



A simple and low-cost technique to initiate single-spore cultures of arbuscular mycorrhizal fungi using a superabsorbent polymer

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Abstract

Most species of arbuscular mycorrhizal fungi (AMF) are propagated with a host plant in a pot culture. However, the soil matrix makes it difficult to monitor the establishment and development of the symbiosis. In vitro culturing using Ri T-DNA transformed roots provides a clear medium and a sterile environment which offsets the constraints of the soil matrix. Nevertheless, the sterile conditions and the Ri T-DNA transformed roots provide very different growing conditions compared to a pot culture. Transparent soil based on superabsorbent polymer (SAP) has the potential of combining the advantages of current in vivo and in vitro culture methods without the constraints associated with either technique (opacity and sterility). Here we describe a SAP-based autotrophic culture as an alternative to current in vivo and in vitro culture methods. This system using two-compartment Petri dishes makes it easy to initiate single-spore cultures and to monitor fungal propagation. The SAP-based autotrophic system allowed the establishment of single-spore cultures of seven species (*Diversispora varaderana*, *Funneliformis geosporus*, *Gigaspora rosea*, *Racocetra fulgida*, *Rhizophagus irregularis*, *R. intraradices* and *Sclerocystis* sp.) from six genera and three families. Cultures were maintained over several months under non-sterile conditions. The Petri dishes avoid the problem of cross contamination and they can be stacked for space optimization. The grains of SAP colonized with new spores were used as inoculum to initiate new cultures in the SAP-based system. The SAP-based autotrophic culture method is a low-cost and low-tech approach, which makes the study of AMF much more accessible.

Keywords Arbuscular mycorrhizal fungi · Single-spore culture · Superabsorbent polymer

1 Introduction

Arbuscular mycorrhizal fungi (AMF) are important soil microorganisms for plant and soil health and have therefore received considerable attention from the perspective of sustainable agriculture (Gianinazzi et al. 2010; Brunnett and Tedersoo 2018; Schaefer et al. 2021). Improving

sustainability in agriculture requires better characterization of plant root symbionts. However, AMF are obligate biotrophs and their propagation requires a permanent association with a host. As a result, AMF are either cultivated with a plant host in pots filled with sterilized soil (in vivo conditions) or with Ri T-DNA transformed roots in Petri dishes filled with sterile medium (in vitro conditions).

Each of these propagation techniques has pros and cons. In vivo conditions are suitable for most of the known AM fungal species, but the opacity of the soil matrix makes impossible to monitor the development of the fungal symbiont. The only way to check for the successful establishment of AM symbiosis is to sieve a soil sample in order to isolate the spores or to observe the presence of intraradical structures within the roots. This is usually done several months following inoculation of the pot culture. If the culture is not initiated from a single spore, more than one AM fungal species can be present in the soil, and taxonomic expertise is required to determine the purity of the pot culture. A pot

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culture inoculated with a single spore is the best way to obtain a pure culture of AMF, as well as a fundamental prerequisite for describing new species. However, pure cultures are difficult to establish as many assays fail due to the limited quantity of fungal inoculum. Therefore, the development of pure cultures is a long and uncertain process under *in vivo* conditions.

In vitro conditions lie at the other end of the spectrum. The development of the fungal symbiosis (i.e., spore germination, root colonization, hyphal propagation and production of new spores) can be monitored live thanks to the transparency of the culture medium. This system streamlines the process of obtaining pure cultures. However, the spores must be sterilized, eliminating potentially important bacteria (mycorrhiza helpers) that participate in the mycorrhizal activity (Xavier and Germida 2003; Frey-Klett et al. 2007). Although Ri T-DNA transformed roots are morphologically, physiologically and metabolically similar to their autotrophic counterparts (Bago and Cano 2005), they may not be the best hosts for most AMF. Indeed, less than 6% (~20/340) of the known species of AMF have been established on Ri T-DNA transformed roots (Rodrigues and Rodrigues 2013) and no more than 10 species are permanently available as *in vitro* cultures from international collections such as the Mycothèque de l'UCLouvain-Belgian Co-ordinated Collections of Microorganisms (MUCL / BCCM) or the Canadian Collection of AMF (CCAMF).

The disadvantages presented by *in vivo* and *in vitro* conditions (i.e., opacity and sterility, respectively) could be avoided by using transparent soils. Transparent soils are made up of particles that become porous and transparent when hydrated. Although these soils have been used in geo-engineering, their use in biology has been limited owing to the toxicity of the material. In contrast, polymeric hydrogels such as Teflon amorphous fluoropolymer or Nafion (Leis et al. 2005) are non-toxic and can be used as a soil or culture medium analog in biological systems. For instance, Nafion precursor beads were used to study root–nematode (O'Callaghan et al. 2018) and root–bacteria interactions (Downie et al. 2012, 2014). Regarding root–AMF interactions, only a brief note reports the use of a polymeric hydrogel to produce '*Glomus intraradix*' inoculum in plastic boxes with strawberry plants as a host (Vestberg and Uosukainen 1992). Abundant sporulation was observed in the matrix of the hydrogel 5 to 7 weeks after inoculation. Hydrogels are superabsorbent polymers (SAPs) that are obtained from acrylamide monomers (Braun et al. 2021). The polymer chains are interconnected by ionic or covalent bonds (cross-linkers) leading to the formation of a three-dimensional network (Saha et al. 2020). This network expands due to the anion–anion electrostatic repulsion resulting from the negatively charged carboxyl groups (Feng et al. 2014; Saha

et al. 2020). Water molecules are absorbed and retained inside the network structure via hydrogen bonding. The water-absorbing capacity usually ranges from 100 to 500 g per gram of SAP, and swollen SAPs are relatively transparent. Moreover, SAPs contain few nutrients for microorganisms, which are unable to biodegrade most polyacrylamides (Braun et al. 2021) in comparison to polysaccharide-based solidifying agents such as agar, alginate, phytigel and gelatin gum.

These properties make the use of SAPs a promising alternative to the *in vivo* and *in vitro* culture techniques currently used to propagate AMF. We therefore developed a SAP-based autotrophic culture and tested its potential to propagate AMF while in symbiosis with a whole plant. Because the SAP-based autotrophic culture contains no nutrients, a fertilizing solution is required to support host growth. Two modified minimal (M) solutions along with reverse osmosis-purified water were therefore used to hydrate the grains of SAP, and their effect on spore germination was assessed. Seven AMF species were cultured on two-compartment Petri dishes using *Plantago lanceolata* as a host, in order to demonstrate the advantages of using the SAP-based autotrophic system to initiate and maintain single-spore cultures of AMF.

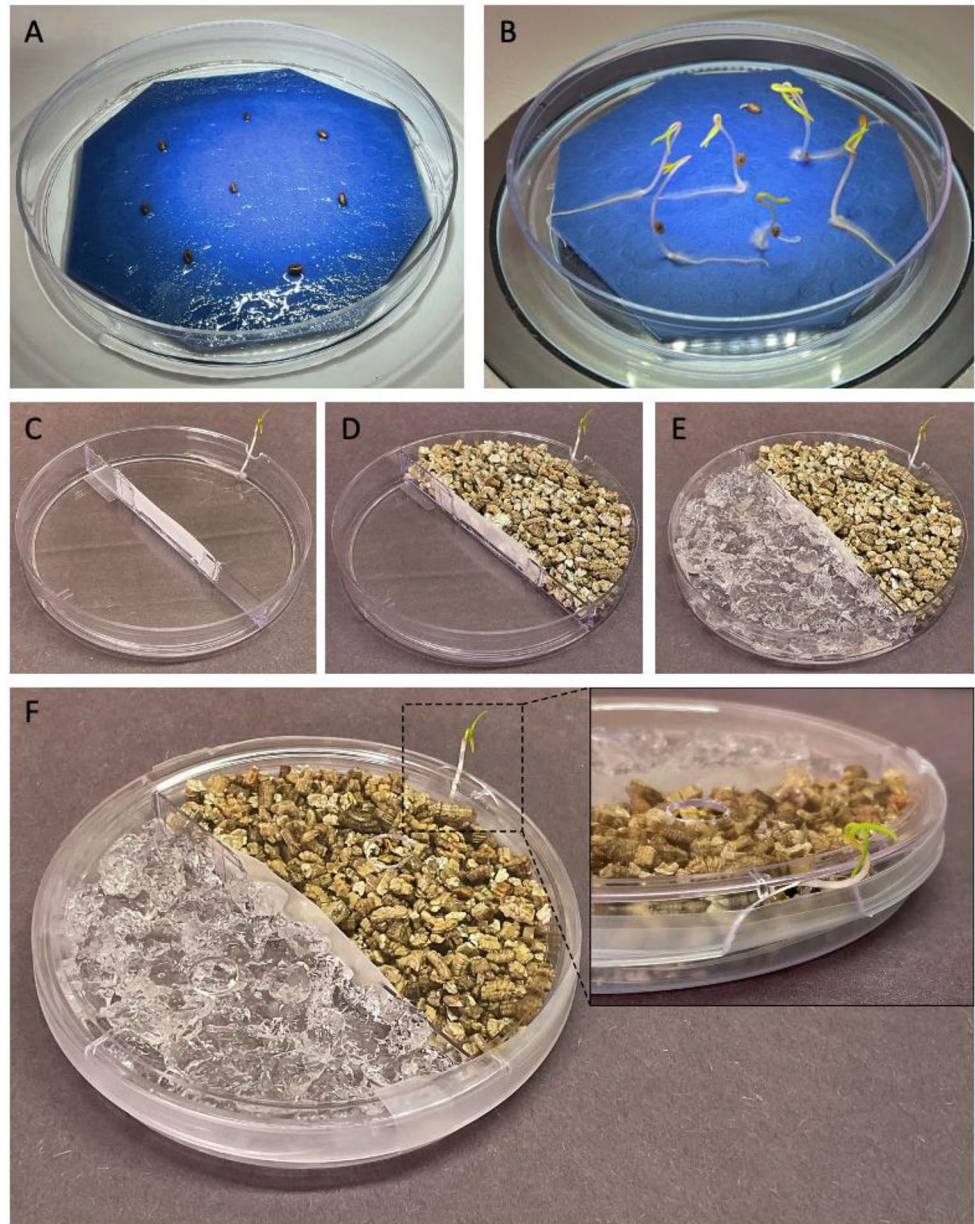
2 Materials and methods

Apart from the equipment used for imaging, the materials required to initiate and maintain single-spore cultures of AMF on SAP medium are not costly and do not need to be autoclaved. The methods used do not involve sterile conditions and the work can be done on a benchtop. The SAP used in this study was poly(acrylamide-co-acrylic acid) potassium salt (see Fig. 1a in Mahon et al. 2020 for the chemical structure). The product is sold under the name HORTA-SORB® MD (CAS registry number 31212-13-2, Horticultural Alliance, LLC, Sarasota FL, USA).

2.1 Nutrient solutions, pH and spore germination assays

The nutrient solutions were derived from the minimal (M) medium recipe described by Bécard and Fortin (1988). The first modified M solution (mMS-1) had the same composition as the M medium, minus the sucrose, vitamins and bacto agar (Table 1). The composition of mMS-1 was then modified to develop mMS-2, taking into account the specificities of the SAP. The concentration of magnesium sulfate heptahydrate (MgSO₄·7H₂O) and calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O) were, respectively, 20 and 10 times lower in mMS-2 because the presence of cations such as

Fig. 1 Preparation of the system used to cultivate arbuscular mycorrhizal fungi (AMF) on superabsorbent polymers (SAP) with a whole plant. A, B) Seeds of *Plantago lanceolata* are germinated on a blotting paper hydrated with modified M solution (mMS-1). C) A single seedling is positioned on the notch cut on the side of the Petri dish. The plastic barrier dividing each compartment was cut and replaced with a nitex membrane (mesh size = 30 – 60 μm). D) Two grams of vermiculite are deposited in the plant compartment. E) The SAP compartment is filled with 15 g of SAP hydrated with mMS-1. F) The Petri dish is wrapped with Parafilm, and each compartment is hydrated weekly through the holes in the lid (See Fig. S1A).



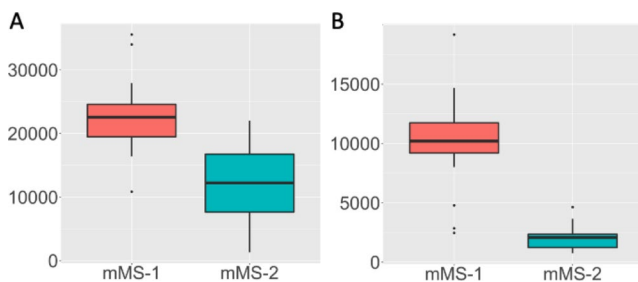
Mg^{2+} and Ca^{2+} tends to decrease the expansion of the polymer network (Saha et al. 2020). Potassium nitrate (KNO_3) and potassium chloride (KCl) were not included in mMS-2 because the SAP used in the SAP-based autotrophic system releases K^+ cations. The concentration of potassium dihydrogen phosphate (KH_2PO_4) was doubled in mMS-2. Finally, ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) and ammonium nitrate (NH_4NO_3) were added to compensate for the nitrogen not provided by KNO_3 . The pH of mMS-1, mMS-2 and reverse osmosis-purified water was measured with a VWR symPHony™ Ag/AgCl pH electrode (SR40C). The pH of the supernatant in SAP hydrated with each of the above-mentioned solutions was measured 12 h following

hydration. The SAP grains were then blended, and the pH was measured 12 h later.

The effect of the SAP hydrated with each of the nutrient solutions on spore germination was assessed using spores of *R. irregularis* (DAOM 197198) isolated from the commercial product Aktiv Potato L (Premier Tech, Rivière-du-Loup, QC, Canada). SAP hydrated with reverse osmosis-purified water (mineral content < 10 ppm) was used as a control. The assay was performed in a 12-well cell culture plate (Cole-Parmer) filled with 2 mL of hydrated and blended SAP to obtain a slick surface. A single spore was deposited in each well and the rate of germination and total hyphal length were recorded after 28 d of incubation at room temperature in the dark. The distance between the longest hyphae

Table 1 Composition of the modified M medium solutions (mMS-1 and mMS-2)

	mMS-1	mMS-2
	mg / L	mg / L
MgSO ₄ ·7H ₂ O	731	36.55
KNO ₃	80	—
KH ₂ PO ₄	4.80	9.60
KCl	65	—
(NH ₄) ₂ SO ₄	—	132
Ca(NO ₃) ₂ ·4H ₂ O	288	28.80
NH ₄ NO ₃	—	130
C ₁₀ H ₁₄ N ₂ FeNaO ₈ (E-6760)	8.00	8.00
KI	0.75	0.75
MnCl ₂ ·4(H ₂ O)	6.00	6.00
ZnSO ₄ ·7(H ₂ O)	2.65	2.65
H ₃ BO ₃	1.50	1.50
CuSO ₄ ·7(H ₂ O)	0.13	0.13
Na ₂ MoO ₄ ·2(H ₂ O)	0.0024	0.0024

**Fig. 2** Boxplots of hyphal length (µm) measured with NeuronJ for the spores that germinated on mMS-1 (n=16) and mMS-2 (n=15) 28 days following inoculation. (A) Total hyphal length. (B) Distance between the two most distant hyphae

observed in two opposite directions was also measured (see Sect. 2.6) to determine the foraging ability of the fungus growing on each type of hydrated SAP.

2.2 Petri dish design

Two-compartment Petri dishes (Corning™ or Kord-Valmark™, 100 mm × 15 mm) were modified as illustrated in Fig. S1A–D. A rotary tool was used to drill two holes in the lid to allow watering of each compartment during culture growth. A notch was cut in the sides of the lid and the bottom of the Petri dish to provide space for the plantlet stem. A rectangular notch was cut in the plastic barrier that divides the two compartments of the Petri dish. Since the rotary tool does not melt the polystyrene, these steps do not generate toxic fumes. Finally, a nitex membrane (Dynamic Aqua-Supply, Surrey, BC, Canada) with 30 or 60 µm mesh was melted on the plastic barrier using a pyrography tool, to allow the hyphae to colonize the SAP compartment and limit the spread of the roots from the vermiculite compartment.

2.3 Culture set-up

Plantlets of *Plantago lanceolata* were used as a host. *Plantago lanceolata* is commonly used to propagate AMF in pot cultures as it tolerates stressful conditions such as low light levels (Walker and Vestberg 1994) and variations in temperature and soil humidity. Seeds (Les Jardins de l'écoumène, Saint-Damien, Québec, Canada) were incubated for 6 d on a blotting paper soaked in mMS-1 to trigger germination (Fig. 1A, 1B). The M medium was modified so that it was free of sugar, vitamins and agar (Table 1). A plantlet with a root length of 2–3 cm was transferred to the Petri dish and the stem was aligned with the notch cut in the side of the Petri dish (Fig. 1C). Two grams of vermiculite (Pro-Mix, Premier Tech Home and Garden Inc, Brantford, ON) were then added to fill the compartment and immobilize the plantlet (Fig. 1D).

The other compartment was filled with 15 g of medium-size grains (1–2 mm) of HORTA-SORB® MD hydrated with mMS-1. SAP particles are also available in two other sizes: 2–4 mm, and 0.2–0.8 mm. The large grains become too large when hydrated and needed to be sliced to fit in the Petri dish. To hydrate the SAP, 5 g of medium-sized grains was sprinkled into a beaker containing 500 mL of modified M medium. It takes 12 h for the larger particles of SAP to be completely hydrated. The refractive index was similar to that of water when the SAP particles were hydrated, making the particles of hydrated SAP almost transparent (Fig. S2). The Petri dishes were then wrapped with Parafilm and incubated for about 12 days (Fig. 1E F) to increase the root biomass in the vermiculite compartment prior to inoculation. The Petri dishes were stacked and maintained in the dark in a tin can to limit the proliferation of green algae (Fig. 3A).

2.4 Fungal material and inoculation

Single-spore cultures were tested with eight species of AMF, representing four families (Claroideoglomeraceae, Diversisporaceae, Gigasporaceae, Glomeraceae), to demonstrate that phylogenetically diverse species of AMF can grow on the SAP-based autotrophic culture system (Table 2). About 20 two-compartment Petri dishes were inoculated with a single spore of each AMF species. Four negative controls (non-inoculated SAP-based autotrophic cultures) were randomly distributed among the 20 cultures. Spores of *R. irregularis* DAOM 197198 were isolated from the commercial product Agtiv Potato L (Premier Tech, Rivière-du-Loup, QC, Canada). Spores of *R. intraradices* were obtained from an in vitro culture by dissolving the medium containing the fungal material with sodium citrate buffer (pH 6.0; at 30 °C, Doner and Bécard 1991). Spores from the other species were isolated from in vivo or in vitro cultures provided

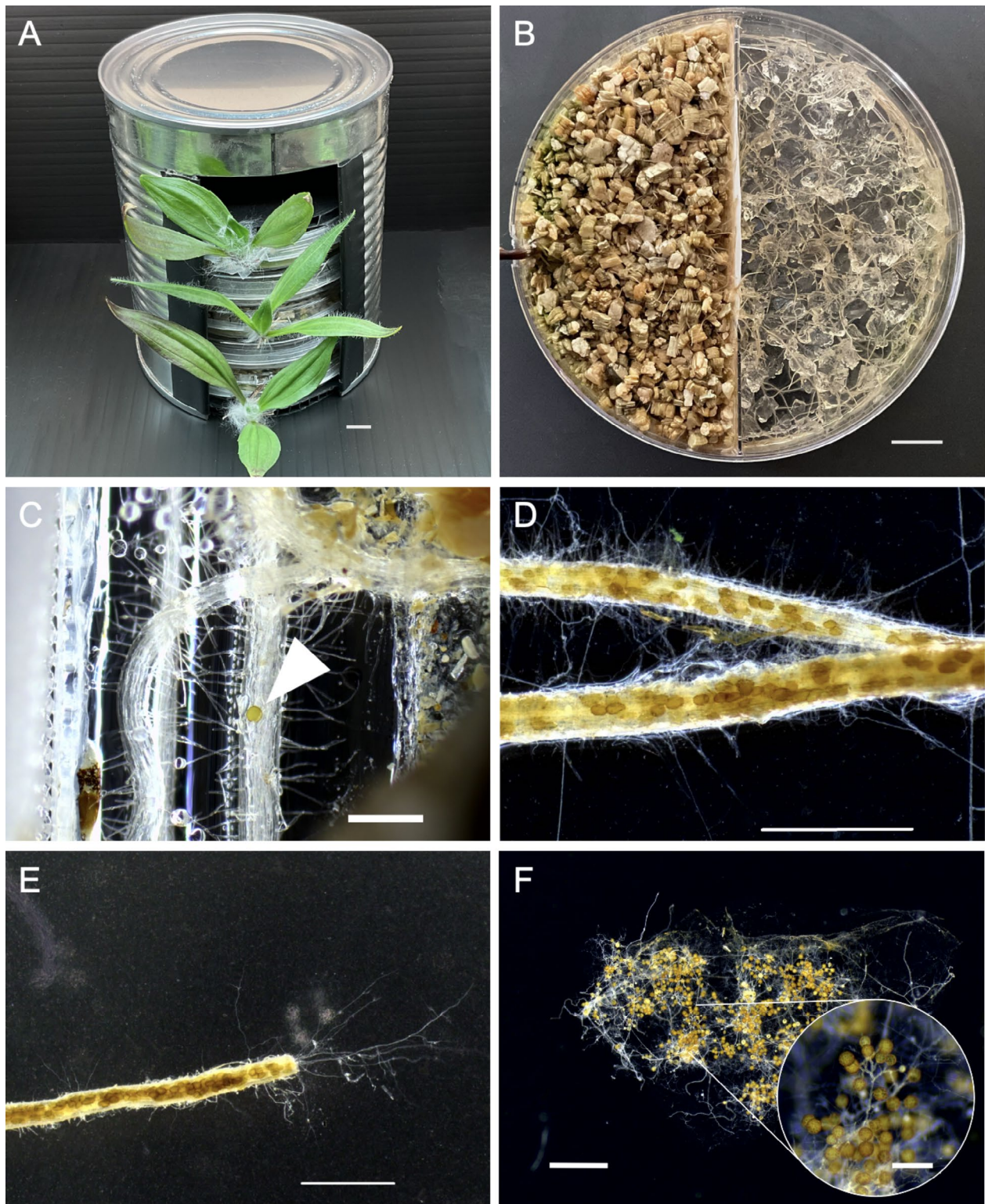


Fig. 3 Cultures of *Rhizophagus irregularis* on SAP in two-compartment Petri dish with *Plantago lanceolata* as a host. (A) Petri dishes can be stacked and maintained in the dark using a tin can to limit the proliferation of green algae, scale bar = 0.8 cm. (B) Six-month-old culture with vermiculite on the left and SAP on the right. Grains of SAP are not rehydrated. (C) Inoculation with a single spore of *R. irregularis*. (D) Roots of *Plantago lanceolata* extracted from the vermiculite

compartment and colonized with *R. irregularis* after six months of culture. (E) Hyphae of *R. irregularis* exiting a broken root. (F) A cluster of *R. irregularis* spores in a grain of SAP observed five months following inoculation with a single spore. The scale bars are 1 cm long in subfigures A and B, 1000 μm in subfigures D, E and F, 500 μm in subfig. C and 300 μm in the inset in subfig. D

Table 2 Species of AMF tested on SAP-based autotrophic culture

Species	CCAMF ID	Other ID	Collector	Collecting date	Sampling location
<i>Claroideoglomus lamellosum</i>	2305D	—	C. Hamel	—	Canada, Saskatchewan
<i>Diversispora varaderana</i>	962E	—	Y. Dalpé	1989-08-26	Canada, Ontario, Lake Superior Provincial Park
<i>Funneliformis geosporus</i>	544F	W671-7	C. Walker	1998-11-20	England, Hampshire, Lymington
<i>Gigaspora rosea</i>	770F	BEG-9	T. Woods	—	USA
<i>Racocetra fulgida</i>	589F	NC303A	S. Stürmer	1997-04-17	USA, North Carolina, Carteret
<i>Rhizophagus intraradices</i> ^a	IVT156	KS906	B.A. Daniels Hetrick	1998-08-03	USA, Kansas
<i>Rhizophagus irregularis</i> ^b	197198	—	C. Plenchette, V. Furlan	—	Canada, Québec, Pont-Rouge
<i>Sclerocystis</i> sp. ^c	2767A	MT106	C. Rosier	2003-03-26	USA, Montana

^a In vitro culture

^b Commercial strain obtained from PremierTech

^c *Sclerocystis* sp. is a contaminant observed in the culture MT106 originally identified as *Ambispora gerdemannii* and received from INVAM.

by the Canadian Collection of AMF. In vivo cultures were maintained in the greenhouse for 8 to 31 months. The soil was a 50/50 mixture of Vermiculite (Perlite Canada Inc., Montréal Saint-Pacôme, QC, Canada) and Turface (Profile Products LLC Buffalo Grove, IL, USA), and the host plant was *Allium ampeloprasum* or *Plantago lanceolata*. About 15 mL of soil from each pot was wet-sieved using four stacked sieves with mesh size 500 µm (top sieve), 300 µm, 150 µm and 38 µm (bottom sieve). Soil material collected on sieves with 150 µm and 38 µm mesh size was used to isolate the spores with a micropipette under a stereomicroscope (Olympus SZX10, Richmond Hill, ON, Canada).

A single spore was then deposited on a root using a Pasteur pipette or precision forceps for the sporocarps of *Sclerocystis* sp. Spores were either directly deposited in the vermiculite compartment or pre-germinated on SAP before being transferred onto a root. In the latter case, the particles of hydrated SAP were blended with an immersion blender until a homogeneous and viscous solution was obtained.

The resulting hydrogel was blurred with microbubbles which disappeared after 24 to 48 h. Spores showing a germinating hypha could be then gently pipetted out of the SAP medium (after adding one mL of nutrient solution to liquefy the medium) and deposited onto a plantlet root in the vermiculite compartment. The site of inoculation could be identified to monitor the connection to the host root and the development of the fungal symbiosis.

2.5 Spore viability

To assess the viability of the spores produced in the grains of SAP, grains from 6-month-old single-spore cultures were used to inoculate new SAP-based autotrophic cultures. A single grain containing hundreds of spores of *R. irregularis* (DAOM 197198) was deposited in the vermiculite compartment. A 6-day-old plantlet of *Plantago lanceolata* was deposited at the same time. Ten Petri dishes were inoculated and incubated for two months. Two non-inoculated Petri dishes were set up as negative controls.

2.6 Imaging and statistical analyses

The development of the host and fungal partners was monitored using a trinocular stereomicroscope (Olympus SZ-6145 TR) equipped with a dark field cartridge (SZX2-CDF) and an Olympus EP50 camera. Hyphal length was measured using the EPview V1.2 software with the NeuronJ plugin (Meijering et al. 2004) as implemented in the Fiji software (Schindelin et al. 2012). The number of spores of *R. irregularis* per grain of SAP was estimated with Fiji software. For each picture of grain, the areas free of spores were erased to avoid false positives. Then the pictures were converted to black and white (8-bit), 'Image/Adjust/Auto Threshold' was set to default, images were converted to black and white with 'Process/Binary/Convert to Mask,' and overlapping spores were discriminated by running the function 'Process/Binary/Watershed'. Spores were counted using 'Analyze/Analyze particles' and the parameters Size and Circularity were set to 80–300 and 0.60–1, respectively. Circularity ranges from 0 (infinitely elongated polygon) to 1 (perfect circle). 'Outlines' was selected from the 'Show' popup menu to display the numbered outlines of the measured particle.

The effect of the nutrient solutions on spore germination was assessed using a randomized complete block design. A total of 72 spores of *R. irregularis* were tested (24 spores per nutrient solution) distributed in six 12-well cell culture plates (Corning™). All the data were checked for normality with the Shapiro-Wilk test and for homoscedasticity with the F-test. Differences in hyphal growth between mMS-1 and mMS-2 were assessed with a t-test. Differences in

hyphal spread between mMS-1 and mMS-2 were assessed with a Wilcoxon test (data were not normally distributed). The same approach was applied to analyze the pH. Five pH readings were performed for each solution (mMS-1, mMS-2 and reverse osmosis-purified water) and each type of SAP (grains or blended) hydrated with each solution. High dynamic range photography, focus merging and panoramas were done using Affinity Photo v1.09. Statistical analyses and graphical outputs were produced with R (R Core Team, 2021).

3 Results

3.1 Impact of the SAP hydrating solutions on spore germination and hyphal growth

The nutrient solution used to hydrate the SAP had no impact on the germination of *R. irregularis* spores; however, it significantly impacted hyphal growth. After 28 d of incubation, a total of 16/24 (67%) and 15/24 (63%) of the spores germinated on mMS-1 and mMS-2, respectively. Germinated spores produced on average 86% more hyphae ($t=4.8492$, $p=1.9e-05$) on mMS-1 ($\bar{x} = 22.9$ mm) than on mMS-2 ($\bar{x} = 12.3$ mm, Fig. 2A). The hyphae also tended to spread five times further away from the germinated spore on SAP hydrated with mMS-1 compared to mMS-2 ($W=234$, $p=3.6e-06$, Fig. 2B). The average distance between the two longest hyphae observed in two opposite directions was 10.1 mm on mMS-1 and 2.1 mm on mMS-2. Only 1/24 of the spores germinated on the SAP hydrated with reverse osmosis-purified water. After 28 d of incubation, this unique germinated spore produced only 2.4 mm of hyphae. These differences could not be explained by the pH level. Each solution, with or without SAP, had similar pH values ($F_{(2,4)}=3.1$, $p=0.15$); however, the pH of each solution increased by 25% in SAP ($F_{(2,4)}=39.7$, $p=0.0023$) and ranged from 6.7 to 7.4 (Fig. S3).

Based on these results, mMS-1 was chosen to hydrate the SAP grains used to establish single-spore cultures. A germination assay on SAP hydrated with mMS-1 was then performed on spores of *C. lamellosum*, *F. mosseae* and *R. fulgida*. *Claroideoglossum lamellosum* had the lowest germination rate with 1/12 spores (8%). The germination rate was intermediate for the spores of *F. mosseae* (2/18 spores, 11%) and the highest for *R. fulgida* (8/24, 33%).

3.2 Establishment of single-spore culture on SAP-based autotrophic system

The SAP-based autotrophic system made it possible to establish single-spore cultures for seven out of eight species

of AMF representing six genera (*Diversispora*, *Gigaspora*, *Racocetra*, *Rhizophagus*, *Sclerocystis*) and three families (Diversisporaceae, Glomeraceae and Gigasporaceae). However, the rate of successful establishment of single-spore cultures varied among the species of AMF tested. *Rhizophagus irregularis* (Fig. 3C to 3F) and *R. intraradices* (Fig. 4A, 4B) were the most efficient species to cultivate, with respectively 19/28 (68%) and 16/20 (80%) Petri dishes showing spores and an extensive extraradical mycelium network in the SAP compartment. In Petri dishes inoculated with *R. irregularis*, the roots of the host plant were heavily colonized (Fig. 3D and E) after three months of culture while hundreds of spores in clusters could be observed in SAP grains (Fig. 3F). The grains of SAP were irregularly colonized since some grains were free of spores while others had hundreds of spores (Fig. S4). A grain of SAP containing at least 1,000 spores ($\bar{x} = 1874$) could be found in 15/28 Petri dishes. The grains of SAP colonized with spores of *R. irregularis* from 6-month-old cultures were used to inoculate new SAP-based autotrophic systems. A total of 10/10 Petri dishes showed spores and hyphae spreading in the SAP compartment, two months following inoculation with the colonized SAP grains.

Regarding the six other species of AMF tested to initiate single-spore cultures, the rate of success ranged from 0 to 59%. Four months following inoculation with a single spore of *Funnelformis geosporus*, a total of 10/17 (59%) Petri dishes were colonized (Fig. 4C, 4D). The spores of *F. geosporus* formed singly in the SAP grains and were dark yellow brown. The inoculation of a single sporocarp of *Sclerocystis* sp. (Fig. 4E) in 20 SAP-based autotrophic systems led three months later to five colonized Petri dishes (25%). Most of the sporocarps were observed in close contact with the roots (Fig. 4F) or on vermiculite particles (Fig. 4G). A couple of sporocarps were observed in the SAP compartment on the few roots that had crossed over the middle barrier. Hyphae and small whitish spores were also produced in the SAP grains (Fig. 4F). 72 days following inoculation with a single spore of *Diversispora varaderoana*, a total of 6/20 (30%) Petri dishes were colonized (Fig. 5A, 5B). Regarding *Racocetra fulgida* (Fig. 5C to 5E), 4/20 (20%) single-spore cultures were obtained. A profusion of auxiliary cells but no spores was observed during the first three months following the inoculation. The auxiliary cells were observed in the vermiculite compartment and a few hyphae explored the SAP compartment. Six months following inoculation, a few spores could be observed in the vermiculite compartment (Fig. 5E). 52 days following inoculation with a single spore of *Gigaspora rosea*, only 1/20 (5%) Petri dish was colonized (Fig. 5F, 5G). Three spores were observed in the SAP compartment and 6 spores in the vermiculite compartment. Auxiliary cells were first

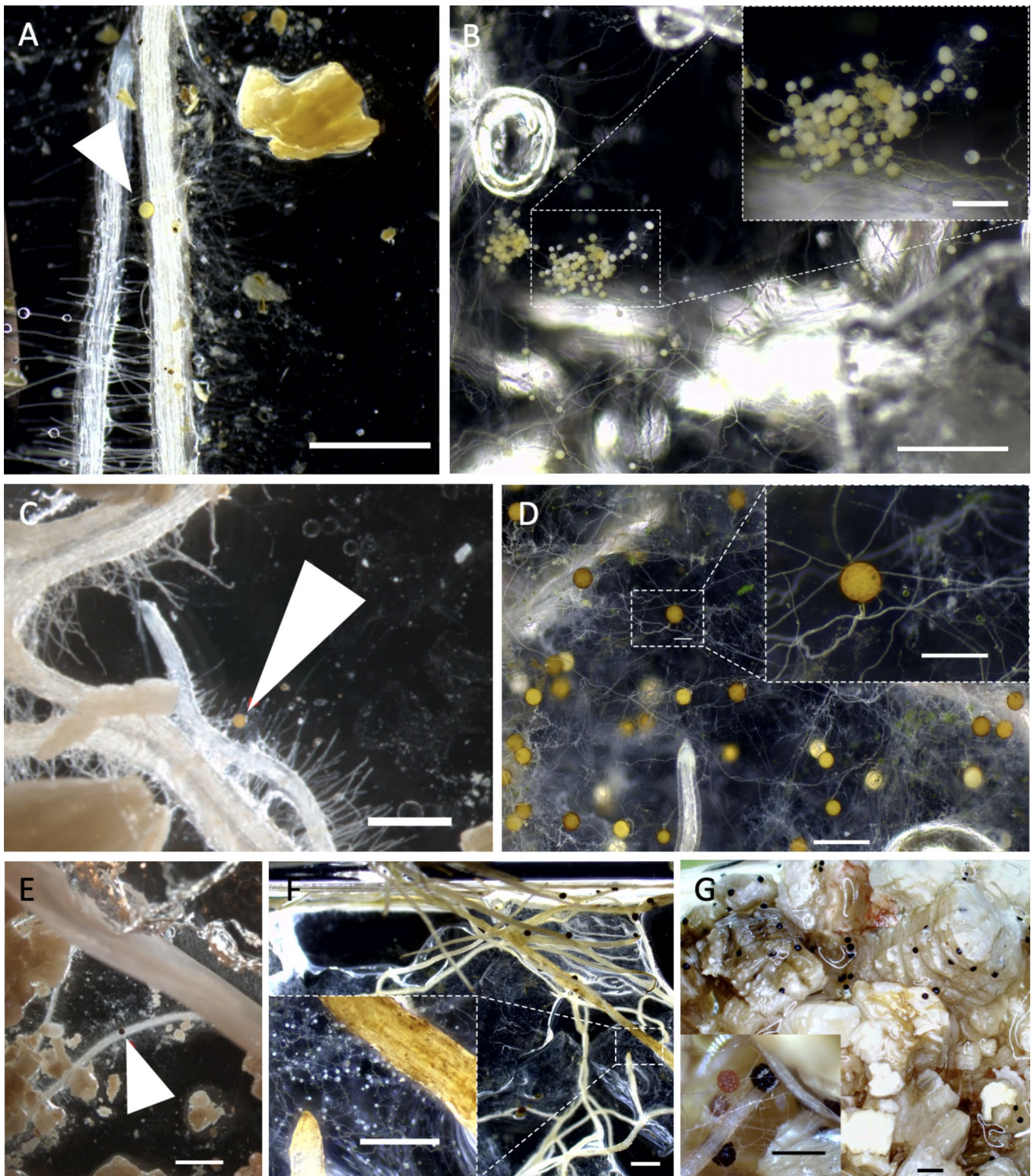


Fig. 4 Single-spore cultures of various AMF species. (A) *Rhizophagus intraradices* at the time of inoculation (scale bar = 1000 µm, vermiculite compartment) and (B) 47 days post inoculation (scale bar = 1000 µm, inset = 250 µm). (C) *Funneliformis geosporus* at the time of inoculation (scale bar = 1000 µm, vermiculite compartment) and (D) 125 days post inoculation (scale bar = 500 µm, inset 250 µm, SAP

compartment). (E) *Sclerocystis* sp. at the time of inoculation (scale bar = 2000 µm, vermiculite compartment) and (F) 89 days post inoculation (scale bar = 1000 µm, inset = 500 µm, SAP compartment). (G) sporocarps of *Sclerocystis* sp. produced in the vermiculite compartment (scale bar = 1000 µm, inset = 500 µm)

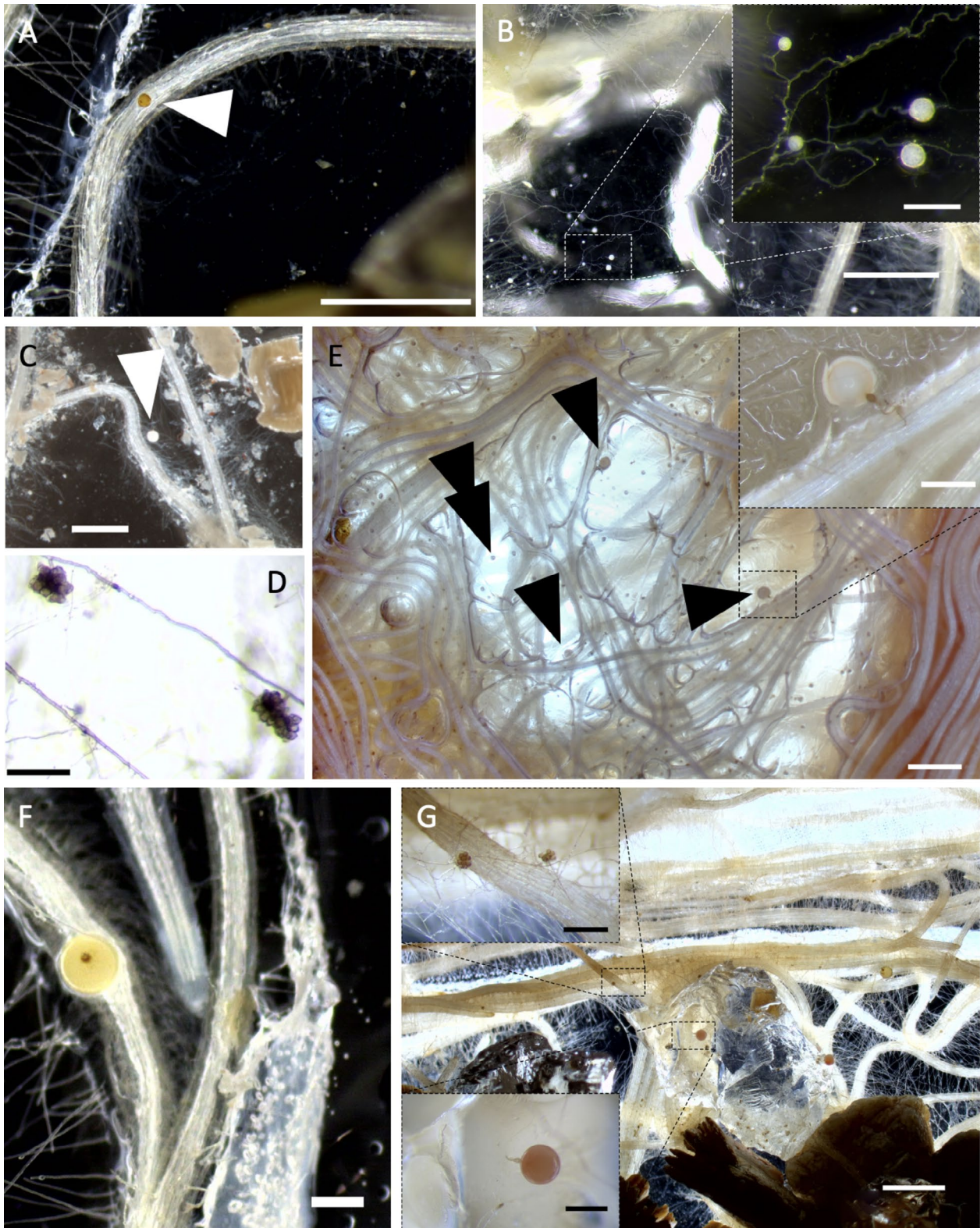


Fig. 5 Single-spore cultures of various AMF species. (A) *Diversispora varaderana* at the time of inoculation (scale bar = 1000 μm) and (B) 72 days post inoculation (scale bar = 1000 μm , inset = 125 μm). (C) *Racocetra fulgida* at the time of inoculation (scale bar = 1000 μm). (D) close-up of the auxiliary cells (scale bar = 100 μm) 190 days post inoculation, and (E) spores observed 190 days post inoculation (scale

bar = 1000 μm , inset = 250 μm). (F) *Gigaspora rosea* at the time of inoculation (scale bar = 250 μm) and (G) 52 days post inoculation (scale bar = 2000 μm , insets = 250 μm). The white single arrows show the single spores at the time of inoculation. The black arrows show the daughter spores of *R. fulgida* and the double black arrow shows one of the many auxiliary cells

observed in the SAP compartment 29 days following inoculation. Lastly, none of the single spores of *Claroideoglomus lamellosum* (20 SAP-based autotrophic systems were inoculated) deposited near the roots of *P. lanceolata* germinated.

Despite the non-sterile conditions of the SAP-based autotrophic system, no major fungal or bacterial contamination was observed spreading in the vermiculite or SAP compartments. While a few yeasts or molds were occasionally observed on SAP grains, the absence of carbohydrates inhibits their spread. After three months of culture, some green algae might be present, but their proliferation, albeit very limited, was essentially localized close to the stem of the host plant due to the presence of diffuse light on the side of the Petri dishes.

4 Discussion

Current *in vivo* and *in vitro* techniques used to cultivate AMF impose a certain number of constraints that the SAP-based autotrophic system overcomes. The system presented here is a very simple technique that can be implemented in any laboratory. It is low-cost, low tech and takes up very little space. Furthermore, it preserves transparency and does not require sterile conditions for monitoring the establishment of symbiosis. These last two features allow live monitoring of the establishment of the symbiosis and benchtop manipulation. As in the case of pot cultures, the use of soil in culturing AMF means that no results can be observed until the soil is wet-sieved or the roots are analyzed to assess the propagation of the fungus. The establishment of the symbiosis cannot be assessed until several weeks after inoculation. The rate of successful establishment depends on the quality and the amount of inoculum used to start the culture. The rate is very low when a single spore is inoculated. This makes it necessary to prepare several culture replicates and take care of them for several weeks without knowing the number of single-spore cultures that will become established. For instance, Tchabi et al. (2010) initiated about 400 cultures inoculated with a single spore (representing nine species of AMF) using a micropipette tip as a micro-pot culture. Seven months later, only 25% of the pot cultures subsequently inoculated with substrate from the micropipette tips resulted in symbiosis establishment. Using the slide method of inoculation (a single nonsterile spore is deposited on a sterilized filter paper mounted on a glass slide and soaked in a solution of plant root exudates and sterilized Hoagland's nutrient solution), Selvakumar et al. (2016) obtained only 6/150 (4%) germinated spores of either *C. lamellosum* or *G. margarita*.

Working under non-sterile conditions considerably simplifies the system set-up and the maintenance of the

cultures. Under *in vitro* conditions, any modification of the plastic wares such as the Petri dishes must be done either with sterile tools under a laminar flow hood or the modified plastic wares were sterilized before the culturing system is assembled. Some autotrophic *in vitro* systems such as the one developed by Voets et al. (2005) require that a hole be drilled in the side of the Petri dish with a flame-sterilized scalpel blade. The Petri dishes are usually made of polystyrene, and melting this material with a hot blade produces toxic fumes. If the plastic wares are modified under non-sterile conditions, they need to be gamma irradiated because plastic cannot be autoclaved. Facilities offering this type of service are not available everywhere, and sterilization of the material must be planned ahead. In autotrophic *in vitro* systems, the hole in the side of the Petri dish also represents an open door for microbial contamination and must be hermetically and permanently sealed with sterilized silicon grease. Similarly, the system developed by Silvani et al. (2019) necessitates working under sterile conditions. This system is set up in a two-compartment Petri dish: one compartment contains a Ri T-DNA transformed carrot root growing on M medium and the other compartment is filled with sterile soil. This system has the disadvantages of both the *in vitro* Petri dish system (sterility) and the *in vivo* pot cultures (opacity). The rationale behind these two culture systems is to combine the monoxenic conditions in Petri dishes with a fully autotrophic host (Voets et al. 2005) and an edaphic compartment similar to natural soils (Silvani et al. 2019). These two elements are essential for creating optimum conditions for propagating AMF. In addition to an autotrophic host and two compartments with distinct edaphic properties, the SAP-based autotrophic system goes further by providing the fungal symbiont with a clean but non-sterile environment. Therefore, it is not necessary to sterilize the spores. Spore sterilization is a laborious step that can impair the viability of the spores, either because the spores do not survive the baths of antibiotics and sodium hypochlorite or because microorganisms important for spore germination are missing. While some bacteria can survive the sterilization process and promote spore germination and pre-symbiotic mycelial growth (Bidondo et al. 2016) Mugnier and Mosse (1987) noticed that “*only when contaminants developed in the agar medium did the spores [of Glomus [sic] mosseae] germinate.*” The production by actinomycetes of volatile compounds such as geosmin, CO₂ and 2-methylisoborneol were found to trigger the germination of *Gigaspora margarita* spores (Carpenter-Boggs et al. 1995). Therefore, the presence of AMF microbial partners is important for the pre-symbiotic steps, and non-sterile conditions may be essential for AMF propagation.

The biotic and abiotic conditions required for spore germination and AMF propagation remain poorly documented

and are likely to vary among species depending on their natural habitats (Daniels and Trappe 1980). In monoxenic cultures, the living components consist solely of the fungus and the Ri T-DNA transformed root and the abiotic conditions are homogeneous. These conditions accommodate only a limited number of species. For instance, the in vitro cultures of AMF available from international collections (BCCM, CCAMF) are strongly biased toward species within the genus *Rhizophagus*. Other species of AMF do not germinate or stop propagating after a few generations. The CCAMF regularly tests the ability of various species isolated from pot cultures to propagate on Ri T-DNA carrot roots. While sterilized spores of species in the genera *Ambispora*, *Diversispora*, *Funneliformis*, *Paraglomus* and *Septoglomus* occasionally germinate, they do not colonize the Ri T-DNA transformed roots. Sterilized spores of species in the genera *Acaulospora*, *Claroideoglomus*, *Dentiscutata* and *Gigaspora* can germinate and colonize the Ri T-DNA transformed roots, but their propagation is unstable and usually does not last for more than two or three subcultures.

In the present study, the rate of germination varied according to the species tested (0–80%) in the SAP-based autotrophic system. The highest germinability of spores was observed with *R. irregularis* and *R. intraradices*. The spores of *R. irregularis* were obtained from the commercial product Agtiv Potato L while those of *R. intraradices* were from an in vitro culture and were likely the same age and physiologically ready for germination. The spores of the other species were obtained from pot cultures maintained in greenhouse over several months (up to three years), and it is therefore possible that the ones used for inoculation varied in age and physiological state. These spores were stored in sterile water at 4 °C for at least four weeks (up to 10 weeks) prior to inoculation in the SAP-based autotrophic system. Juge et al. (2002) showed that cold stratification longer than 14 days significantly increased spore germination for *R. irregularis* (DAOM 197198). However, cold stratification is not the only factor known to increase spore germination. Tchabi et al. (2010) showed that a three-months storage under air-dried conditions contributed to successfully achieve single-spore cultures for the nine species of AMF, including *Acaulospora* and *Claroideoglomus* species. None of the spores of *C. lamellosum* germinated in the SAP-based autotrophic system. It is not known whether the storage conditions prior to inoculation or the biotic and abiotic conditions of the SAP-based autotrophic system were adequate or if the spores were viable. Selvakumar et al. (2016) also observed a very low rate of germination with only a single germinated spore of *C. lamellosum* out of 150 single-spore trials (these 150 single-spore trials also included spores of *G. margarita* and the exact number of spores from each species was not reported). In the present study, the average rate

of colonization was 36% following the inoculation with a single spore. The analysis of the pH showed that the conditions on SAP were relatively neutral (6.7 to 7.4), although pH tends to increase over time. If this becomes an issue, the pH could be stabilized with 2-[N-morpholino]ethanesulfonic acid (MES) buffer or SAP can be simply replaced with new polymer. The pH values recorded in SAP are known to support and promote germination (Dalpé et al. 2005).

Racocetra fulgida and *Sclerocystis* sp. preferentially propagated in the vermiculite compartment, at least during the first three months following the inoculation. This suggests that the grains of SAP are not a universal substrate for the propagation of AMF, and some AMF species may require different conditions. Other types of SAP can also be used, as not all the commercial SAP products are equivalent. The SAP-based autotrophic system is customizable and can be easily adapted to accommodate a large diversity of AMF. For instance, the propagation of AMF with different host plants and nutrient solutions can be easily tested. Recent studies showed that *R. irregularis* (Sugiura et al. 2020) and *R. clarus* (Tanaka et al. 2022) can grow on medium supplied with fatty acids or lipids under asymbiotic conditions. The hydration of the grains of SAP with a solution containing palmitoleic acid or myristate could improve the spore production in the SAP-based autotrophic culture. However, this could also foster contamination under non-sterile conditions. With the mMS-1 or mMS-2 solution, contaminants (green algae, bacteria, fungi) were occasionally observed but they could not spread due to the shortage in nutrients.

5 Conclusion

The SAP-based autotrophic system is a reliable and affordable technique for initiating single-spore cultures and it has a higher rate of success than some older methods. While more species of AMF should be tested, this technique is a promising tool for the production of single-spore cultures and viable inoculum for subcultures. This system has the potential to produce spore inoculum either in hydrated or dehydrated grains and it could facilitate the conservation of AMF. The transparency and non-sterile conditions of this culture system make it a versatile tool for studying the biology of AMF (spore germination, competition between species, host preference) under various experimental conditions. Finally, in addition to their water storage function, the grains of SAP can also be used as biofertilizer since the SAP-based autotrophic system has also the potential to produce thousands of spores of *R. irregularis* using basic laboratory equipment.

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