World J Microbiol Biotechnol
DOI 10.1007/s11274-009-0065-8

SHORT COMMUNICATION

Transformed soybean (*Glycine max*) roots as a tool for the study of the arbuscular mycorrhizal symbiosis

Laura Fernández · Vanesa Silvani · Josefina Bompadre · Mariana Pérgola · Alicia Godeas

Received: 15 February 2009 / Accepted: 7 May 2009

Abstract Ri T-DNA transformed roots have been used effectively in studying the interaction between various plant hosts and arbuscular mycorrhizal (AM) fungi. We investigated the in vitro monoxenic symbiosis between the AM fungus *Glomus intraradices* and transformed soybean roots (TSRs). Comparisons were made between TSR system and plants of the same genotype. The extraradical fungal structures generated in vitro culture showed normal development. Straight runner hyphae branched into short simple branched absorbing structures and spores were initiated. AM symbiosis was confirmed by the presence of arbuscules and vesicles in cortical cells of the TSRs. The frequency of intraradical colonization in TSRs was higher than in plants grown in soil, whereas the intensity values of intraradical colonization in TSR cultures were similar to those in whole plants. These results show that TSR cultures were able to support the growth and characteristic development of *G. intraradices*.

Keywords Transformed soybean roots · Monoxenic culture · *Glomus intraradices*

Introduction

Soybean (*Glycine max* (L.) Meer.) is the major crop in global agricultural production, widely cultivated in the US, Brazil, Argentina, China, and India. In Argentina, the soybeans continues to increase its importance as the principal agricultural crop and export product, with the result of more cultivatable land being devoted to its growth (Secretaría de Agricultura, Ganadería, Pesca y Alimento 2008).

There is a need to better understand the mechanisms of mycorrhizal symbioses in order to manage the use of AM fungi in agricultural practices (Labour et al. 2003). Although there is some available information concerning the establishment of AM symbioses in soybeans (Bethlenfalvay et al. 1982; Schenck and Smith 1982), monoxenic in vitro root cultures colonized by an AM fungus have not been reported.

Transformed root organ cultures (ROCs) are frequently used to simplify the study of host-AM fungal interactions (Bago et al. 1998; Fortin et al. 2002). However, host-AM monoxenic cultures are criticized because of their non-natural characteristics (Bago and Cano 2005). Comparisons of results obtained with ROCs and whole plants carrying the same genotype grown in soil could address this criticism.

Although induction of hairy roots on soybean explants mediated by *A. rhizogenes* for the study and propagation of soil nematodes has been described (Savka et al. 1990; Cho et al. 2000), they have never been used for the establishment of soybean-AM symbiosis in vitro. This system could simplify the study of interaction responses between soybean roots and AM fungi; furthermore its development could be easily monitored under this culture condition.

The main objective of this approach was to make use of the transformed soybean roots (TSRs) model system for the establishment of monoxenic culture and characterization of colonization by the AM fungus *Glomus intraradices* Schenck & Smith. A secondary objective was to compare the internal colonization of the root system of plants of the

L. Fernández (✉) · V. Silvani · J. Bompadre · 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
e-mail: laurafbidondo@yahoo.com.ar

Published online: 22 May 2009

same genotype grown in greenhouse conditions with that of the TSRs. We further demonstrated the advantage of the split-plate system (St-Arnaud et al. 1996) for the cultivation of AM species characterized by their fast growth rate and slower growth of soybean ROCs.

Materials and methods

Plant material and inoculation procedure

Seeds of three soybean genotypes, XP8900RG, AG043RG and NX4430RG (NIDERA S.A.), with a long, medium and short crop cycle duration, respectively were used. Seeds were surface-sterilized with 70% v/v of ethanol solution for 15 min, 20% v/v of sodium hypochlorite solution plus Tween 20 (0.1%) for 20 min, and rinsed three times with sterile distilled water. Seeds were then transferred with sterile tweezers to a Petri plate containing drops of 0.35% w/v Gel-Gro® (ICN Biochemicals, Aurora, OH, USA) plus 0.03% w/v $MgSO_4$ (pH 6; Silvani et al. 2008), and incubated in continuous (70 µE per $s.m^{-2}$) at 25°C for 3 days to induce their germination and cotyledon development .

Inoculation with *A. rhizogenes* strain LBA9402 was carried out to induce soybean hairy roots according to Cho et al. (2000). Cells of strain LBA9402 harbor a Ri plasmid with the oncogene *rolC*, one of the genes that promote root formation (Palazón et al. 1998). Inoculum of LBA9402 was prepared by growing the bacteria in shake liquid Luria Bertani (LB) medium for 48 h at 30°C. The abaxial surface of 3-day-old cotyledons was wounded several times with a sterile scalpel previously dipped into the overnight bacterial culture, and wounded cotyledons were then incubated with the abaxial side up on moist filter paper at 25°C in the dark. After 3 days of co-culture, cotyledons were transferred to Petri plates containing MXB medium, composed of MS basal nutrient salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968) and 2% sucrose (pH 5.7) solidified with 0.35% w/v Gel-Gro®. Ampicillin (1,000 mg ml^{-1}) was then added to inhibit the growth of *A. rhizogenes*. Three passages with antibiotics were made on the same medium in order to obtain bacteria-free roots. Some of the cotyledons were inoculated with an autoclaved bacterial culture and used as controls; as well as root apexes collected from germinating seedling grown in MXB solid medium were used as control treatment.

Emerging roots were excised when they reached 15–20 mm in length and placed individually on Petri plates (10 cm in diameter) filled with fresh MXB solid medium without antibiotics to initiate the establishment of each root line. The growth and adaptation of the initial lines to the in vitro culture conditions were compared. Their geotropism was evaluated by incubating the Petri plates placed upside down; premature aging and development of calli were qualitatively evaluated during an 8-week period in MXB medium. In addition, root growth was observed in minimum medium (MM) traditionally used for AM fungal cultures (Bécard and Fortin 1988). Four replicates for each root line were incubated at 25°C in the dark.

Polymerase chain reaction (PCR) analysis of TSRs

After complete removal of *A. rhizogenes*, hairy root tissues were harvested and genomic DNA was extracted using the CTAB procedure (Doyle and Doyle 1990). Plasmid DNA of strain LBA9402 was isolated using the SDS/alkaline-lysis method (Sambrook et al. 1989) and used as a positive control for PCR analysis. Each DNA sample was used as the template to run PCR for testing the presence of the *rolC* gene, one of the oncogenes involved in the hairy root induction process (Tanaka et al. 2001). The oligonucleotide primers for amplification were *rol1* 5′-ATG GCT GAA GAC GAC CTG TGT-3′(Tm: 64°C) and *rol2* 5′-GCC GAT TGC AAA CTT GC ACT C-3′(Tm: 64°C). The PCR mixture consisted of 200 ng of template DNA, 0.4 µM of each primer, 200 µM dNTPs, 2.5 mM $MgCl_2$, 2U *Taq* polymerase, and *Taq* buffer (1:10 v/v) in a total volume of 25 µl. PCR conditions for amplification of the *rol*C gene consisted of an initial denaturing cycle (94°C, 5 min), 30 amplification cycles (94°C, 1 min; 60°C, 1 min; 72°C, 1.5 min), and a final elongation cycle (72°C, 5 min). After amplification, PCR products were examined by electrophoresis on a 1.0% w/v agarose gel, and detected by fluorescence under UV light after staining with ethidium bromide.

Inoculum of *G. intraradices* (GA5)

To obtain viable spores and mycelia of *G. intraradices* a monoxenic culture of strain GA5 provided by *Banco Glomeromycota* In Vitro (BGI, FCEyN, University of Buenos Aires, Argentina) was routinely grown in association with Ri T-DNA transformed carrot roots in MM and incubated in an inverted position at 25°C in the dark (Bécard and Fortin 1988). A co-culture in two-compartment Petri plates with the distal root-free side lacking sucrose was used for enhancing spore production as described by St-Arnaud et al. (1996). After 3 months, spores in the distal compartment and colonized roots from the proximal compartment were recovered and both forms of inoculum were used for colonization of soybean roots.

In vitro two-compartment culture system

Two-compartment 9-cm-diameter Petri plates were used as growth systems for both symbiosis partners. An appropriate

growth medium (MXB) for soybean root culture was placed in one of the compartments and a fresh actively growing 10-cm-long root explant was added, while the other compartment was filled with MM for an optimal AM fungal development. Soybean roots were allowed to grow in both compartments. Inoculation of TSRs was carried out by placing a 1-cm^3 plug of medium from the distal compartment (containing ~200 spores and abundant external hyphae) and a 1-cm^3 plug from the proximal compartment (containing fragments of colonized roots, 300 spores and external hyphae) in the vicinity of the soybean root explants on MM side. Cultures were incubated at 25°C in the dark for 8 weeks.

Spore germination, re-growth of intraradical hyphae, and development of the extraradical mycelium network were assessed periodically during incubation, and possible contaminations were monitored. Non-destructive observations were carried out under a stereobinocular microscope and the lengths of roots and AM fungal extraradical mycelium were measured for each plate using the grid intersect method (Marsh 1971). The number of newly formed spores was also assessed by counting 10 cells of 1 cm^2 for each replicate.

Establishment of the AM symbiosis was checked at the 8th week; roots from the MM side were carefully removed, cleared in KOH (10% w/v 15 min, 90°C) and stained with trypan blue in lactic acid (0.02% 10 min, 90°C; modified from Phillips and Hayman 1970). Intraradical colonization was quantified by examination of 50 randomly selected root pieces, in groups of ten, and the frequency (*%F*) of mycorrhizal colonization was calculated as the percentage of root segments containing hyphae, arbuscules or vesicles (Declerck et al. 2004). Also, the intensity (*%I*) of colonization was estimated as described by Declerck et al. (1998). All measurements were taken under a Nikon light binocular microscope at a 100× magnification.

In vivo assay

Additionally, seeds of the AG043RG genotype, were surface-sterilized and germinated on moist filter paper for 48 h. Soybean seedlings were grown in pots with 500 g of an autoclaved (100°C for 1 h, three consecutive days) mixture of 1:1:1 soil, perlite, vermiculite, and were inoculated by placing the same amount of inoculum of GA5 than in vitro assay (1-cm^3 plugs containing colonized roots, hyphae and spores from a previus monoxenic culture) on the roots of each soybean seedlings. Every pot was irrigated with 50 ml of Hewitt (1952) nutritive solution twice and routinely grown under greenhouse conditions with ambient natural light and temperature conditions. Mycorrhizal colonization was microscopically assessed at the 8th week as previously described.

Image production and data analysis

Photographs of soybean hairy root cultures, extraradical structures, and intraradical development of strain GA5 were taken using a Nikon Coolpix950 digital camera. Images were digitalized and assembled using Adobe Photoshop CS3.

Results of intraradical colonization in TSRs and soybean whole plants were analyzed using the Fisher's protected LSD test ($P < 0.05$).

Results

Selection and culture of TSRs

Callus development was observed at some infection sites of the cotyledon explants inoculated with bacteria, and roots began to differentiate from this tissue after 10 days (Fig. 1a). Six TSR lines were obtained from AG043RG seeds (SM1–SM6), five from XP8900RG seeds (SL1–SL5) and three from NX4430RG seeds (SS1–SS3). These lines were able to grow on solid hormone-free medium without exogenous bacterial development after the *A. rhizogenes* transformation and purification process.

After 8 weeks of culture, some roots of each line showed either a slow growth rate or no growth at all next to the excision from their original explants, whereas others continued to grow vigorously. All the hairy roots lines tested, exhibited a plagiotropic growth. In addition, some roots with different degrees of aging, including development of calli and yellowing, were observed at this time.

Otherwise, no roots emerged from cotyledons in control treatments. Comparisons observed between untransformed and transformed soybean roots resulted that, TSRs lines produced more numerous lateral root branches with white root apexes, instead of untransformed roots that did not grow at all at this time in MXB without addition of phytohormones.

Considering the fast expansion on solid MXB medium as well as the slow yellowing and callus formation of the TSR SM3 line (Fig. 1c) in comparison with the other root lines, the SM3 line was selected to characterize and compare the *G. intraradices* interaction in in vitro and in vivo conditions. Amplification of the *rol*C gene was positive for the selected TSR SM3 line (Fig. 1b).

The TSRs SM3 lines cultivated in MM were less vigorous than those cultivated in MXB. Also, morphological differences were observed in both media; the root apex was wider in MXB (315 ± 19.15 µm) than in MM (200 ± 16.33 µm), the elongation zone seemed to be shorter, and root hairs more dense in MXB than in MM (Fig. 1d–g).

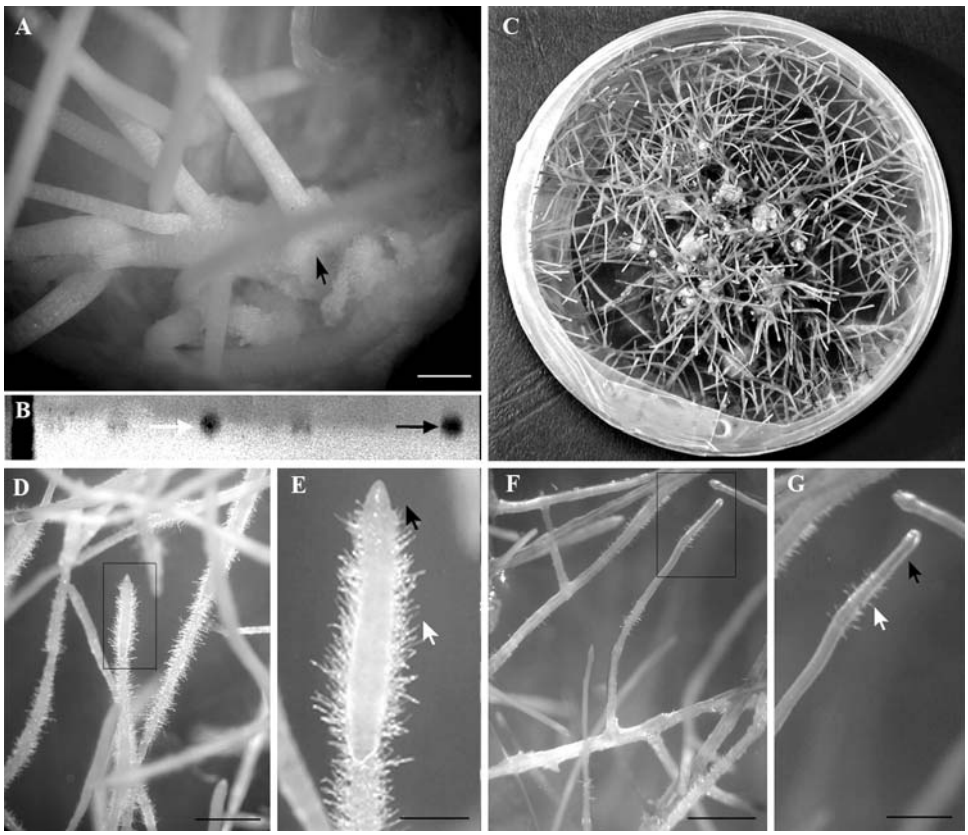

Fig. 1 **a** Induction of soybean hairy roots from cotyledon callus tissue (*black arrow*) 10 days after infection with *A. rhizogenes* (Bar: 2 mm). **b** PCR detection of *rolC* (550 bp) DNA from SM3 transformed roots (*white arrow*); DNA from plasmid LBA9402 (*black arrow*). **c** Culture of SM3 after bacterial decontamination 8 weeks after inoculation in fresh MXB. Petri dish is 10 cm in diameter. **d** Line SM3 of soybean hairy roots after 8 weeks growth in MXB medium. **f** Line SM3 of soybean hairy roots after 8 weeks growth in MM medium (**d** and **f** Bar: 600 µm) **e** Detail of root in box of (**d**) showing short elongation zone (*black arrow*) and many root hairs (*white arrow*). **g** Detail of root in box of (**f**) showing long elongation zone (*black arrow*) and few root hairs (*white arrow*) (**e** and **g** Bar: 300 µm)

Mycorrhizal receptiveness and development

Both types of AM fungal inocula used generated new hyphae that spread and contacted the TSRs. The development of extraradical mycelium began with straight runner hyphae forming short simple branched absorbing structures (BAS; Fig. 2a–c). At 8 weeks, the length of the AM extraradical mycelium was 418.45 ± 270.65 cm/cm^2, an average of six new healthy spores per cm^2 was produced, and the length of TSRs growing in MM was 349.81 ± 83.63 cm/cm^2.

Pre-infective hyphae generated from GA5 inoculum grew parallel to roots and formed several appressoria and many entry points; arbuscules and intraradical longitudinal hyphae were also developed (Fig. 2d–f). However, the presence of vesicles was less commonly observed. At the time of harvest, the frequency (*%F*) of soybean root colonization in MM by strain GA5 was 66 ± 20.74 and the intensity (*%I*) of colonization was 24.04 ± 13.41. Similar results (Table 1) were obtained for intensity (*%I*) of intraradical colonization of the AG043RG soybean whole plants grown in greenhouse conditions (20.60 ± 1.05), and the frequency (*%F*) was lower under in vivo conditions (24.14 ± 0.607) than in in vitro cultures of TSR SM3.

Discussion

Numerous roots were successfully generated from soybean cotyledons after applying the transformation method mediated by *A. rhizogenes* as previously described by Cho et al. (2000). The variability in growth shown in the various roots lines in this study, including the poor growth of some lines, could have been due to variations in the site of insertion, the T-DNA copy number, and the genome of the originally transformed cells (Tanaka 1997).

The described transformation protocol has some advantages: it is simple and requires little time allowing the possibility of obtaining TSR cultures able to subculture for many generations and to host root endophytes such as AM fungi.

It has been estimated that an unbalanced hormonal condition in hairy roots caused by the integration of genes from *A. rhizogenes* into the genome of infected plant tissues may affect the normal functioning of AM symbioses (Barker and Tagu 2000). Our attempts to obtain in vitro untransformed soybean roots have been unsuccessful. The failure to subculture them could be due to the rapid aging of excised soybean roots on solid media. As a result, genetic transformation of soybean roots was necessary to keep continuous cultures.

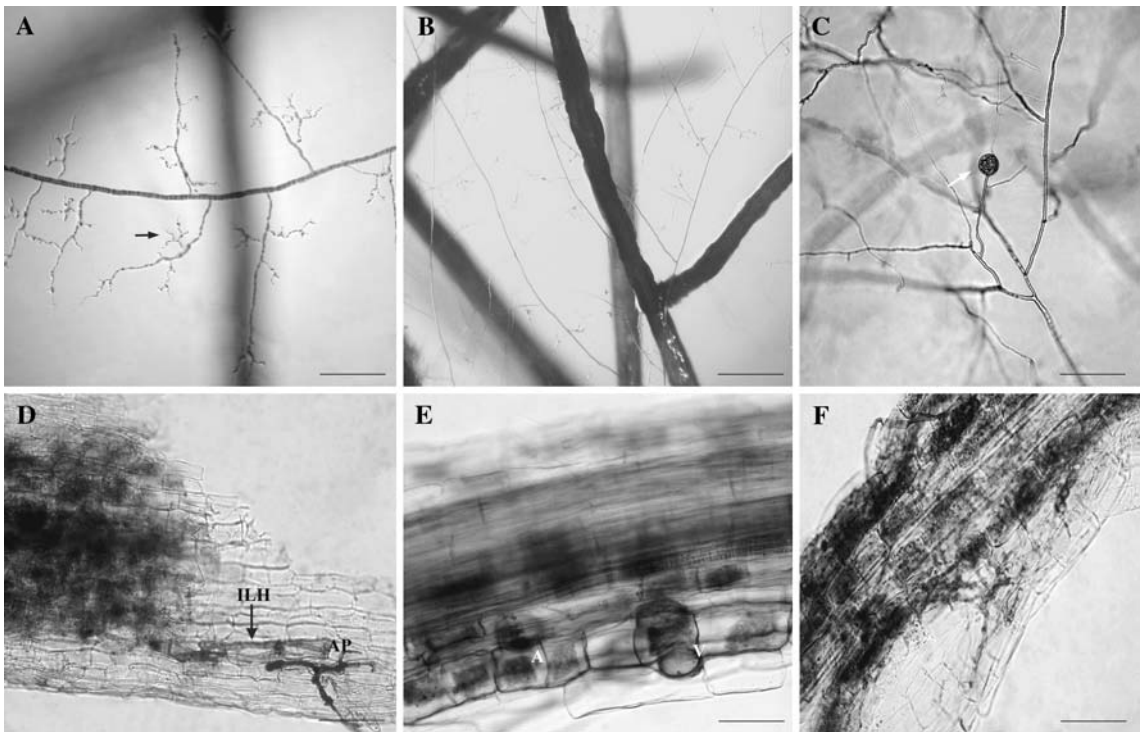

Fig. 2 **a–c** Extraradical structures of *G. intraradices* (GA5) formed in association with transformed soybean roots (*TSRs*), line SM3 in MM medium. **a** Runner hyphae producing lateral simple branched absorbing structures (*BAS*) (*black arrow*). **b** Extraradical mycelium developing next to TSRs. **c** newly formed spore with a normal appearance (*white arrow*) (All bars: 100 μm). **d–e** Intraradical colonization of *G. intraradices* (GA5) on TSRs in vitro. **f** Intraradical colonization of *G. intraradices* (GA5) on soybean roots grown in pots. *A* arbuscules, *AP* appressoria, *ILH* intraradical longitudinal hyphae, *V* vesicles. (Bars **d** and **e**: 150 μm, Bar **f**: 120 μm)

Table 1 Comparison between intraradical colonization values by GA5 in transformed soybean roots in vitro cultures and in soybean whole plants grown in greenhouse conditions

Intraradical colonization	Soybean AG043RG	
	TSR SM3 line	Soybean whole plants
%F	66 ± 1.14 a	24.14 ± 0.607 b
%I	24.04 ± 0.73 a	20.60 ± 1.05 a

Each number is the mean of five observations ± standard error. Mean values in the same row followed by different letters are significantly different (LSD test, $P < 0.05$)

Most in vitro experiments characterizing AM symbiosis have been carried out with carrot (*Daucus carota* L.) hairy root lines established by Bécard and Fortin 1988. Hairy root cultures of other species including tomato (*Solanum lycopersicum* L.; Labour et al. 2003) and legumes such as *Medicago truncatula* Gaertn. (Boisson-Dernier et al. 2001), *Pisum sativa* L. (Balaji et al. 1994) and *Trifolium repens* L. (de Souza and Berbara 1999), have also been developed. All of them have been genetically transformed by *Agrobacterium rhizogenes* with the insertion of T-DNA into the plant genome.

To date, only few legume transformed roots have been achieved and associated with AM fungus under in vitro conditions (Cranenbrouck et al. 2005). In spite of the increasing importance of soybean crops in agricultural production and the benefits of microorganisms such as AM fungi, to our knowledge, there are no reports of monoxenic TSR cultures of AM fungi. In the present study, we obtained TSRs that successfully hosted *G. intraradices* and established an AM symbiosis with high rates of root colonization. Moreover, no differences were found in the morphological features of intra-and extraradical fungal structures in comparison with the monoxenic culture of *G. intraradices* fungus previously described by Cano and Bago (2006). Therefore, this finding has added a new interesting legume model plant and demonstrated that TSRs may serve as another tool for AM symbiosis research.

The use of two-compartment Petri plates allowed an adequate development of TSRs on the MXB side and a normal symbiosis establishment on the MM side. The AM fungus *G. intraradices* strain GA5 associated with TSRs showed a limited development of extraradical mycelium and sporulation in comparison with other reports using different species of ROCs (Bago et al. 1998, 2006). Besides the moderate external colonization by *G. intraradices*, TSRs showed high internal colonization when compared to previous results on both untransformed and transformed

tomato roots (Chabot et al. 1992; Labour et al. 2003). The explanation for the differences found in both intra and extraradical fungal development is not clear. Possibly, the different behaviors observed between ROCs could be related with the selected explants, the physiological state of the host root, or the culture conditions; therefore, these parameters must be optimized in each culture system chosen to advance future research (Fortin et al. 2002).

Considering that TSRs were able to support colonization by *G. intraradices*, this system could be another useful tool for introducing AM fungal species that have never been cultured in in vitro conditions. In addition, preliminary results have shown that TSRs SM3 may also be suitable to be effectively colonized by other AM species such as *Gigaspora decipiens* Hall and Abbott (unpublished results).

We also found comparable results between TSRs in vitro and whole plants in in vivo conditions using the same plant genotype. The intensity values of colonization were similar in both systems, with a comparable development of intraradical structures by strain GA5. The enhanced frequency of colonization by AM fungi observed in TSRs could be explained by the closer proximity of propagules to the transformed roots in the limited space of Petri plates compared with the high extension of soybean roots growing in pots at the same time of harvest. Nevertheless, the values of frequency of intraradical colonization reached were similar with those reported in another published study for soybean plants growing under greenhouse conditions at the same harvest time (Nwoko and Sanginga 1999).

In conclusion, the ability of *G. intraradices* strain GA5 to complete its life cycle in association with the SM3 line soybean roots under in vitro conditions enhances the interest of this cultivation system for future biological studies and agronomic applications in this widely extended crop. Furthermore, the TSRs provide the opportunity to study interactions such as symbiotic associations with other rhizosphere microorganisms.

Acknowledgments We wish to thank to UBA, CONICET and ANCYPT for financial support. The authors acknowledge to Dr. Josefina Albergina of the University of Buenos Aires and to Dr. Elizabeth Agostini of the University of Rio Cuarto for technical assistance.

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