



Short communication

A method to obtain monosporic cultures of arbuscular mycorrhizal fungiS. Fracchia^a, A. Menendez^a, A. Godeas^a, J.A. Ocampo^{b,*}^a*Department Ciencias Biológicas, 4° II Pabellón, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina*^b*Department of Microbiology, Estación Experimental del Zaidín, Professor Albareda 1, 18008 Granada, Spain*

Received 11 April 2000; received in revised form 24 July 2000; accepted 30 November 2000

Abstract

We describe here a simple technique for obtaining monosporic cultures of AM fungi. *Gigaspora rosea*, *Gi. sp.*, *Glomus mosseae* and *G. sp.* were used. One surface-sterilized spore was transferred to a 5-cm diameter Petri dish with 10 ml of 10 mM MES buffer, plus 0.04 g of Gel-Gro. The contents of a vermiculite–perlite (1/1, v/v) dish with 2-week-old clover seedlings were transferred onto the Gel-Gro medium with the germinated spore. All plants inoculated with single germinated spores developed mycorrhizas. With this system, non-destructive observation of development of hyphae, formation of fan-like structures, hyphal contact and penetration of AM fungi into the plant root was possible. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Arbuscular mycorrhizas; *Gigaspora*; *Glomus*; Monosporic culture

Arbuscular mycorrhizas (AM), the most widespread symbioses on earth, are receiving attention because of the increasing range of their application in sustainable agriculture and ecosystem management (Bethelenfalvay and Schuepp, 1994). Since AM fungi are obligate symbionts most experiments have been done by using pot culture inoculum derived from surface-disinfected spores of a single AM fungus on a host plant grown in a sterilized medium. However, since many pot cultures of AM fungi have been generated from multiple spores, they may contain more than one strain of fungus. Single spore culture isolates of AM fungi can be a valuable resource, not only for plant growth experiments, but also for taxonomic and biochemical studies. Several techniques for establishing single spore isolate used germinated and ungerminated spores (Hepper, 1984; Brundrett and Juniper, 1995). However, these techniques did not allow the observation of development of hyphae, formation of a fan-like structure, hyphal contact and penetration of AM fungi into the plant root, establishing that the plant had become colonised. New approaches to the study of the biology of AM fungi have also been developed involving growing these fungi in Ri T-DNA transformed roots cultures in which some AM fungus species develop profusely and form viable spores (Becard and Piche, 1990). Transformed roots were obtained by inoculation with *Agrobacterium rhizogenes* carrying the Ri T-plasmid. However,

these techniques are labour consuming, are limited to few AM fungus species, and tend to have a low rate of success.

We describe here a simple technique for obtaining monosporic cultures of AM fungi that allows non-destructive observation of penetration of plant roots by the mycelia produced by one single spore.

Plant seedlings were grown in 5-cm diameter Petri dishes with 10 ml of autoclaved (120°C, 20 min) vermiculite–perlite mixture (1/1, v/v). The vermiculite and perlite were previously sieved through 500 µm mesh. Clover (*Trifolium repens*), known to be good host for AM fungi, was used as a test plant. Seeds were surface-sterilised with 10% sodium hypochlorite for 2 min and 30 seeds were sown on each vermiculite:perlite Petri dish. Plants were grown in a chamber with Sylvania incandescent and cool-white lamp 400–700 nm, with a 16/8 h day/dark cycle at 25/19°C and 50% relative humidity. Plants were regularly watered and fed with 1 ml of a diluted (1/2) Long-Ashton nutrient solution (Hewitt, 1952).

AM spores were isolated by the wet sieving (700, 250, 100 and 50 µm meshes) and decanting technique (Gerde-mann, 1955). The clamidospores of *Glomus mosseae* (Fracchia et al., 1998), and *Glomus sp.* that had clamidospores which were morphologically different from *Glomus mosseae*, and the azygospores of *Gigaspora sp.* were isolated from soil of the Ciudad Universitaria farm (Buenos Aires, Argentina). The soil (pH 6.5, 1/1, soil/water method) contained (mg kg⁻¹): 34.9 P (NaHCO₃-extractable), 127 N, and 23 K, and consisted of (%): sand, 40.8; silt, 43; clay,

* Corresponding author. Fax: +34-58-129600.

E-mail address: jocampo@eez.csic.es (J.A. Ocampo).

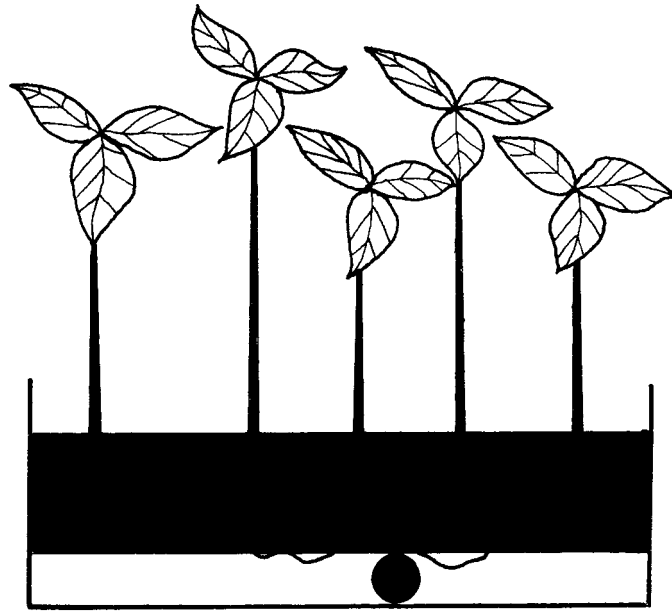


Fig. 1. Vermiculite–perlite in dish (1) with 2-week-old clover seedlings (2) upon Gel-Gro medium (3) with one germinated spore (4).

15.5 and 1.1 organic matter. *Trifolium repens*, *Shorgum halepensis* and *Wedelia glauca* were the most common native plants of this soil. The azygospores of *Gigaspora rosea* were obtained from the Banque Européenne des Glomales (BEG no. 9). All spores were surface sterilized with 50 ml of sterile water plus 1 g of chloramine T together with 20 μg of streptomycin and a trace of surfactant (Mosse, 1962). The spores were rinsed with sterile water. One surface-sterilized spore was transferred with a sterilized Pasteur capillary pipette to a 5-cm diameter Petri dish with 10 ml of 10 mM 2-(N-morpholin) ethane sulphonic acid (MES) buffer (pH7) plus 0.04 g of Gel-Gro (ICN Biochemicals, Aurora, OH, USA). There were 25 replicates each per AM strain. Petri dishes were incubated at 25°C for 8 days and spore germination and hyphal development were observed under a binocular microscope. Petri dishes contaminated with other microorganisms were discarded. Ten Petri dishes with hyphal length about 5 mm for *Glomus* or 2 cm for *Gigaspora* strains were selected. The contents of a vermiculite–perlite dish with 2-week-old clover seedlings were transferred onto the Gel-Gro medium with the germinated spore (Fig. 1). There were controls without spores. The hyphal development was observed every 2 days through the bottom of the Petri dish under the binocular microscope. After 3 weeks of growth in the chamber, the content of one dish was put into one pot. There were 10 replicate pots for controls and for each AM fungal species. The pots contained 500 ml of soil that was steamed at 100°C three times at 24 h intervals and mixed (1/1, v/v) with sterilized (120°C, 20 min) quartz sand. The soil from Ciudad Universitaria farm was the same as described before. Plants were watered from below and fed with a Long-Ashton nutrient solution at 10 ml per week (Hewitt, 1952). After 4 and 8 weeks plants

were harvested, the roots in the 10 replicate pots were cleared and stained (Phillips and Hayman, 1970), and the percentage of root length colonisation was assessed by the grid line intersect method (Giovannetti and Mosse, 1980). The means were compared using standard errors of means.

Germination of *Gi. rosea* and *Gi. sp.* were observed after 2 days of inoculation and hyphal length reached 15–20 mm after 3–4 days. *G. mosseae* and *G. sp.* germinated after 4–5 days and the hyphal length was near 5 mm after 7–8 days. When the Gel-Gro with the germinated spores was in contact with the clover roots, a fan like structure of the hyphae was observed after 10 days for *Glomus* and after 7 days for *Gigaspora*. At this time, hyphal contact with the plant root was observed (Fig. 2).

No root colonization of control plants in Petri dishes with vermiculite and Gel-gro but without spores was observed. All plants inoculated with single germinating spores developed mycorrhizas. After 4 weeks of growth in soil pots, roots of clover plants reached colonization values of 22 ± 3 , 20 ± 4 , 17 ± 3 and $16 \pm 4\%$ (mean \pm standard error, $n = 10$) when inoculated with *Gi. rosea*, *Gi. sp.*, *G. mosseae* and *Glomus sp.*, respectively. After 8 weeks, clover plants had 50 ± 6 , 52 ± 8 , 48 ± 4 and $45 \pm 7\%$ (mean \pm standard error, $n = 10$) of root colonised by *Gi. rosea*, *Gi. sp.*, *G. mosseae* and *Glomus sp.*, respectively. After 4 and 8 weeks of growth, control plants had no root colonization.

This method permits the making of a monosporic culture of AM fungi with a high percentage of success. The viscosity of the Gel-Gro allowed optimal spore germination and contact of the spores with the plant root. With this system the observation of development of hyphae, formation of a fan-like structure, hyphal contact and penetration of AM fungi into the plant root was possible. This monoxenic

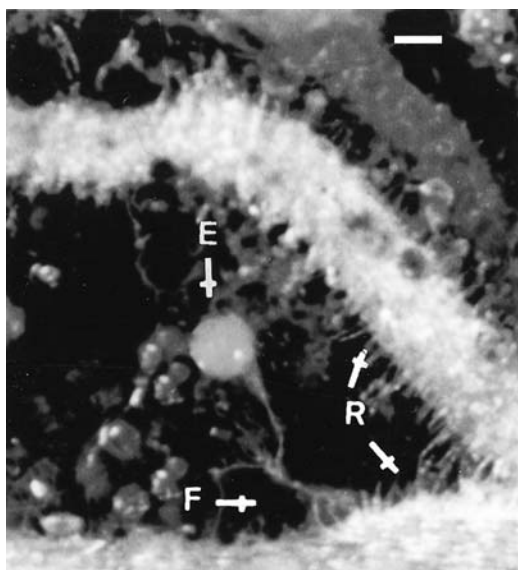


Fig. 2. Fan-like structure of hypha (F) of *Gigaspora rosea* (E) in contact with clover roots (R). Scale bar represents 150 μm .

culture technique developed could be useful for studying preinfection stages, since the plants could be examined frequently under low magnification and the growth of the fungus in the rhizosphere observed. Material for a fine structure study on the early stages of infection could be obtained in this way since the time of formation of any particular entry point can be established fairly accurately. These observations also allow one to establish that the test plant became colonised with a high probability of success. Since a single spore can successfully infect a plant, this provides a way of starting a pure line of an AM fungus by transplanting the infected seedling to a soil and allowing new spores to form (Menge, 1984). In this way intraspecific variation in

the fungus can be studied, and this would provide a valuable technique for taxonomic studies.

References

- Becard, G., Piche, Y., 1990. Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and nonhost Ri T-DNA transformed roots. *Canadian Journal of Botany* 68, 1260–1264.
- Bethelenfalvay, G.J., Schuepp, H., 1994. Arbuscular mycorrhizas and agro-system stability. In: Gianinazzi, S., Schuepp, H. (Eds.). *Impact of Arbuscular Mycorrhizas in Sustainable Agriculture and Natural Ecosystems*. Birkhauser Verlag, Basel, pp. 117–131.
- Brundrett, M., Juniper, S., 1995. Non-destructive assessment of spore germination of VAM fungi and production of pot cultures from single spores. *Soil Biology & Biochemistry* 27, 85–91.
- Fracchia, S., Mujica, M.T., García-Romera, I., García-Garrido, J.M., Martín, J., Ocampo, J.A., Godeas, A., 1998. Interactions between *Glomus mosseae* and arbuscular mycorrhizal sporocarp-associated saprophytic fungi. *Plant and Soil* 200, 131–137.
- Gerdemann, J.W., 1955. Relation of a large soil-borne spore to phycomycetous mycorrhizal infections. *Mycologia* 47, 619–632.
- Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* 84, 489–500.
- Hepper, C.M., 1984. Isolation and culture of VA mycorrhizal (VAM) fungi. In: Powell, C.L., Bagyaraj, D.J. (Eds.). *VA Mycorrhiza*. CRC Press, Boca Raton, FL, pp. 95–112.
- Hewitt, E.J., 1952. Sand water culture methods used in the study of plant nutrition. Commonwealth Agricultural Bureau, Technical Communication No. 22.
- Menge, J.A., 1984. Inoculum production. In: Powell, C.L., Bagyaraj, D.J. (Eds.). *VA Mycorrhiza*. CRC Press, Boca Raton, FL, pp. 187–204.
- Mosse, B., 1962. The establishment of vesicular arbuscular mycorrhiza under aseptic conditions. *Journal General Microbiology* 27, 509–520.
- Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* 55, 158–161.