Mycorrhiza (1999) 8:335–338

SHORT NOTE

B. Vimard · M. St-Arnaud · V. Furlan · J.A. Fortin

Colonization potential of in vitro-produced arbuscular mycorrhizal fungus spores compared with a root-segment inoculum from open pot culture

Accepted: 25 December 1998

Abstract A reliable inoculum, free from other microorganisms, to produce arbuscular mycorhizal (AM) plants is of the greatest importance when studying the interaction between AM plants and soil microorganisms. We investigated the colonization of leeks from monoxenic in vitro-produced *Glomus intraradices* spores. The isolated spores were produced using a two-compartment in vitro growth system previously described. A spore suspension was used as inoculum and was compared to the inoculum potential of endomycorrhizal root segments of pot-grown leek (*Allium porrum* L.) plants. The leeks were grown in a controlled environment and two types of sterilized growth media were tested: calcined montmorillonite clay and a soil mix. Root colonization progressed faster in the soil mix than in the clay. However, in this medium, after an initial delay, root colonization from in vitro-produced spores was essentially the same as that observed with the root-segment inoculum, reaching 44% and 58% respectively, after 16 weeks. Leek roots colonized by the monoxenically-produced spores harbored only the studied AMF fungi while the roots colonized from the root segments were substantially contaminated by other fungi.

Key words Inoculum · Co-culture · Spore · Soil · Glomales · AM

B. Vimard (✉) · M. St-Arnaud · J.A. Fortin
Institut de Recherche en Biologie Végétale,
Université de Montréal and Jardin Botanique de Montréal,
4101 East, Sherbrooke St.,
Montréal, Québec, Canada H1X 2B2
Fax: +1-514-872-9406, e-mail: vimardb@magellan.umontreal.ca,
Tel.: +1-418-877-1867

V. Furlan
Centre de Recherche, Agriculture et Agroalimentaire Canada,
Sainte-Foy, Québec, Canada G1V 2J3

Introduction

Arbuscular mycorrhizal fungi (AMF) propagules exist as spores, living hyphae, isolated vesicles, mycorrhizal root segments or colonized soil (Biermann and Linderman 1983; Diop et al. 1994; Sylvia and Schenck 1983). Until now, root segments and spores isolated from open pot culture (Gilmore 1968) of AM-inoculated plants have been the usual source of AM inoculum for research purposes (Ferguson and Woodhead 1982). However, this type of inoculum requires a large space for production and is prone to contamination even with good phytosanitary care (Ames and Linderman 1978). Production of propagules under aseptic conditions remains one of the most promising ways of obtaining the high quality pathogen-free inoculum desirable for research purposes.

In vitro culture of AMF was achieved for the first time in the early 1960s (Mosse 1962). Since this pioneering work, the use of Ri T-DNA-transformed *Daucus carota* L. (carrot) roots as the host has permitted increases in spore production of *Glomus mosseae* (Mugnier and Mosse 1987), *Gigaspora margarita* (Bécard and Fortin 1988) and *Glomus versiforme* (Declerck et al. 1996). Isolation of the fungal symbiont from the plant root partner, using a two-compartment Petri dish, is the most successful way to date of achieving large production of spores (Fortin et al. 1996; St-Arnaud et al. 1996) of *Glomus intraradices*, making these propagules even more attractive for mass-production of inoculum.

It is essential to develop a suitable method to assess root colonization potential of such propagules. The purpose of this paper was to evaluate the ability of in vitro-produced *Glomus intraradices* spores to colonize *Allium porrum* L. (leek) seedlings roots, compared with vesicle-bearing root segments in two different substrates.

Materials and methods

Experimental design

Seedlings of *A. porrum* L. were grown in pots inoculated with AM-colonized root segments or in vitro produced spores. The root colonization by *Glomus intraradices* Schenck & Smith was studied during 16 weeks in calcined montmorillonite clay (Turface, IMC Imcore, Mundelein, Ill.) or in a soil mix. The experimental design was a split plot in five blocks with the two growth media randomized among the main plots and the two inoculum types randomized among the subplots. The different blocks were inoculated sequentially at weekly intervals. Treatments were replicated five times.

Inoculum preparation

The root-segment inoculum consisted of AM leek roots from open pot culture colonized at 82% of root length with *G. intraradices* Schenck & Smith (DAOM 181602), mixed with the growth medium in which plants were grown; this contained 60 spores/g fresh wt. The same isolate of *G. intraradices* was grown in vitro on Ri T-DNA-transformed *D. carota* L. roots in a two-compartment Petri dish as described by St-Arnaud et al. (1996). The colonized gel from the root-free compartment of a 7-month-old colony was aseptically cut into 1-cm^2 pieces to prepare the aseptic spore inoculum. Gel pieces were stirred and solubilized in 100 ml of a citrate buffer solution (Doner and Bécard 1991) at 30 °C for 15 min. The mycelium suspension was filtered over a 0.45-μm nylon mesh (Micron Separation Inc., Fisher Scientific). The spores collected on the mesh were rinsed with sterile distilled water, transferred into a sterile blender and homogenized in 30 ml of sterile distilled water for 40 s at low speed in order to separate clumped spores. The homogenate concentration was adjusted to 15 spores/ml. The spore viability was determined using 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide, MTT bioassay (An and Hendrix 1988) on four subsamples each time a new spore inoculum was prepared (each experimental block) and the mean viability was calculated.

Leek seedlings preparation

Seeds of *A. porrum* 'Bleu de Solaise' were surface sterilized for 20 min in 1.8% sodium hypochloride solution, rinsed twice with sterile distilled water and sown in 30-cm^2 plastic flats filled with sterile Turface (autoclaved twice with a 2-day interval for 1 h at 121 °C) and then saturated with Long Ashton nutrient solution (Hewitt 1966). Seedlings were allowed to grow for 3 weeks before use.

Inoculation of plants, growth conditions and measurements

Plastic pots (15 cm) were disinfected by soaking overnight in a 1.8% sodium hypochlorite solution and thoroughly rinsed with water before use. Pots were filled with 1.2 l of Turface or with a soil mix prepared by mixing equal parts of a peat-based medium (Pro-Mix, Tourbières Premier, Rivière-du-Loup, Canada), Perlite, Turface and soil from a leek field (61% sand, 22% limestone, 17% clay, 4% organic matter, pH 5.7; Yao Konan 1996). Both media were autoclaved twice for 1 h (121 °C) with a 2-day interval in 4-l plastic autoclavable bags, and then saturated with Long Ashton nutrient solution. This procedure has been repeatedly used in our laboratory to kill AMF propagules in growth substrates. Plants subsequently grown without AMF inoculation never show signs of AM colonisation. Therefore, in order to save space in growth cabinets, non-inoculated controls were omitted in this specific experiment. In the pots for the root-segment inoculum treatment, 10 g fresh wt. of inoculum was uniformly spread to form a layer 2 cm under the substrate surface, and 35 leek seedlings were transplanted in each pot. In the pot for the in vitro spores inoculum, the seedlings were inoculated by dipping the roots in the spore suspension before transplanting and spreading 100 ml of the spore suspension into the substrate.

The plants were maintained in a growth cabinet, and received 120 ml of Long Ashton nutrient solution per pot every second week until the end of the experiment; they were watered as needed with distilled water between fertilization. Fluorescent lamps were operated for a 16-h day at a photon flux of 450 $\mu E \cdot m^{-2} \cdot s^{-1}$ (PAR). Relative humidity was 60–70% and temperatures were 23 °C (day) and 16 °C (night). The five different experimental blocks were potted and inoculated at 1-week intervals. Three seedlings were randomly harvested from each pot 4, 8, 12 and 16 weeks after inoculation. The root system of each seedling was examined separately in order to obtain three estimates of root colonization in each pot at each harvest. The roots were washed under tap water, cut into 1-cm sections, cleared in 10% KOH and stained with acid fuchsin (Kormanik and McGraw 1982). The percentage of root length colonized by *G. intraradices* or by unidentified contaminant fungi was determined using the gridline intersect method (Giovannetti and Mosse 1980) under a dissecting microscope at ×80 magnification. Only the root segments containing arbuscules or vesicles were considered colonized by *G. intraradices*.

Statistical analyses

Statistical analyses were performed with General Linear Model Procedures of SAS software (SAS Institute Inc 1992). The effects of substrate and inoculum treatments were analyzed by ANOVA. Rank transformations were performed on percentages of *G. intraradices*-colonized root length and on percentages of root length harboring unindentified fungal mycelium 4 weeks after inoculation, while arcsine transformation (Draper and Smith 1981) was performed on the percentages of root length harboring unidentified fungal mycelium 16 weeks after inoculation in order to meet the requirements of the tests. AM colonization of roots 4 weeks after inoculation was not statistically analyzed because no transformation satisfied the requirements of the test. The statistical model used included block, inoculum, substrate and the required interaction effects, but for the purpose of the multiple comparisons of root colonization levels between the four inoculum by substrate combinations, these two treatments were considered as four levels of one single treatment and a posteriori comparisons were done by Tukey's studentized range tests.

Results

In vitro-produced spores were highly viable with a mean of 96.4% of spores stained in the MTT bioassay. Inoculum type and growth media had significant effects ($P < 0.05$) on leek root colonization 8–16 weeks after inoculation. No significant interaction was found between inoculum type and growth media.

Four weeks after inoculation, the plants which received the root-segment inoculum were colonized at a level of 20% of root length in Turface and at 30% in the soil mix substrate, while those inoculated with the spores had 0.2% of colonized root length in the soil mix but showed no traces of AMF colonization in Turface (Table 1). All treatments showed traces of colonization by 8 weeks. Sixteen weeks after inoculation, plants grown with the spore inoculum in the soil mix medium had an AMF colonization level similar to those with the root-segment inoculum in both substrates.

Table 1 Effect of substrate and inoculum type on root colonization of leek seedlings by *Glomus intraradices* and unidentified contaminant fungi. Values in each column followed by the same letter are not significantly different at $P<0.05$ by ANOVA

Substrate	Inoculum type	Colonization by *G. intraradices* (%) Weeks after inoculation				Colonization by contaminant fungi (%) Weeks after inoculation			
		4[a]	8	12	16	4	8	12	16
Turface	Root segments	20.2	34.6b	34.2c	40.5b	16.1b	19.6b	17.2b	11.8b
	In vitro spores	0.0	0.5a	0.3a	0.0a	1.5a	4.2a	0.8a	0.7a
Soil mix	Root segments	30.8	54.0c	65.8d	58.3c	26.3b	11.4b	9.8b	8.1b
	In vitro spores	0.2	4.5a	18.5b	43.5b	1.1a	0.6a	1.1a	1.1a

[a] Data from the 4-week sampling for the root length colonized by *G. intraradices* were not submitted to ANOVA analysis because transformation did not lead to a normal distribution of the residuals

Inoculum type had a significant effect ($P<0.01$) on percentages of leek root length harboring unidentified fungi at all harvesting times, while growth media had no effect. No interaction was found between inoculum type and growth media.The use of the root-segment inoculum led to a mean colonization with unidentified fungi of 14–16%, while the use of the in vitro-produced spore inoculum generated roots with 1–1.8% of root length containing visually detectable contamination (Table 1).

Discussion

Until now, monoxenically-produced spores had never been conclusively tested for their ability to perform well in soil (Lovato et al. 1995). We demonstrate high viability of in vitro-produced spores in this experiment, as well as their ability to induce AM colonization in a soil medium. The aseptically-produced inoculum yielded endomycorrhizal leek plants with 44% of root length colonized by *G. intraradices* 16 weeks after inoculation, which is comparable to the level reached with a root-segment inoculum. The use of an in vitro-produced spore inoculum insures that only the AMF studied is inoculated in designed treatments.

One possible major drawback of in vitro-produced spore inoculum is the need to maintain subcultures on agar media. Questions may be raised about the infectivity and genetic stability of these cultures after many years. Laiho (1970), as well as Marx and Daniel (1976), have shown that ectomycorrhizal fungi can lose their ability to colonize plant roots after several years of subculture. Recently, Plenchette et al. (1996) reported a decrease in infectivity of *in vitro*-produced *Glomus versiforme* spores and mycorrhizal root pieces to colonize leek plant roots after only three generations. Infectivity loss during successive generations was not directly estimated in this experiment. However, subculturing *G. intraradices* did not seem to adversely affect spore viability or infectivity. The in vitro-produced spores used were harvested from a 30th subculture, yet viability was very high and root colonization, after an initial delay, was comparable to non-in vitro-produced inoculum providing soil substrate was used. The delay observed before the spore inoculum began to colonize leek plants may be related to an innate dormancy of spores, since viability was higher than 95% as measured with the MTT test. Similar differences between spores and root segments were previously reported by Warner and Mosse (1980) and Biermann and Linderman (1983). It was suggested that the root segments were readily infective because they contain active hyphae with intraradical vesicles, structures that probably needed no physiological modification and had no nutritional or hormonal deficiencies (Powell 1976). On the other hand, spores may require a dormancy period before they can germinate and produce infective hyphae. Tommerup (1983) concluded that newly formed spores of *Glomus* spp. have an innate dormancy of 6 weeks in wet soil and that no environmental condition will shorten this maturation period before germination. This is in accordance with our results of an initial lag period of 4 weeks. Recent work in our laboratory demonstrating that a 6-week cold treatment of a *G. intraradices* spore suspension eliminates this delay before germination (Nantais 1997) increases interest in spores as inoculum. The results obtained with the spore inoculum emphasize the importance of the growth medium in AMF inoculation. Almost no colonization was observed with the in vitro-produced spore inoculum in Turface, while in the soil mix this inoculum led to the same infection level as the root-segment inoculum. The use of soil mix growth media to produce endomycorrhizal leeks in pot culture also clearly shortened the time needed to achieve a high level of AM colonization, regardless of inoculum type. These results are in accordance with previous reports (Biermann and Lindermann 1983; Schubert et al. 1990).

In conclusion, monoxenically-produced spores as AMF inoculum in a soil mix growth medium yielded an AM colonization level similar to a root-segment inoculum 16 weeks after inoculation, and with a much lower level of fungal contaminantion. This inoculum is thus more suitable for large scale production as well as for biochemical and molecular investigations of the AM symbiosis.

Acknowledgements We wish to thank S. Daigle for good advice on experimental design and statistical analysis, and M. Tessier and L. Nantais for linguistic revision of the manuscript. This research was supported by an NSERC operating grant to J.A. F.

References

Ames RN, Linderman RG (1978) The growth of easter lily (*Lilium longiflorum*) as influenced by vesicular-arbuscular mycorrhizal fungi, *Fusarium oxysporum*, and fertility level. Can J Bot 56:2773–2780

An ZQ, Hendrix JW (1988) Determining viability of endogonaceous spores with a vital stain. Mycologia 80:259–261

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Biermann B, Linderman RG (1983) Effect of container plant growth medium and fertilizer phosphorus on establishment and host growth response to vesicular-arbuscular mycorrhizae. J Am Soc Hortic Sci 108:962–971

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

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

Doner LW, Bécard G (1991) Solubilization of gellan gels by chelation of cations. Biotechnol Tech 5:25–28

Draper NR, Smith H (1981) Applied regression analysis. Wiley, New York

Ferguson JJ, Woodhead SH (1982) Increase and maintenance of vesicular-arbuscular mycorrhizal fungi. In: Schenck NC (ed) Methods and principles of mycorrhizal research. APS, St. Paul, Minn, pp 47–54

Fortin JA, St-Arnaud M, Hamel C, Chavarie C, Jolicoeur M (1996) Aseptic in vitro endomycorrhizal spore mass production. US Patent Number 5,554,530

Gilmore AE (1968) Phycomycetous mycorrhizal organisms collected by open-pot culture methods. Hilgardia 39:87–105

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 84:489–500

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Commonwealth Agricultural Bureau, London

Kormanik PP, McGraw AC (1982) Quantification of vesicular-arbuscular mycorrhizae in plant roots. In: Schenck NC (ed) Methods and principles of mycorrhizal research, APS St. Paul, Minn, pp 37–45

Laiho O (1970) *Paxillus involutus* as a mycorrhizal symbiont of forest trees. Acta For Fenn 106:1–13

Lovato PE, Schuepp H, Trouvelot A, Gianinazzi S (1995) Application of arbuscular mycorrhizal fungi (AMF) in orchard and ornamental plants. In: Varma A, Hock B (eds) Mycorrhiza. Springer, Berlin Heidelberg New York, pp 443–467

Marx DH, Daniel WJ (1976) Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. Can J Microbiol 22:338–341

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Nantais LM (1997) Optimization of arbuscular mycorrhizal inoculum through selection and increased production of *Glomus intraradices* propagules. MSc thesis, University of Montréal

Plenchette C, Declerck S, Diop TA, Strullu DG (1996) Infectivity of monoaxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri-T-DNA-transformed carrot roots. Appl Microbiol Biotechnol 46:545–548

Powell CL (1976) Development of mycorrhizal infections from Endogone spores and infected root segments. Trans Br Mycol Soc 66:439–445

SAS Institute Inc (1992) The SAS System for Windows 3.10, release 6.08. SAS Institute Inc, Cary, N.C.

Schubert A, Mazzitelli M, Ariusso O, Eynard I (1990) Effects of vesicular-arbuscular mycorrhizal fungi on micropropagated grapevine: influence of endophyte strain P fertilization and growth medium. Vitis 29:5–13

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332

Sylvia DM, Schenck NC (1983) Application of superphosphate to mycorrhizal plants stimulates sporulation of phosphorus-tolerant vesicular-arbuscular mycorrhizal fungi. New Phytol 95:655–661

Tommerup IC (1983) Spore dormancy in vesicular-arbuscular mycorrhizal fungi. Trans Br Mycol Soc 81:37–45

Warner A, Mosse B (1980) Independent spread of vesicular arbuscular mycorrhizal fungi in soil. Trans Br Mycol Soc 74:407–410

Yao Konan M (1996) Influence de différentes espèces de champignons endomycorhiziens sur la croissance et le rendement de cultivars d'oignon (*Allium cepa*) soumis à différentes conditions culturales. MSc thesis, University of Laval