ELSEVIER

Soil Biology & Biochemistry 40 (2008) 1259–1263

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Short Communication

A simple method to obtain endophytic microorganisms from field-collected roots

V.A. Silvani*, S. Fracchia, L. Fernández, M. Pérgola, A. Godeas

Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 4to piso Pabellón 2, 1428 Buenos Aires, Argentina

Received 31 August 2007; received in revised form 14 November 2007; accepted 20 November 2007
Available online 9 January 2008

Abstract

We describe a simple technique for obtaining endophytic microorganisms from field-collected roots. Roots of plant species were surfaced-sterilized, cut into pieces, and then each root piece was transferred to drops of Gel-Gro medium. The number of segments with bacteria and fungi was quantified and those with bacteria and dark septate endophytic (DSE) fungi were plated onto tryptic soy agar (TSA) and malt extract agar (MEA), respectively. When arbuscular mycorrhizal fungi (AMF) grew from root segments, they were inoculated into Ri T-DNA-transformed carrot root cultures. Eight different species of bacteria and five dark septate endophytes were isolated. Thirty AMF isolates were identified and continuous pure cultures established. This easy and inexpensive approach allowed us to culture various endophytic microorganisms in an *in vitro* system saving time and space.

Keywords: Root endophytes; AMF; DSE; Bacteria; *In vitro* culture; Field roots

Root tissues form a morphologically, physically, and chemically complex microcosm that provides habitats for diverse communities of microorganisms (Sieber and Grünig, 2006). Roots are colonized by bacteria, endophytic fungi, and mycorrhizal fungi (Brundrett, 2006). Many groups of such organisms are important as sources of novel biologically active secondary metabolites and could be used as microbial inoculants to improve plant growth and plant health (Linderman, 1994; Adhikari et al., 2001; Schulz et al., 2002; Mandyam and Jumpponen, 2005; Kloepper and Ryu, 2006). The isolation procedure is a critical and important step in obtaining these microorganisms.

In this paper, we propose a simple technique to obtain root bacterial and fungal endophytes from field roots, with special emphasis on how to acquire monoxenic cultures of arbuscular mycorrhizal fungi (AMF).

The study area was located in a natural field near Ciudad Universitaria (Buenos Aires, Argentina). The rhizosphere had the following characteristics: pH 6.5 (1/1, soil/water method), 34.9 mg kg^{-1} P ($NaHCO_3$-extractable), 127 mg kg^{-1} N, and 23 mg kg^{-1} K and consisted of sand 40.8%, silt 43%, clay 15.5%, and 1.1% organic matter.

The following plant species, *Picris echioides*, *Sonchus oleraceus*, *Bidens laevis*, *Solidago chilensis*, *Hypochoeris* sp., *Ricinus communis*, *Melilotus alba*, *Trifolium repens*, *Medicago lupulina*, *Paspalum dilatatum*, and *Sorghum halepense* were collected and immediately processed. Root samples of at least two individuals of each plant species were stained with trypan blue solution according to the method of Phillips and Hayman (1970), and the percentage of mycorrhizal root length was calculated by the grid line intersect method (Giovannetti and Mosse, 1980). Only plant roots with high colonization were used. The remaining roots were washed under tap water and adhered particles of soil were removed using a fine paintbrush.

Healthy roots with visible intraradical structures, such as vesicles (Fig. 1A), melanized septate hyphae and microsclerotia (Fig. 1B), were selected with the aid of a stereomicroscope, surface-sterilized under a laminar-flow bench with 3% (v/v) NaOCl and Tween 80 (3 drops ml^{-1}) for 3 min in Falcon® tubes, rinsed three times with sterile distilled water and cut into 3-mm pieces. Each root fragment was transferred with sterile tweezers to a Petri

*Corresponding author. Tel.: +54 11 4576 3300.
E-mail address: vsilvani@bg.fcen.uba.ar (V.A. Silvani).

0038-0717/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2007.11.022

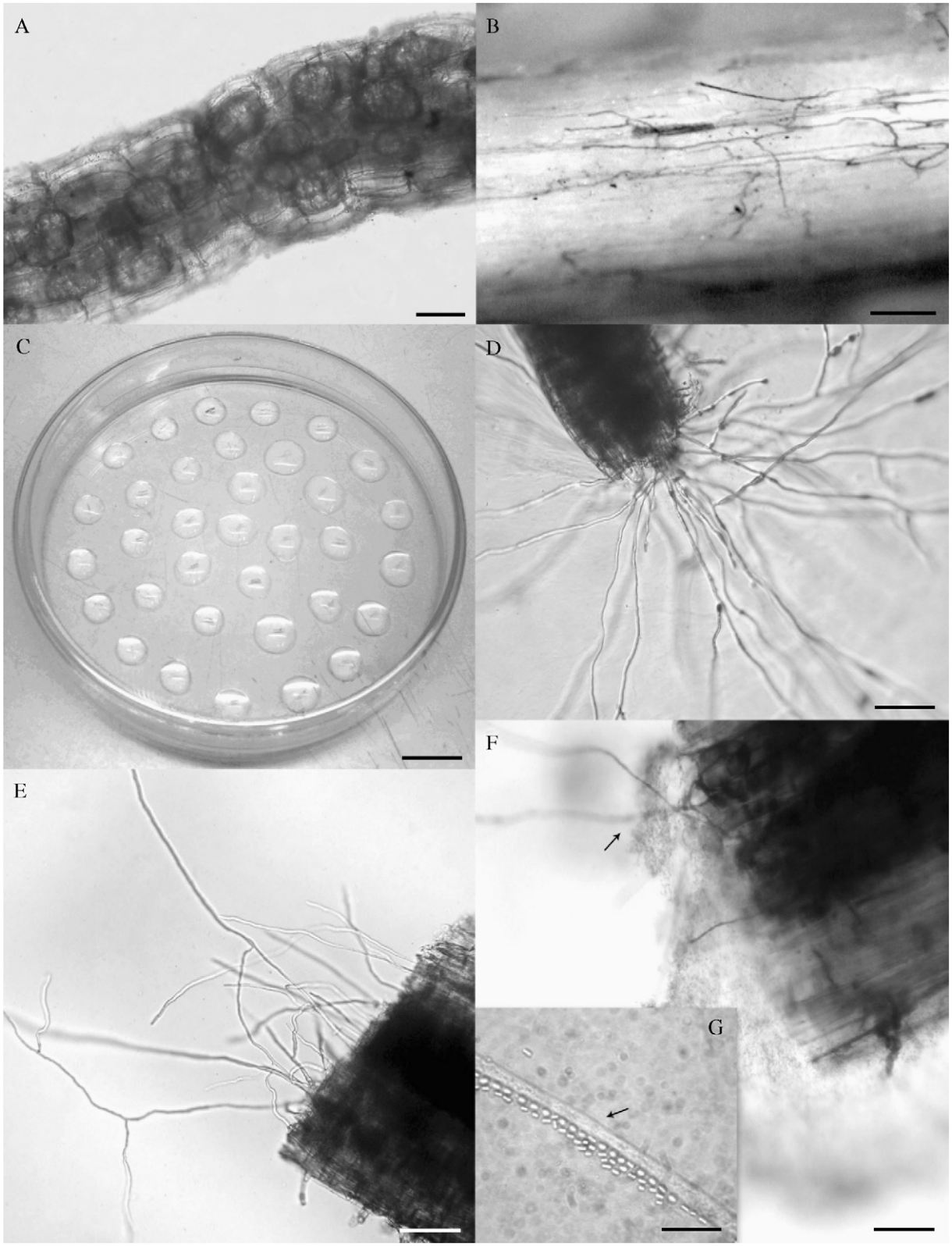

Fig. 1. (A) Fragment of field-collected root of *T. repens* with vesicles and spores of AMF bar = 100 μm. (B) Microsclerotia and dark septate hyphae in a field root segment of *P. dilatatum*. Bar = 200 μm. (C) Petri dish containing drops of Gel-Gro medium with incubated root segments. Bar = 2 cm. (D) DSE hyphae arising from root fragment of *P. dilatatum*. Bar = 200 μm. (E) AMF hyphae arising from cut end of root fragment of *R. communis*. Bar = 200 μm. (F) Bacteria and AM hyphae (arrow) emerging from root fragment of *S. chilensis*. Bar = 200 μm. (G) *Bacillus* sp. growing along the AMF hyphae (arrow). Bar = 20 μm.

dish (diameter, 15 cm) containing 35–40 drops of 0.35% (w/v) Gel-Gro® (ICN Biochemicals Aurora, OH, USA) plus 0.03% $MgSO_4$ (pH 6) (Fig. 1C). Root fragments were incubated at 25 °C in the dark, and after 4 days, each fragment was checked for microorganisms emerging from cut ends using a binocular microscope (32× and 60× magnification) (Fig. 1D–F). When re-growth took place, root fragments with bacteria or dark septate endophytic (DSE) fungi were planted in tryptic soy agar (TSA) or malt extract agar (MEA), respectively. Root fragments with AMF only (Fig. 1E) were inoculated in Ri T-DNA transformed carrot root cultures (T-roots) and incubated in an inverted position at 25 °C in the dark (Bécard and Fortin, 1988; Declerck et al., 1998; Fortin et al., 2002).

Development of extraradical mycelia and spores of AMF was monitored every week. As soon as the first spores were differentiated, they were transferred to fresh T-roots for continuous culture.

A variation in this method allowed us to decrease the bacterial contamination in both AMF and DSE isolations (Table 1). The disinfected roots were submerged in a solution of antibiotics (Streptomycin 200 mg l^{-1}, Ampicillin 200 mg l^{-1} and Tetracycline 100 mg l^{-1}) for 15 min, and then rinsed three times in sterile water. The Gel-Gro medium was supplemented with the antibiotic solution (1.25 ml l^{-1}) and used as above.

We found colonization by AMF and DSE in most collected plant species (Table 1). Members of the Asteraceae and *R. communis* showed a higher percentage of AMF colonization than grasses. Regrowth of AMF from root segments of *S. oleraceus*, *B. laevis*, and *R. communis* and emergence of numerous non-septated straight hyphae

Table 1
Percentage values of mycorrhizal root length of plant species, incubated root fragments with AMF and/or other fungi, bacteria, AMF and/or other fungi plus bacteria, root fragments with hyphal re-growth of AMF as the only endophyte, and number of isolates obtained in the *in vitro* system with two different treatments of surface sterilization

Host	Mycorrhizal root length (%)	Root fragments with (%)			Root fragments with AM hyphal regrowth (%)	No. of isolates obtained *in vitro* pure cultures
		AMF and/or other fungi	Bacteria	AMF and/or other fungi + bacteria		
Sterilization treatment: 3% NaOCl						
Picris echioides	74	28.4	11	43.3	6.2	3
Sonchus oleraceus	85	39.6	13.8	36.2	24.2	4
Bidens laevis	52.6	15	27.7	10.9	0.5	1
Solidago chilensis	79.3	13.4	31.3	26.8	1	0
Trifolium repens	87.4	10.3	8.2	6.2	0	0
Medicago lupulina	65.8	1.5	35	3.6	0	0
Melilotus alba	51	5.7	15.5	61.5	0	0
Ricinus communis	76	7.6	47.2	15.5	1	1
Sorghum halepense	61	3.5	22.8	61.4	0	0
Paspalum dilatatum	59.5	13.4	19	66.4	1.6	0
Sterilization treatment: 3% NaOCl + antibiotic						
Picris echioides	74	67.8	3.2	9	20.8	0
Hypochoeris sp.	75	46	2	2	10	0
Bidens laevis	65.4	30.3	0	1.2	9.2	6
Solidago chilensis	65.5	75.2	0	3.7	3.8	0
Trifolium repens	89	64	2.5	3.8	0	0
Medicago lupulina	54.7	24.2	0.6	0.6	9	6
Melilotus alba	45.7	16	12	10	6	2
Ricinus communis	68.5	39.5	0.5	1	9.1	6
Sorghum halepense	47.8	40.7	0	0	0	0
Paspalum dilatatum	50.9	84.9	0	6.4	0.5	1

from the cut ends of root pieces were observed already after one day of incubation (Fig. 1E). Root segments from *M. alba*, *M. lupulina*, *P. dilatatum* and *P. echioides* developed fewer hyphae after 3–4 days. No regrowth of AMF from roots of *T. repens* and *S. halepense* was observed. This finding shows that roots of different plant species with the same origin not always generate a similar number of fungal isolations and that not all root-inhabiting AMF are easily established in monoxenic cultures (Dalpe et al., 2005).

Since a high number of root segments with endophytic bacteria were found using hypochlorite as the disinfectant solution, we consider that this method is a powerful means for the isolation of endophytic bacteria. Bacteria associated with AMF mycelium from inside the roots of many host plants species (Fig. 1F and G) were growing together after the isolation; therefore, this system could allow us to study the interactive relationships between both endophytic microorganisms, where species interaction can be isolated and controlled. For example, adding antibiotics, as it was mentioned above, in the root disinfection process and the Gel-Gro medium increased the number of root fragments with AMF hyphae regrowth and other fungi, most likely because of the antagonistic interactions between bacteria and fungi (Krechel et al., 2002; Cao et al., 2005; Dalpe et al., 2005).

Seven out of 11 collected plant species were useful to obtain *in vitro* AM cultures. We obtained five *Glomus intraradices* strains from *S. oleraceus* and *R. communis*, and 25 *Glomus* sp. from *P. echioides*, *B. laevis*, *M. lupulina*, *M. alba*, *R. communis* and *P. dilatatum*, thus demonstrating that intraradical structures of AMF in root pieces were good inocula to establish monoxenic cultures. The AMF completed their life cycle in the *in vitro* cultures and maintained their viability.

Furthermore, we isolated DSE from roots of *P. dilatatum*. Diversity studies based on sampling and staining roots have shown that DSE are found in most monocot species (Peterson et al., 2004; Weishampel and Bedford, 2006).

The results presented above verify that the presence of intraradical vesicles, melanized septate hyphae, and microsclerotia facilitates the selection of colonized roots by AMF and DSE (Diop et al., 1994; Klingner et al., 1995; Diop, 2003).

However, successful sterilization is critical, since it must be sensitive enough to recover endophytes and to eliminate organisms present in the rhizoplane (Hallmann et al., 2006).

The methodology proposed is inexpensive and permits sampling of many roots at the same time. Culturing the root fragments in little drops of clear and colorless gellan gum in a single Petri dish provides an easy non-destructive way to observe endophytic microorganism regrowth under a binocular microscope. Moreover, drops act like a physical barrier for endophytic bacterial and fungal growth, thus preventing cross-contamination. The high-strength gel allows an optimal growth of mycelium, and the adding of antibiotics permits the obtention of a high number of monoxenic cultures.

Our method could be useful both to elucidate the composition and ecology of different root endophytic microorganisms, including AMF, and to use field-collected roots as starter inocula for *in vitro* cultures of mycorrhizal fungi and other microorganisms.

In addition, it overcomes many of the difficulties present in the classical method of root-trap culture with model host plants, because it reduces the time lapse required to obtain monoxenic cultures. Another advantage over the classical root-trap cultures is that the inoculum is originated from the living intraradical mycelia, thus reflecting the diversity of root-infecting AMF. Finally, however, we believe that both methods are complementary for understanding fungal endophytic diversity, because not all AMF species have the capacity to complete their life cycle with success under monoxenic conditions.

We thank the UBA, CONICET and ANCYPT for financial support and R. Larry Peterson for his critical review of this manuscript. Comments and suggestions of two anonymous reviewers have greatly improved this manuscript.

References

Adhikari, T.B., Joseph, C.M., Yang, G., Phillips, D.A., Nelson, L.M., 2001. Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice. Canadian Journal of Microbiology 47, 916–924.

Bécard, G., Fortin, J.A., 1988. Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytologist 108, 211–218.

Brundrett, M.C., 2006. Understanding the roles of multifunctional mycorrhizal and endophytic fungi. In: Schulz, B.J.E., Boyle, Ch.J.C., Sieber, T.N. (Eds.), Microbial root endophytes. Springer, Berlin, pp. 107–132.

Cao, L., Qiu, Z., You, J., Tan, H., Zhou, S., 2005. Isolation and characterization of endophytic streptomycete antagonists of *Fusarium* wilt pathogen from surface-sterilized banana roots. FEMS Microbiological Letters 247, 147–152.

Dalpe, Y., de Souza, F.A., Declerck, S., 2005. The monoaxenic culture of arbuscular mycorrhizal fungi as a tool for systematics and biodiversity. In: Declerck, S., Strullu, D.-G., Fortin, J.A. (Eds.), In vitro Culture of Mycorrhizas. Springer, Berlin, pp. 31–48.

Declerck, S., Strullu, D.G., Plenchette, C., 1998. Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90, 579–585.

Diop, T.A., 2003. In vitro culture of arbuscular mycorrhizal fungi: advances and future prospects. African Journal of Biotechnology 12, 692–697.

Diop, T.A., Plenchette, C., Strullu, D.G., 1994. Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5, 17–22.

Fortin, J.A., Bécard, G., Declerk, S., Dalpé, Y., St-Arnaud, M., Coughlan, A.P., Piché, Y., 2002. Arbuscular mycorrhiza on root-organ cultures. Canadian Journal of Botany 80, 1–20.

Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring VA mycorrhizal infection in roots. New Phytologist 84, 489–500.

Hallmann, J., Berg, G., Schulz, B., 2006. Isolation procedures for endophytic microorganisms. In: Schulz, B.J.E., Boyle, Ch.J.C., Sieber, T.N. (Eds.), Microbial Root Endophytes. Springer, Berlin, p. 299.

Klingner, A., Hundeshagen, B., Kernebeck, H., Bothe, H., 1995. Localization of the yellow pigment formed in roots of gramineous plants colonized by arbuscular fungi. Protoplasma 185, 50–57.

Kloepper, J., Ryu, Ch.M., 2006. Bacterial endophytes as elicitors of induced systemic resistence. In: Schulz, B.J.E., Boyle, Ch.J.C., Sieber, T.N. (Eds.), Microbial Root Endophytes. Springer, Berlin, pp. 32–52.

Krechel, A., Faupel, A., Hallmann, J., Ulrich, A., Berg, G., 2002. Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Meloidogyne incognita* (Kofoid and White) Chitwood. Canadian Journal of Microbiology 48, 772–786.

Linderman, R.G., 1994. Role of VAM fungi in biocontrol. In: Pfleger, F.L., Linderman, R.G. (Eds.), Mycorrhizae and Plant Health. APS Press, St. Paul, pp. 1–26.

Mandyam, K., Jumpponen, A., 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. Studies in Mycology 53, 173–189.

Peterson, L.R., Massicotte, H.B., Melville, L.H., 2004. Mycorrhizas: Anatomy and Cell Biology. NRC Research Press, Ottawa and CABI Publishing, pp. 57–79.

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. Transaction British Mycological Society 55, 158–161.

Schulz, B., Boyle, C., Draeger, S., Römmert, A., 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research 106, 996–1004.

Sieber, T.N., Grünig, Ch.R., 2006. Biodiversity of fungal root-endophyte communities and populations, in particular of the dark septate endophyte *Phialocephala fortinii* s.1. In: Schulz, B.J.E., Boyle, Ch.J.C., Sieber, T.N. (Eds.), Microbial Root Endophytes. Springer, Berlin, pp. 107–132.

Weishampel, P.A., Bedford, B.L., 2006. Wetland dicots and monocots differ in colonization by arbuscular mycorrhizal fungi and dark septate endophytes. Mycorrhiza 16, 495–502.