et al. 2006) was demonstrated. However, with both systems, high-level colonization took several weeks, and produced nearly full-grown mycorrhizal plants before trialing. One reason could be attributed to the time needed for the fungal spore inoculum to germinate, grow, contact, and colonize the root. It is obvious that any system allowing the fast and homogenous mycorrhization of seedlings within a few days is highly desirable.

Under in vitro conditions, excised roots or autotrophic plants are usually inoculated with isolated spores or root pieces showing mycelial re-growth, or a combination of both (see review in Declerck et al. 2005). In nature, also the AM fungal mycelium growing from colonized roots represents an important source of inoculum for the colonization of neighboring plants, due to the several hyphae apexes ramifying from the colony (Friese and Allen 1991). Several authors (Friese and Allen 1991; Giovannetti et al. 2004) demonstrated the capacity of such hyphae to interlink plants, creating an indefinite web of mycelium. Recently, Cano et al. (2008) successfully obtained freshly colonized excised roots in vitro using bi-compartmental Petri plates with a mycorrhizal donor root organ developing in a root compartment. The colonization percentage was 25% after 1 week and remained constant thereafter. However, to our knowledge, no in vitro system has ever considered the AM fungus arising from an autotrophic donor plant as a source of inoculum to colonize autotrophic receiver seedlings in vitro.

In the present study, we developed an in vitro mycorrhization system adapted to seedlings, by using the symbiotic phase of the fungus as inoculum. The mycorrhization system is detailed as well as the capacity of the new mycorrhizal seedlings to reproduce the fungal life cycle and mass produce mycelium and spores.

Materials and methods

Biological material

A strain of *Glomus intraradices* Schenck and Smith MUCL 41833 grown in ROC with Ri T-DNA-transformed carrot (*Daucus carota* L.) roots was purchased from GINCO (BCCM/MUCL, Microbiology unit, Université catholique de Louvain, Belgium, http://www.mbla.ucl.ac.be/ginco-bel). The strain was provided in a Petri plate (90 mm diam.) on the modified Strullu Romand (MSR) medium (Declerck et al. 1998 modified from Strullu and Romand 1986) and solidified with 3 g l^{-1} GelGro™ (ICN, Biomedicals, Inc., Irvine, CA, USA). The strain was subsequently sub-cultured following the method described in Cranenbrouck et al. (2005). Several thousand spores were obtained in a period of 5 months.

Seeds of *M. truncatula* Gaertn. cv. Jemalong strain A 17 (SARDI, Australia) were surface-sterilized by immersion in sodium hypochlorite (8% active chloride) for 10 min, rinsed in deionized sterile (121°C for 15 min) water and germinated in Petri plates (90 mm diam.) filled with 35 ml MSR medium lacking sucrose and vitamins, and solidified with 3 g l^{-1} GelGro™. Twenty-five seeds were plated per Petri plate. The Petri plates were incubated in the dark at 27°C. Seeds germinated within 1–2 days and seedlings were ready to use after 4 days.

Experimental design: the mycelium donor plant in vitro culture system

A system was designed to demonstrate (1) the capacity of an AM extraradical mycelium extending from an autotrophic donor plant to colonize seedlings and (2) the ability of these colonized seedlings to reproduce the fungal life cycle. The system was adapted from the autotrophic in vitro culture system developed by Voets et al. (2005). Briefly, bi-compartmental Petri plates (90 mm diam.) were used to physically separate a root compartment (RC) from a hyphal

compartment (HC). A small opening (±2 mm diam.) was made in the base and the lid of the Petri plates at the side of the RC using the tip of a heated forceps. Both compartments of the Petri plates were filled with 20 ml of MSR medium lacking sucrose and vitamins, solidified with 4 g l^{-1} Gel Gro™. Both compartments of the Petri plates were separately filled under an inclination of 4°, allowing the culture medium in both compartments to reach the top of the partition wall, while the level of the medium remained low at the side of the small opening of the RC.

Four-day-old *M. truncatula* seedlings were transferred to the RC (Fig. 1a), with the roots placed on the surface of the medium and the shoot extending outside the plate. Approximately 100 spores of *G. intraradices*, extracted from the ROC following solubilization of the MSR medium (Doner and Bécard 1991), were inoculated in the vicinity of the roots (Fig. 1a). The Petri plates were then sealed with Parafilm (Pechiney, Plastic Packaging, Chicago, IL 60631, USA) and the openings carefully plastered with sterilized (121°C for 15 min) silicon grease (VWR International, Belgium) to avoid contaminations. Petri plates were subsequently wrapped with opaque plastic bags to keep the AM fungi and *M. truncatula* roots in the dark, while the shoots developed under light conditions. The systems were transferred to a growth chamber under controlled conditions (22/18°C (day/night), 70% relative humidity, photoperiod of 16 h day^{-1} and an average photosynthetic photon flux of 225 µmol m^{-2} s^{-1}).

Starting from week 3, 10 ml MSR medium, lacking sucrose and vitamins, and cooled to 40°C in a water bath, was added weekly to the RC. This medium was added to provide the plants with nutrients and to maintain the

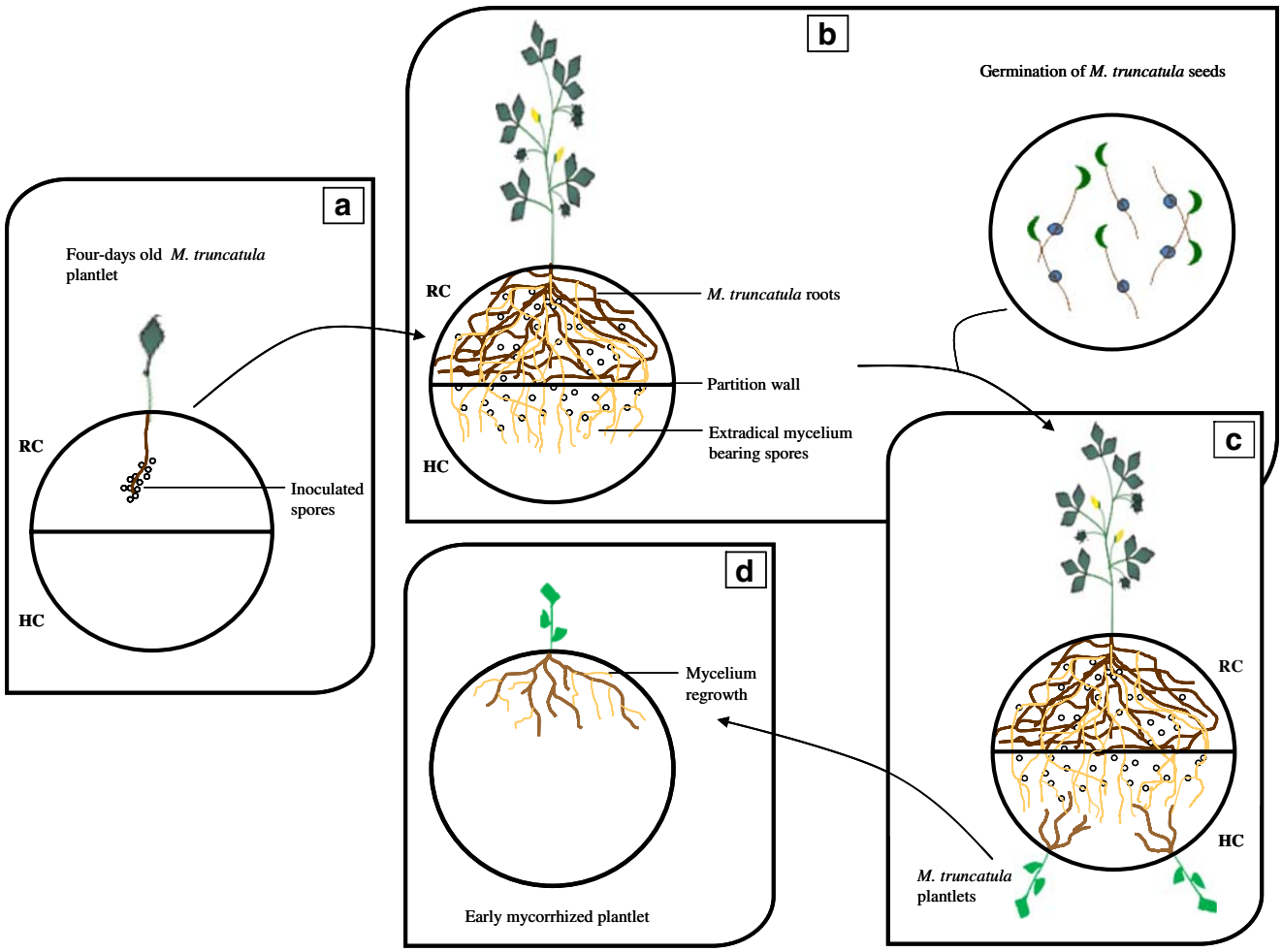

Fig. 1 Schematic representation of the mycelium donor plant (MDP) in vitro culture system developed in bi-compartmental Petri plates for fast mycorrhization of seedlings. RC (root compartment), HC (hyphal compartment). **a** *Medicago truncatula* plantlet associated with *Glomus intraradices* in an in vitro culture system on the modified Strullu Romand (MSR) medium, lacking sucrose and vitamins. **b** *M. truncatula* plant after 8 weeks of association in the MDP in vitro culture system, in which a profuse extraradical mycelium bearing spores was produced in the HC. **c** Two 4-day-old *M. truncatula* seedlings were inserted in the system to obtain fast mycorrhization of the roots. **d** The mycorrhized seedlings were grown on MSR medium to analyze the re-growth of the AM fungus

medium at the level of the top of the partition wall, facilitating hyphae to cross from the RC to the HC. Roots that passed the partition wall were trimmed.

After a period of 8 weeks, four parameters were estimated: (1) the number of hyphae crossing the partition wall from RC to HC, (2) the surface of extraradical mycelium covering the HC, (3) the mycelium length in the HC, and (4) the number of spores produced in the HC (Fig. 1b). The surface of the extraradical mycelium covering the HC was estimated using a transparent plastic sheet placed on the bottom of the Petri plates and the surface area in the HC covered by the extraradical mycelium traced on this sheet. The surface was then estimated as compared to the whole surface of the HC. The extraradical mycelium length and number of spores developed in the HC were estimated following the method detailed in Voets et al. (2005). These data, together with the surface of the HC covered by the extraradical mycelium, were obtained under a dissecting microscope (Olympus SZ40, Olympus Optical GmbH, Germany) at ×10–40 magnifications. The hyphae crossing the partition wall were counted under a compound bright-field light microscope (Olympus BH-2, Olympus Optical GmbH, Germany) at ×125 and ×250 magnifications.

After this 8-week period, two new small openings (±2 mm diam. each), separated 4.5 cm from each other, were made in the base and the lid of the Petri plates, at the side of the HC. The Petri plates were slightly opened at this side and two small openings were made using the tip of a heated forceps. One 4-day-old *M. truncatula* seedling was inserted in each opening following the same methodology as described above (Fig. 1c). Petri plates were then sealed carefully and incubated under the same conditions as described above.

One replicate consisted of a mycelium donor plant (MDP) in vitro culture system developed in a bi-compartmental Petri plate, with an 8-week-old mycorrhizal *M. truncatula* plant in the RC and two 4-day-old nonmycorrhizal *M. truncatula* seedlings in the HC, with their roots in contact with the dense extraradical mycelium covering the HC. Thirty MDP systems were randomly divided into five groups, with six replicates per group. For each MDP in vitro culture system, one seedling from the HC was used to estimate the root colonization dynamics while the other seedling was used to evaluate the spore production and extraradical mycelium development arising from *M. truncatula* seedlings (Fig. 1d). These parameters were evaluated after 3, 6, 9, 12, and 15 days of contact of the seedling with the extraradical mycelium in the HC.

Time course root colonization of *M. truncatula* seedlings grown in an extraradical mycelium network

After 3, 6, 9, 12, and 15 days of contact with the mycelium network in the HC, one *M. truncatula* seedling per MDP in vitro culture system (i.e., six replicates) was harvested. The intraradical root colonization was estimated after staining. Roots of each seedling were cleared in 10% KOH at 80°C for 10 min, rinsed with distilled water, and stained with a blue ink solution (1% HCl with 1% blue ink (Parker); Vierheilig et al. 1998) at 80°C for 15 min. Thirty randomly selected root pieces (10 mm length) were mounted on microscope slides and examined for AM fungal colonization under a compound bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at ×50 or ×125 magnifications. Frequency (%*F*) and intensity (%*I*) of root colonization were estimated using the method of Declerck et al. (1996). In addition, vesicles were counted in each root fragment.

Spore production and extraradical mycelium development from *M. truncatula* seedlings grown for increased time in mycelium networks

After 3, 6, 9, 12, and 15 days of contact with the mycelium network in the HC, the second seedling developing in each MDP in vitro culture system (i.e., six replicates) was carefully removed and subsequently transferred to mono-compartmental Petri plates (90 mm diam.), which contained the same MSR medium lacking sucrose and vitamins as described above. Spore production and extraradical mycelium development was monitored weekly for 4 weeks, following the methodology detailed in Voets et al. (2005). Stem length, number of leaves, and root length of each seedling were measured weekly. Root length was estimated following the same methodology as hyphal length.

Statistical analyses

Percentages of root colonization (%*F* and %*I*) were arcsine $\sqrt{(x/100)}$-transformed and tested for normal distribution. Data were subsequently subjected to the Tukey's honest significant difference (HSD) test in order to identify the significant differences ($P \leq 0.05$) between colonization times. Data analysis was performed with the statistical package Statistica (StatSoft Inc 2001). Data of spore production and hyphal growth for the different colonization times (3 to 15 days) at the different weeks after association were analyzed using a Kruskal–Wallis test followed by Wilcoxon tests.

Results

Development of plants and fungi in the MDP in vitro culture systems

Spores started to germinate within 10 days after inoculation, and the first contact points between hyphal tips and

roots were established shortly thereafter. From this moment, the AM extraradical mycelium developed profusely in the RC. The first hyphae that crossed the partition wall were observed at week 6. Once the hyphae reached the HC, a dense mycelium was produced, colonizing this compartment at a growth rate of 5 mm day^{-1} (data not shown). After 8 weeks, 97±1% of the surface of the HC was covered by a dense mycelium (Fig. 2a, b). The number of hyphae that crossed the partition wall separating the RC from the HC was high (Fig. 2c), i.e., 335±35, and an average of 4,109±207 cm of hyphae was formed in the HC, bearing 7,442±658 spores. Thirty Petri plates were selected and randomly divided into five groups of six plates. No statistical differences were noted between the five groups of Petri plates, for the surface covered by the mycelium in the HC (p value=0.063), the number of hyphae crossing the partition wall from RC to HC (p value=0.086), the hyphal length in the HC (p value=0.135), and the number of spores produced in the HC (p value=0.179). The plants showed normal growth with ramified stems and continuous production of new leaves.

After insertion of the 4-day-old plantlets in the extraradical mycorrhizal network, plantlets continued growth with the development of new roots and leaves. At contact between the hyphae and the root system of the seedlings, multiple appressoria were formed from root apices to subapical zones and behind.

Time course root colonization of *M. truncatula* seedlings grown in an extraradical mycelium network

After 3, 6, 9, 12, and 15 days of contact with the seedlings in the mycelium network, one seedling per MDP in vitro culture system was harvested and the intraradical colonization estimated (Fig. 3). No significant differences were found for the frequency of mycorrhization (i.e., the number of root fragments bearing mycorrhizal structures) between the different colonization times. However, the intensity of

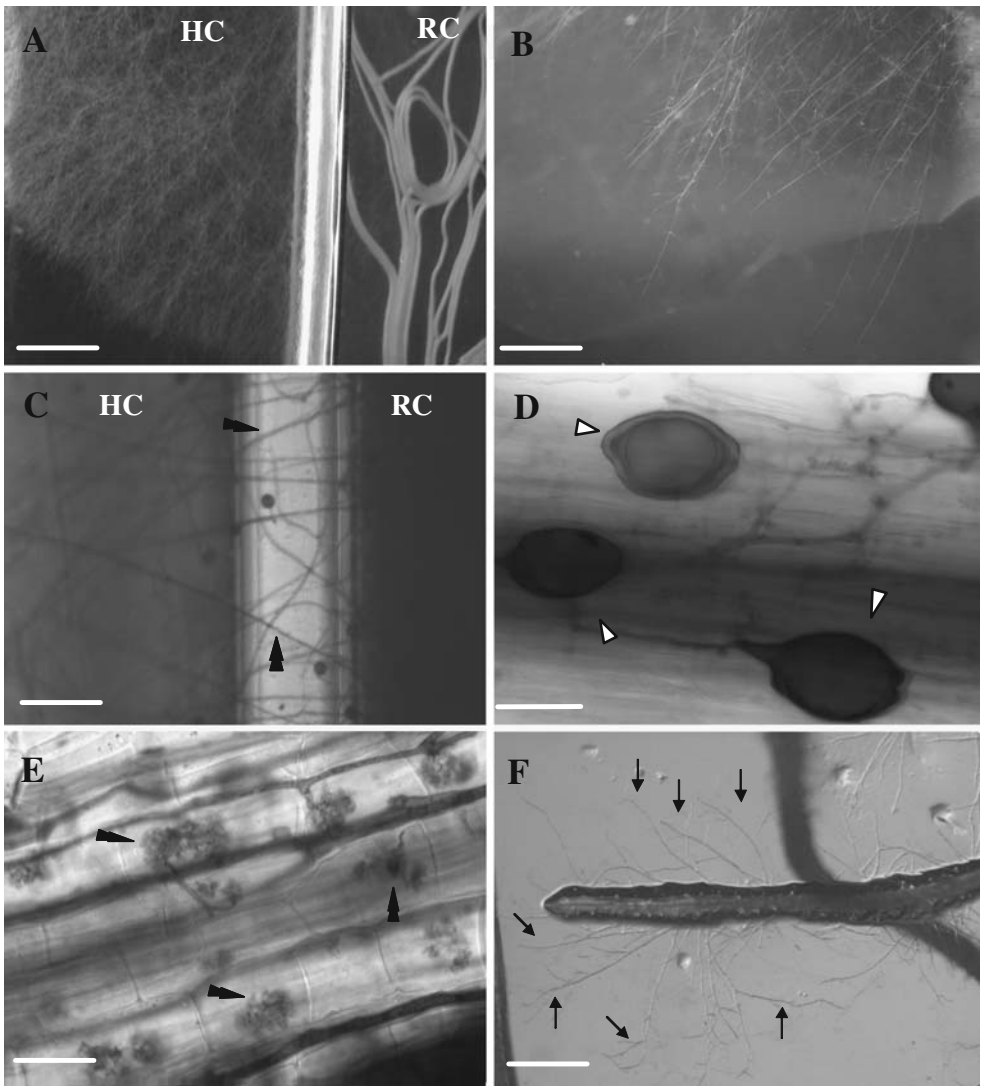

Fig. 2 Growth patterns obtained with the mycelium donor plant (MDP) in vitro culture system. **a** Dense extraradical mycelium front developing in the hyphal compartment (*HC*) and coming from the root compartment (*RC*), scale bar=350 µm. **b** Close up of the hyphal front showing numerous hyphae extending on the surface of the HC, scale bar=200 µm. **c** Numerous hyphae crossing the partition wall separating the RC from the HC and extending in the HC (*double black arrowheads*), scale bar 100 µm. **d** Vesicles in the roots of the receiver plants developing in the HC (*white arrowheads*), scale bar=50 µm. **e** Arbuscules in the roots of the receiver plant developing in the HC (*double black arrowheads*), scale bar=80 µm. **f** Hyphal re-growth from hyphae attached to the roots as well as from new emerging hyphae (*black arrows*), scale bar=200 µm

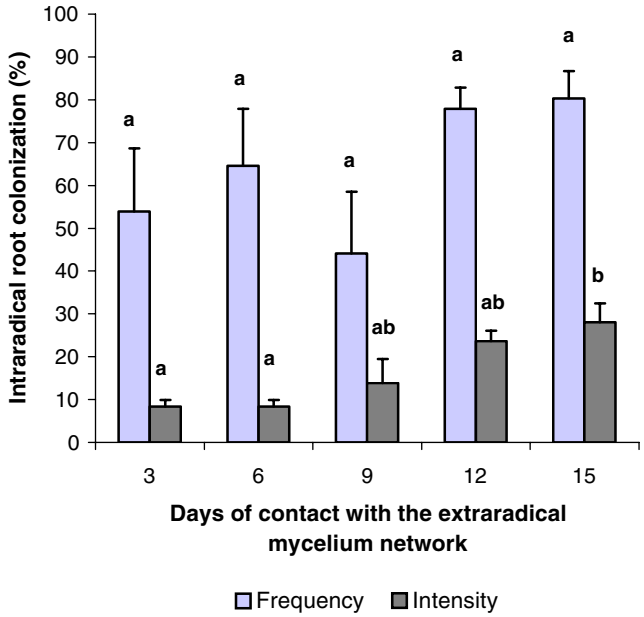

Fig. 3 Intraradical root colonization of mycorrhizal *Medicago truncatula* seedlings after 3, 6, 9, 12, and 15 days of growth in a mycelium network extending from a mature *M. truncatula* plant. Histograms represent means of six replicates +SE. Within frequency and intensity percentage, *values followed by a different letter* differ significantly at $P<0.05$ (Tukey's HSD)

mycorrhization (i.e., the percentage of the cortex colonized by the AM fungus) was significantly higher after 15 days of contact with the extraradical mycelium network, as compared to the seedlings grown in contact with the extraradical mycelium network for a period of 3 and 6 days (Fig. 3). The seedlings that were grown for 9 and 12 days in contact with the extraradical mycelium network had intermediate values.

The seedlings in contact for 3 days with the extraradical mycelium network presented only hyphae in their roots. No arbuscules or vesicles were detected. In the roots of the seedlings that were grown in the extraradical mycelium network for 6 days, vesicles were sparsely present. An average of 0.43±0.32 vesicles/cm root was counted. The number of vesicles increased slightly on day 9 (1.15±0.32 vesicles/cm root) and day 12 (1.35±0.25 vesicles/cm root) and were significantly more abundant on day 15 (7.10±1.66 vesicles/cm root; Fig. 2d). Arbuscules were also observed on day 6 and their abundance increased with time, although not formerly estimated (Fig. 2e).

Spore production and extraradical mycelium development from *M. truncatula* seedlings grown in mycelium networks

With the exception of the seedlings in contact with the extraradical mycelium network for 3 days, all the seedlings belonging to the other treatments showed hyphal re-growth and spore production 48 h after the transfer of the seedlings onto fresh MSR medium. Hyphal re-growth was observed starting from intact and injured hyphae attached to the roots as well as from new emerging hyphae (Fig. 2f). During the whole experiment, seedlings that were grown in contact with the extraradical mycelium network for 12 and 15 days produced at least three times as much mycelium (Fig. 4) and spores (Fig. 5) than the seedlings that were grown in contact with the extraradical mycelium for 6 and 9 days. At the end of the experiment, the highest values for spore production and mycelium length were found for seedlings that were grown in contact with the extraradical mycelium network for 12 and 15 days.

The early colonized seedlings showed normal growth and development during 4 weeks of the experiment. Small differences of shoot length, root length, and number of leaves were observed between treatments.

Discussion

In this study, we demonstrated that an AM fungus symbiotically attached to an autotrophic donor plant is a powerful source of inoculum, allowing for the fast and heavy in vitro mycorrhization of seedlings. The seedlings were colonized within 3 days of growth in the extraradical mycelium network. Within 6 days of contact, they were able to reproduce the fungal life cycle after transfer onto fresh medium, with the production of a dense extraradical mycelium bearing a high number of spores.

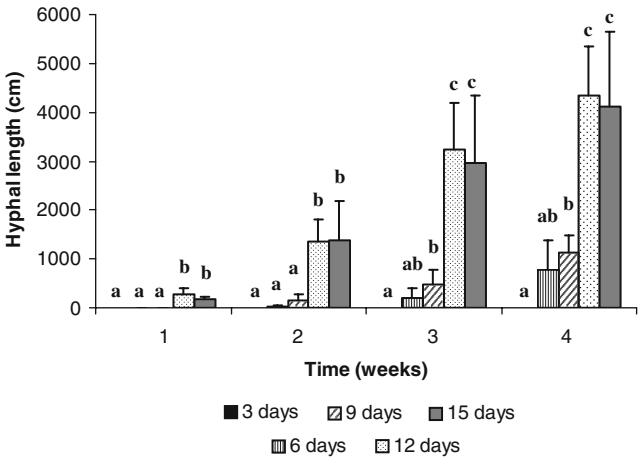

Fig. 4 Dynamics of hyphal growth of *Glomus intraradices* associated with *Medicago truncatula* seedlings after transplanting onto fresh MSR medium. *M. truncatula* seedlings were grown in contact with an extraradical mycelium extending from a mature *M. truncatula* plant for 3, 6, 9, 12, or 15 days before transplanting. Histograms represent means of six replicates +SE. Within inoculation treatment, *values of hyphal growth followed by a different letter* differ significantly at $P<0.05$ (Wilcoxon)

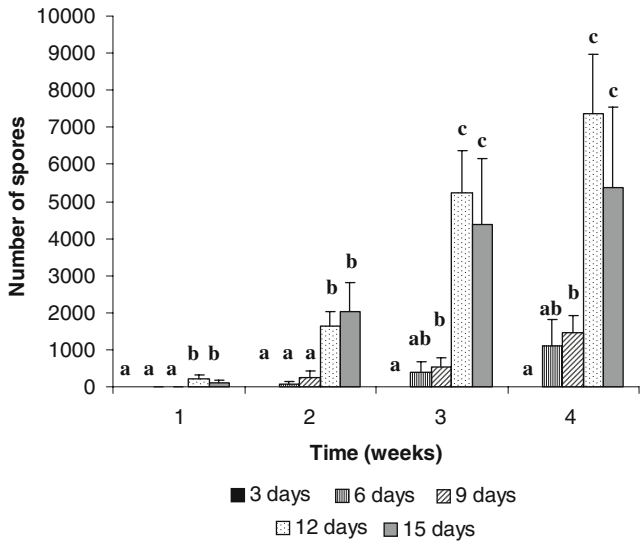

Fig. 5 Dynamics of spore production of *Glomus intraradices* associated with *Medicago truncatula* seedlings after transplanting onto fresh MSR medium. *M. truncatula* seedlings were grown in contact with an extraradical mycelium extending from a mature *M. truncatula* plant for 3, 6, 9, 12, or 15 days before transplanting. Histograms represent means of six replicates +SE. Within inoculation treatment, *values of spore production followed by a different letter* differ significantly at $P<0.05$ (Wilcoxon)

Colonization of roots by AM fungi can arise from different sources of inoculum, i.e., spores, root fragments, and hyphae (Biermann and Linderman 1983; Hart and Reader 2002; Klironomos and Hart 2002). In vitro, most results were reported with isolated spores (see review in Fortin et al. 2002; de Souza and Declerck 2003) and to a lesser extent with mycorrhizal root pieces (Strullu and Romand 1986; Diop et al. 1994a, b; Declerck et al. 1996, 1998) or isolated vesicles (Declerck et al. 1998). The importance of hyphae growing from living mycorrhizal roots has also been reported for a long time as the principal source of inoculum for connecting plants together via the AM fungal mycelium. Read et al. (1985), Friese and Allen (1991), and Hart and Reader (2005) emphasized the importance of such extraradical mycelium networks to initiate rapid colonization of seedlings and recently Cano et al. (2008) used this characteristic with ROC. Starting from a mycorrhizal ROC developing in a RC, they successfully achieved colonization of young root tissues developing in the HC. Mean colonization of 25% was obtained within a period of 1 week but only increased slightly thereafter. This capacity was exploited here, for the first time in vitro with autotrophic plants, to accelerate the mycorrhization process of seedlings. Extensive root colonization in the seedlings' roots (i.e., 54% of root length) was observed within 3 days of growth in the extraradical mycelium network and this percentage only slightly increased thereafter. Arbuscules and vesicles started to be produced from day6. The presence of these structures continuously increased until the last observation on day15. The results on root colonization contrasted with previous in vitro experiments conducted with plants (Voets et al. 2005; Dupré de Boulois et al. 2006), where multiple spores were used as the source of inoculum. In these studies, the first contact points between hyphae and roots were observed after approximately 2 weeks and root colonization levels reached 50% in about 8 weeks (Voets et al. 2005; Dupré de Boulois et al. 2006). This difference could mainly be attributed to the density of infective hyphae actively growing from the roots. In our experiment, a highly dense mycelium, consisting of hundreds of growing hyphae, grew towards the root system and spread between the roots of the seedlings, causing uniform colonization of the roots. This extraradical mycelium attached to the donor root system extensively explored and exploited the volume of the HC. On the contrary, multiple germinating spores have limited independent growth capacity and therefore more localized colonization, reliant on the root proximity. It is admitted that high propagule density reduces the length of the lag phase (i.e., before colonization is detected) in the curve of percentage colonization versus the time and may be linked with a rapid spread of the fungus in the root system (Smith and Read 1997). The rapid increase in colonization (after 3 days) observed in our experiment was therefore related to the high number of infective hyphae and their capacity to extend far beyond the host root to colonize new roots. The fast root colonization within the first 3 days was followed by a slight increase in root length colonization during which the spread of the fungus was presumably nearly equivalent to the growth of the roots. Although the frequency of root colonization did not differ significantly with time, the intensity of root colonization, characterized by the development of numerous arbuscules and vesicles, increased with time and was significantly higher in the plants that grew for 15 days in the extraradical mycorrhizal network. These structures were first observed within a period of 6 days following contact of the root system with the extraradical mycelium.

After transfer onto fresh medium, extensive re-growth of mycelium and spore production were obtained with seedlings grown in contact with the extraradical mycelium network of the donor plant for at least 6 days. On the contrary, only restricted growth was obtained with seedlings grown in contact with the extraradical mycelium for 3 days. It is unclear which stage of colonization is required to trigger profuse extraradical mycelium re-growth. Mosse and Hepper (1975) and Hepper (1981) observed considerable extraradical mycelium growth following appressoria formation and it seemed that arbuscules were not vital to initiate fungal re-growth. This was in contradiction with Bécard and Piché (1989) who claimed that arbuscules are

an absolute necessity for hyphal re-growth. In our experiment, arbuscules were observed starting from day 6. However, it is difficult to link re-growth to the exclusive presence of arbuscules since the first vesicles were also observed on day 6. These thick-walled structures are important storage organs that play a significant role as propagules in root fragments (Declerck et al. 1998) and possibly also in living roots. In addition, it has been observed that intraradical hyphae are able to grow out of the roots and explore the environment (Bago and Cano 2005). Absence of re-growth on day 3 could possibly be related with the absence of these structures. In our experiment, we also observed that extraradical hyphae attached to the mycorrhizal roots were capable of initiating re-growth through intact or injured sections. This may represent another important mechanism of fungal re-growth in transplanted mycorrhizal seedlings. The capacity of injured hyphae to re-growth was recently demonstrated in vitro by de la Providencia et al. (2007). These authors demonstrated that artificially injured extraradical hyphae were able to re-grow, repair, explore the surrounding environment, and colonize new roots. This was further supported by the recent "cut'n-go" technique developed by Cano et al. (2008). This technique consisted in cutting the growth medium in the RC and therefore the hyphae crossing from HC to RC to synchronize re-growth of the extraradical mycelium in the HC. These authors demonstrated that after removal of the medium and refilling with fresh medium, a compact colony front colonized the fresh culture medium, therefore arising from new hyphae and injured hyphae. Therefore, it seems from our results that extraradical mycelium re-growth may be related to "exiting hyphae" (as defined by Friese and Allen 1991) from colonized roots and also through existing intact or injured extraradical mycelium that are physically attached to the roots.

Spore production started within 1 week after the transfer of the mycorrhizal plantlets onto fresh medium. On average, the highest numbers of spores that were produced within a period of 4 weeks were reached with the seedlings grown in association with the mycelium network for a period of 12 ($73\times10^2\pm16\times10^2$ spores) and 15 ($54\times10^2\pm22\times10^2$ spores) days. With some exceptions obtained in ROC studies (Declerck et al. 2001; Elsen et al. 2003), spore production was generally very low with either plants (Dupré de Boulois et al. 2005, 2006; Voets et al. 2005) or root organs (Declerck et al. 1996; Rufyikiri et al. 2003) in such a short period. It is obvious that young actively growing seedlings having a homogeneously and heavily colonized root system, with the presence of dense internal hyphae together with arbuscules and vesicles, are a perfectly adapted starter inoculum for the mass production of spores of *G. intraradices*-like strains.

The MDP in vitro culture system proposed here allowed the fast, extensive, and homogenous colonization of plant roots at the seedling stage. Based on a high density of mycelium, with hundreds of hyphae developing in the HC, colonization levels reached more than 50% in seedlings after 3 days of contact with the mycelium. These colonization levels have never been obtained in any other in vitro study before. This system offers wide research and application possibilities. Various economically important monocots (maize, banana) as well as herbs (clover, plantago) and shrubs (vineyard) have been successfully colonized in their seedling stage using this system with *M. truncatula* as donor plant (S. Declerck, pers. comm.). This might offer a broad range of research possibilities for which either homogenous highly colonized seedlings or mass-produced in vitro inoculum is necessary.

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References

Bago B, Cano C (2005) Breaking myths on arbuscular mycorrhizas in vitro biology. In: Declerck S, Strullu DG, Fortin JA (eds) In vitro culture of mycorrhizas. Springer, Berlin, pp 111–138

Bago B, Azcon-Aguilar C, Piché Y (1998) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus Glomus intraradices grown under monoxenic conditions. Mycologia 90:52–62. doi:10.2307/3761011

Bago B, Pfeffer PE, Zipfel W, Lammers P, Shachar-Hill Y (2002) Tracking metabolism and imaging transport in arbuscular mycorrhizal fungi. Plant Soil 244:189–197. doi:10.1023/A:1020212328955

Bécard G, Fortin JA (1988) Early events of vesicular arbuscular mycorrhiza formation on Ri-T-DNA transformed roots. New Phytol 108:211–218. doi:10.1111/j.1469-8137.1988.tb03698.x

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. doi:10.1111/j.1469-8137.1983.tb03472.x

Cano C, Dickson S, González-Guerrero M, Bago A (2008) In vitro cultures open new prospects for basic research in arbuscular mycorrhizas. In: Varma A (ed) Mycorrhiza. Springer-Verlag, Berlin, pp 627–654

Cranenbrouck S, Voets L, Bivort C, Renard L, Stullu DG, Declerck S (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, Berlin, pp 341–348

Dalpé Y, Declerck S (2002) Development of *Acaulospora rehmii* spore and hyphal swellings under root-organ culture. Mycologia 94:850–855. doi:10.2307/3761699

de la Providencia IE, de Souza FA, Fernandez F, Delmas NS, Declerck S (2005) Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytol 165:261–271. doi:10.1111/j.1469-8137.2004.01236.x

de la Providencia IE, Fernandez F, Declerck S (2007) Hyphal healing mechanism in the arbuscular mycorrhizal fungi *Scutellospora reticulata* and *Glomus clarum* differ in response to severe physical stress. FEMS Microbiol Lett 268:120–125. doi:10.1111/j.1574-6968.2006.00572.x

de Souza FA, Berbara RLL (1999) Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia 91:343–350. doi:10.2307/3761379

de Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012. doi:10.2307/3761908

de Souza FA, Declerck S, Smit E, Kowalchuk GA (2005) Morphological, ontogenetic and molecular characterization of *Scutellospora reticulata* (Glomeromycota). Mycol Res 109:697–706. doi:10.1017/S0953756205002546

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

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585. doi:10.2307/3761216

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M (2000) *Glomus proliferum* sp nov.: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia 92:1178–1187. doi:10.2307/3761485

Declerck S, D'Or D, Cranenbrouck S, Le Boulenge E (2001) Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. Mycorrhiza 11:225–230. doi:10.1007/s005720100124

Declerck S, Strullu DG, Fortin JA (eds) (2005) In vitro culture of mycorrhizas. Springer, Berlin

Diop TA, Plenchette C, Strullu DG (1994a) Dual axenic culture of sheared-root inocula of vesicular–arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22. doi:10.1007/BF00204015

Diop TA, Plenchette C, Strullu DG (1994b) In vitro culture of sheared mycorrhizal roots. Symbiosis 17:217–227

Doner LW, Bécard G (1991) Solubilisation of gellan gels by chelation of cations. Biotechnol Tech 5:25–28. doi:10.1007/BF00152749

Dupré de Boulois H, Delvaux B, Declerck S (2005) Effects of arbuscular mycorrhizal fungi on the root uptake and translocation of radiocaesium. Environ Pollut 134:515–524. doi:10.1016/j.envpol.2004.08.015

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

Elmeskaoui A, Damont J-P, Poulin M-J, Piché Y, Desjardins Y (1995) A tripartite culture system for endomycorrhizal inoculation of micropropagated strawberry plantlets in vitro. Mycorrhiza 5:313–319. doi:10.1007/BF00207403

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

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20. doi:10.1139/b01-139

Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphea in the soil–inoculum types and external hyphal architecture. Mycologia 83:409–418. doi:10.2307/3760351

Giovannetti M, Sbrana C, Avio L, Strani P (2004) Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. New Phytol 164:175–181. doi:10.1111/j.1469-8137.2004.01145.x

Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344. doi:10.1046/j.0028-646X.2001.00312.x

Hart MM, Reader RJ (2005) The role of the external mycelium in early colonization for three arbuscular mycorhizal fungal species with different colonization strategies. Pedobiologia (Jena) 49:269–279. doi:10.1016/j.pedobi.2004.12.001

Hepper C (1981) Techniques for studying the infection of plants by vesicular–arbuscular mycorrhizal fungi under axenic conditions. New Phytol 88:641–647

Hernandez-Sebastia C, Piché Y, Desjardins Y (1999) Water relations of whole strawberry plantlets in vitro inoculated with *Glomus intraradices* in a tripartite culture system. Plant Sci 143:81–91. doi:10.1016/S0168-9452(99) 00014-X

Hernandez-Sebastia C, Samson G, Bernier PY, Piché Y, Desjardins Y (2000) *Glomus intraradices* causes differential changes in amino acid and starch concentrations of in vitro strawberry subjected to water stress. New Phytol 148:177–186. doi:10.1046/j.1469-8137.2000.00744.x

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

Koch AM, Kuhn G, Fontanillas P, Fumagalli L, Goudet J, Sanders IR (2004) High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. Proc Natl Acad Sci U S A 101:2369–2374. doi:10.1073/pnas.0306441101

Louche-Tessandier D, Samson G, Hernandez-Sebastia C, Chagvardieff P, Desjardins Y (1999) Importance of light and CO_2 on the effects of endomycorrhizal colonization on growth and photosynthesis of potato plantlets (*Solanum tuberosum*) in an in vitro tripartite system. New Phytol 142:539–550. doi:10.1046/j.1469-8137.1999.00408.x

Mosse B, Hepper C (1975) Vesicular arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223. doi:10.1016/0048-4059(75) 90088-0

Nielsen JS, Joner EJ, Declerck S, Olsson S, Jakobsen I (2002) Phospho-imaging as a tool for visualization and noninvasive measurement of P transport dynamics in arbuscular mycorrhizas. New Phytol 154:809–819. doi:10.1046/j.1469-8137.2002.00412.x

Pawlowska TE, Taylor JW (2004) Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature 427:733–737. doi:10.1038/nature02290

Pawlowska TE, Douds DD, Charvat I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556. doi:10.1017/S0953756299008801

Read DJ, Francis R, Finlay RD (1985) Mycorrhizal mycelia and nutrient cycling in plant communities. In: Fitter AH (ed) Ecological interactions in soil. Blackwell Scientific, Oxford, pp 193–217

Rufyikiri G, Thiry Y, Wang L, Delvaux B, Declerck S (2002) Uranium uptake and translocation by the arbuscular mycorrhizal fungus

Glomus intraradices, under root organ culture conditions. New Phytol 156:275–281. doi:10.1046/j.1469-8137.2002.00520.x

Rufyikiri G, Thiry Y, Declerck S (2003) Contribution of hyphae and roots to uranium uptake and translocation by arbuscular mycorrhizal carrot roots under root-organ culture conditions. New Phytol 158:391–399. doi:10.1046/j.1469-8137.2003.00747.x

Smith SE, Read DJ (eds) (1997) Mycorrhizal symbiosis, 2nd edn. Academic, San Diego

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

StatSoft Inc (2001) Statistica® release 6. Statsoft Incorporation, Tulsa

Strullu DG, Romand C (1986) Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. C R Acad Sci 303:245–250

Vierheilig H, Coughlan AP, Wyss U, Piché Y (1998) Ink and vinegar, a simple staining technique for arbuscular mycorrhizal fungi. Appl Environ Microbiol 64:5004–5007

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, Declerck S (2006) *Glomeraceae* and *Gigasporaceae* differ in their ability to form mycelium networks. New Phytol 172:185–188. doi:10.1111/j.1469-8137.2006.01873.x

Voets L, Goubau I, Olsson PA, Merckx R, Declerck S (2008) Absence of carbon transfer between *Medicago truncatula* plants linked by a mycorrhizal network, demonstrated in an experimental microcosm. FEMS Microbiol Ecol 65:350–360. doi:10.1111/j.1574-6941.2008.00503.x