In vitro Culture of Sheared Mycorrhizal Roots

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Abstract

Sheared-roots of two vesicular-arbuscular mycorrhizal (VAM) fungi, *Glomus intraradices* (Schenck et Smith) and *Glomus versiforme* (Karsten) Berch developed *in vitro* extensive extraradical structures in two media during three months without supplemented living host partner. The water agar medium (Difco bacto-agar) was more suitable to sporulation than minimal M medium where the mycelium was more profuse. An average of 60 mature spores of *Glomus intraradices* were produced in each Petri dish and were able to complete their life cycle in re-association with tomato root explants. The long-term behaviour of the extraradical phase from 0.5 cm of vesicular-arbuscular mycorrhizal root pieces is discussed regarding different hypotheses advanced for the achievement of pure cultures of VAM fungi.

1. Introduction

In recent years, there have been many attempts to cultivate obligate vesicular-arbuscular mycorrhizal (VAM) fungi in pure culture. Resting spores were used as starter material in most studies (Mosse and Hepper, 1975; Mugnier and Mosse, 1987; Bécard and Fortin, 1988). Growth of VAM fungi always

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ceased when the hyphal link to the root was broken. Factors such as nutrition (Hepper, 1983), chemical treatments (Gianinazzi-Pearson et al., 1989; Bécard and Piché, 1989, and genetical factors (Burggraaf and Beringer, 1989; Bécard and Pfeffer, 1993; Bianciotto and Bonfante, 1993) have been studied to explain the lack of growth of the extraradical phase of VAM fungi. Commonly used culture media revealed the positive roles of low amounts of phosphorus, the detrimental action of sodium sulfate on symbiosis establishment (Hepper, 1979) and the inhibiting effect of zinc and manganese concentrations in many commercial agars on spore germination (Hepper and Smith, 1976). Low amounts of natural or synthetized flavonoids positively stimulated fungal development (Gianinazzi-Pearson et al., 1989; Morandi, 1989; Chabot et al., 1992a). Otherwise, certain amino acids (Hepper and Jacobsen, 1983), volatile compounds, particularly carbon dioxide and root exudations, promote hyphal elongation (Carr et al., 1985; Elias and Safir, 1987; Bécard and Piché, 1989).

During the last decade, Strullu and Romand (1986, 1987) and Strullu et al. (1991) reported the ability of intraradical forms of *Glomus* spp. to initiate a saprotrophic growth in *in vitro* culture. On agar medium, isolated vesicles and hyphae produced vegetative mycelium able to form new typical VAM infections. However, no observations have yet been published for the long-term *in vitro* behaviour of the extraradical phase from sheared VAM roots. Using 0.5 cm long segments of two VAM roots, we tested (1) the *in vitro* ability of their extraradical phase to grow on two solidified media without host plants or living roots; (2) the long-term outcome of the extraradical phase; and (3) the effectiveness of spores produced.

2. Materials and Methods
Fungal materials

Allium porrum roots heavily colonized by *Glomus intraradices* and *Glomus versiforme* under greenhouse conditions were removed after six months. Selected VAM roots were delicately washed prior to surface desinfection. Surface sterilization was achieved in an ultrasonic apparatus under laminar flux by successive baths in ethanol 96 ℃ (10s), 6% calcium hypochlorite (1 min), 2% chloramine T (10 min) and 0.2% sulfate streptomycine plus 0.1% sulfate gentamycine (10 min). Then VAM roots were cut with scissors into 0.5 cm segments. The sheared-root inocula contained approximately 90 to 130 intraradical forms (chlamydospores or vesicles) of both fungi. The average diameter of intraradical chlamydospores of *G. intraradices* was 59 μm while that of intraradical vesicles of *G. versiforme* reached 43 μm. The experimental unit consisted of a culture of 0.5 cm leek roots colonized by *G. intraradices* or

G. versiforme in a Petri dish (5.5 cm diameter) containing either 15 mL of dis tilled water gelled with 0.8% Bacto-agar or the minimal M medium (Bécard and Fortin, 1988) which contained in mgL^{-1} distilled water: $MgSO_4 \cdot 7H_2O$, 731; KNO_3, 80; KCl, 65; KH_2PO_4, 4.8; $Ca(NO_3)_2 \cdot 4H_2O$, 288; $NaFeEDTA$, 8; KI, 0.75; $MnCl_2 \cdot 4H_2O$, 6; $ZnSO_4 \cdot 7H_2O$, 2.65; H_3BO_3, 1.5; $CuSO_4 \cdot 5H_2O$, 0.13; $Na_2MoO_4 \cdot 2H_2O$, 0.0024; glycine, 3; thiamine, 0.1; pyrodoxine, 0.1; nicotinic acid, 0.5; myoinositol, 50; sucrose, 10000 and Bacto-agar 8000. The pH of the media was adjusted to 5.5 before sterilization at 121 °C for 15 min. Petri dishes were then placed in the dark at 27 °C.

Tomato root culture

Tomato seeds (*Solanum lycopersicon* Mill. Var Saint-Pierre) were surface-sterilized in hydrogen peroxide (15%) for 3 min. They were then rinsed in sterile distilled water before germinating on water agar in the dark at 27 °C. The tips (2 cm) of emerged radicles were transferred to SR medium (Strullu and Romand, 1986). The composition of this medium in mgL^{-1} was as follows: $MgSO_4 \cdot 7H_2O$, 368; KNO_3, 303; KH_2PO_4, 44; $Ca(NO_3)_2 \cdot 4H_2O$, 2040; $NaFeEDTA$, 65; $MnSO_4 \cdot 4H_2O$, 2.23; $ZnSO_4 \cdot 7H_2O$, 0.29; H_3BO_3, 1.86; $CuSO_4 \cdot 5H_2O$, 0.24; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.035; thiamine, 1; pyrodoxine, 1; nicotinic acid, 1; cyanocobalamine, 0.5; calcium panthothenate, 1; sucrose, 15000 and Bacto-agar 8000. The pH of the medium was adjusted to 5.5 before sterilization at 121 °C for 15 min. Fast-growing tomato roots were cloned by regular subcultures on this medium in the dark at 27 °C.

Assessment of fungal development

Ten replications of the experimental unit were made for each fungus. Hyphal length, number and viability of newly formed spores were assessed for 90 days. The linear growth of the main germinating hyphae was assessed for a week in eight random Petri dishes (four replicates per treatment by measuring four fat-growing hyphae daily). Hyphal growth (mm/per time unit) was estimated using a 2 mm grid in which the number of intersections between hyphae and the gridlines was converted to hyphal length, according to the procedure of Bécard et al. (1992). The spores produced were counted directly in the Petri dishes (magnification 160×). Viability of the newly-produced spores was evaluated by testing their germinative capacity on water agar medium for 10 days. The spores were removed from the media using a sharp scalpel. To test their ability to regenerate VAM symbiosis, some germinating spores were associated with 7 cm segments of isolated tomato roots on minimal M medium. Both

experiments on spore germination and on dual culture were also conducted at 27 °C in the dark.

3. Results

Denaturing root process

Both surface sterilization by chemical treatments and the shearing process greatly reduced the metabolic activity of the VAM root pieces. The cutting of their apical tips stopped differentiation of root cells and consequently their growth. However, the intraradical forms of the VAM fungi did not loose their lipid contents.

Hyphal growth from sheared roots of VAM fungi

All media were suitable for hyphal growth from sheared VAM roots (Fig. 1A and Fig. 1B). The growth curves were linear. *G. versiforme* grew faster than *G. intraradices* in all Petri dishes. Final growth on M medium was slightly higher than on water Bacto-agar: average length of hyphæ of *G. versiforme* was over 9 cm in both media while that of *G. intraradices* fluctuated from 7.5 cm on Bacto-agar medium to 8.1 cm on M medium.

Morphological features of the extraradical phase

Regeneration of hyphæ started from wounded VAM roots within 3 to 6 days after incubation (Fig. 2). Extraradical fungal structures showed many differences in their morphological features and directions. The average number of germinated hyphæ per cut root ends varied with VAM fungus but often fluctuated from five on agar medium to over ten on M medium. Hyphæ of *G. intraradices* regularly grew straight on agar medium compared to M medium where they were sometimes sinuous. Hyphæ produced by sheared-roots colonized by *G. versiforme* were more prolific than those of sheared-roots colonized by *G. intraradices*. Both agar and M media showed similar growth patterns of the main hyphæ of *G. versiforme*; the germinating hyphæ were weak and often sinuous. According to their emergence zone, hyphal diameters varied greatly. For *G. intraradices* the main hyphæ bearing arbuscule-like branches reached 16 μm in diameter, the secondary branches, 8 to 10 μm, and the tertiary ones, 4 μm. The diameters of the main and the ramified hyphæ of *G. versiforme* were similar and reached 10 and 6 μm respectively. Hyphal biomass was also greater on M medium than on agar medium. However, extensive hyphal biomass covered each Petri dish three months after incubation by both VAM fungi.
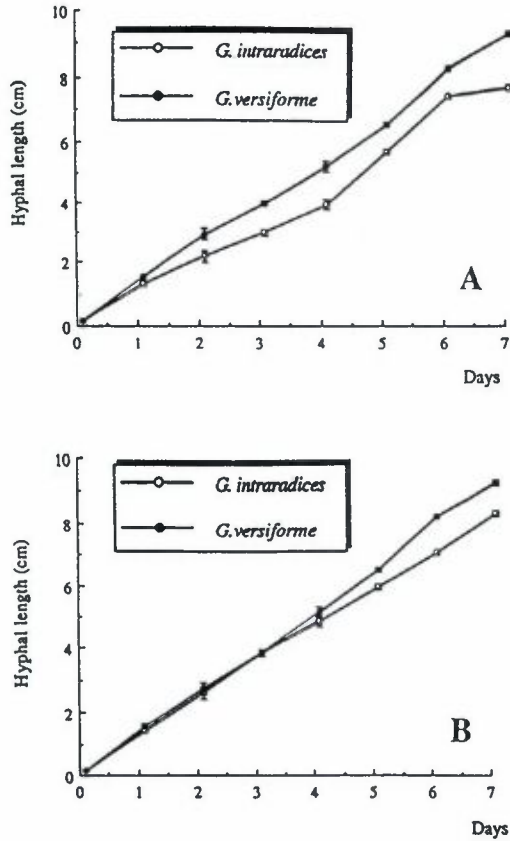
Figure 1. Hyphal growth (cm) from germinating sheared roots of *Glomus* spp. during 7 days
of cultivation on water agar (A) and minimal M (B) media. Vertical bars indicate
standard deviations.

Three days after germination, the VAM fungi often began to sporulate.
First spores were seen on the lateral hyphae. The rate of spore formation was
slow at the beginning. The maximum number of spores was 63 after three
months on agar medium for *G. intraradices* (Figs. 2, 3). The sporulation of
this fungus (Table 1) was much smaller on M medium despite the extensive
hyphal network (less than 4 times on water agar medium on the 90th day). The
diameter of the hyaline globular vegetative spores of *G. intraradices* depended
on their developmental status (Fig. 4). Mature spores with dense lipid oil
droplets reached 65 to 80 μm in diameter while detectable spores in formation
attained 20–25 μm. These differences linked to development were found every
day of the experiment. Otherwise, the good mycelium growth of *G. versiforme*
was accompanied by poor sporulation (Fig. 5). Only a few spores (60 μm) in
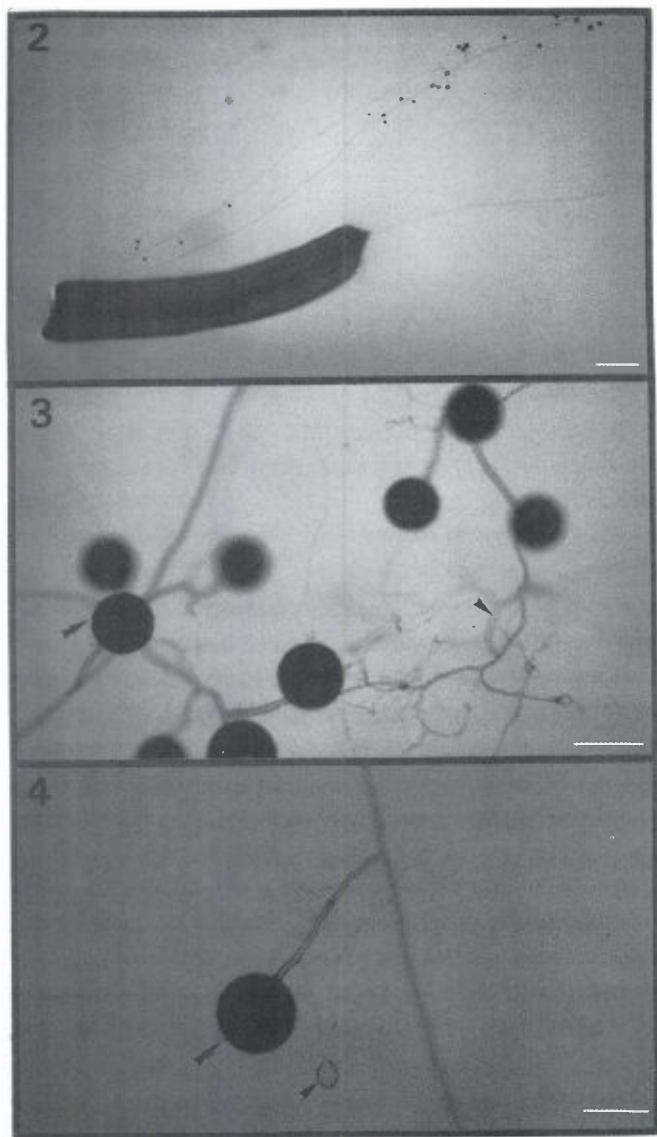diameter) were observed in plates containing agar medium and none on M

Figure 2. *In vitro* sporulation of sheared roots of *G. intraradices* on water agar medium. Note numerous spores formed on branching hyphae. Scale bar = 0.7 mm.

Figure 3. Extraradical phase of *G. intraradices* on water agar medium showing dense mycelia (arrow) and spores (double arrow). Scale bar = 100 μm.

Figure 4. Magnification of spores of *G. intraradices* formed on M medium. Note the young spore (arrow) and mature spore (double arrow) with dense contents. Scale bar = 100 μm.
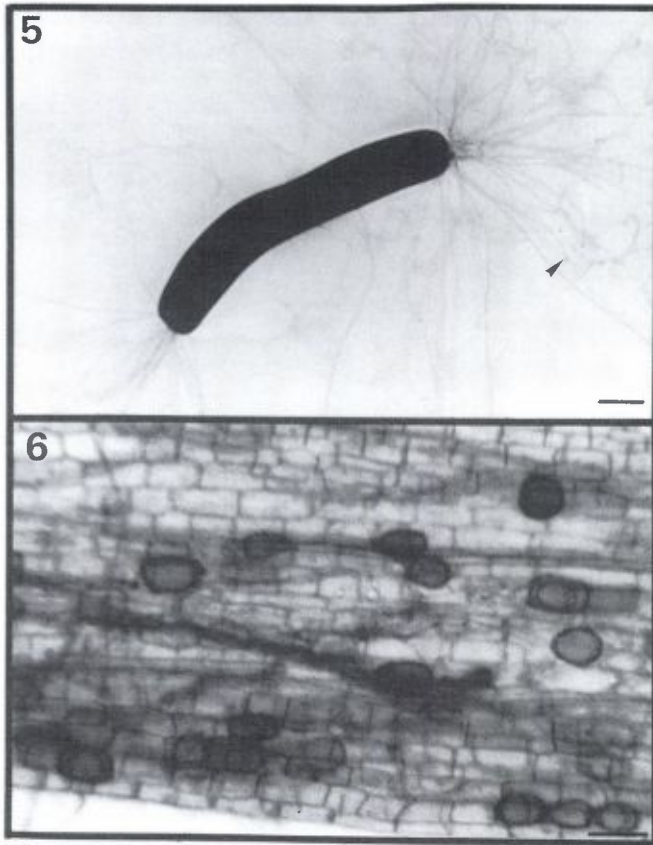
Figure 5. Extraradical phase from sheared root of *G. versiforme* on water agar medium. Note the extensive biomass of regenerating hyphae and a few spores (arrow). A similar pattern of germination without sporulation of *G. versiforme* was found on M medium. Scale bar = 0.7 mm.

Figure 6. Portion of tomato root colonized *in vitro* by newly produced spores of *G. intraradices* after 1 month. Note vesicles and hyphae found inside the strained root. Scale bar = 100 μm.

medium (Table 1). All newly-produced spores kept their cytoplasmic contents up to three months.

Viability of produced spores

Newly-produced spores readily germinated after aseptic transfer on water agar medium. For both fungi, 85% of the spores germinated between 3 to 6 days after transfer. At day 10, the germination reached 95%. Spores often

Table 1. *In vitro production of spores from sheared roots of* Glomus *spp. in two different media*

	Sheared roots of *Glomus versiforme*		Sheared root of *Glomus intraradices*	
Months	Water agar	Minimal M	Water agar	Minimal M
1	5b	0a	24c	6b
2	6b	0a	42b	9b
3	10a	0a	63a	14a

Values in each column followed by the same letter are not significantly different (P< 0.05 Newman-Keus test).

produced a single germ tube which developed many lateral branches. The branches usually became septate and ceased to grow after a few days if they were not in the vicinity of a host partner. In association with tomato root explants, they exhibited profuse mycelium and formed typical mycorrhizal infections (Fig. 6).

4. Discussion

The present paper is the first report of sporulation of VAM fungi on water agar medium without supplemented nutrients in *in vitro* conditions. More than 60 mature spores were produced from sheared-roots of *G. intraradices*. Water agar medium was suitable for a sustainable growth of hyphae in the presence of a decaying root. Vigorous regenerating hyphae predominantly originated from intraradical forms. The growth patterns of hyphae were similar to those obtained in dual root organ cultures. The results show that a relationship exists between hyphal development and the type of growth medium. The absence of a living host partner did not inhibit the *in vitro* ability of *Gigaspora margarita* to replicate DNA and undergo nuclear divisions (Bécard and Pfeffer, 1993; Bianciotto and Bonfante, 1993). This probably occurs in the case of *Glomus* spp. leading to extensive hyphal establishment in a nutrient-deficient medium such as water Bacto-agar.

The M medium was developed from White's medium after observing the detrimental effects of high concentration of nitrogen, carbon and sucrose (Bécard and Fortin, 1988). This medium was previously found to be suitable to support the life cycle of VAM fungi (Chabot et al., 1992b; Diop et al. (1992a, 1994)). Under the conditions we used, the best sporulation of sheared-roots was obtained with water agar. Different hypotheses were advanced to

explain the sporulation of *Glomus* spp. on the media. Stresses observed in *in vitro* cultivation systems induced variable behaviour of the VAM fungi. Water Bacto-agar seemed to be more stressing than M medium and fast sporulation in the former medium was a survival adaptation of VAM fungi. *G. intraradices* was more sensitive to stressed environment than *G. versiforme*. Diop et al. (1992a,b) reported that on M medium, *G. margarita* developed first an extensive hyphal biomass and later an abundant sporulation when growing conditions became limited.

The intraradical forms retained their lipid contents and germinated well irrespective of the decrease in metabolic activity of the root explants by both surface sterilization and the shearing process. This is not surprising as intraradical vesicles in dead and decaying infected roots have been found to maintain their viability by germinating in a similar manner as extraradical spores; VAM fungi developed a saprotrophic phase in these roots (Tommerup and Abbott, 1981). The low level of exudates from surface-sterilized excised roots (with reduced metabolic activity) may have been sufficient to support development of VAM fungi. The results confirm suggestions that the quality rather than the quantity of root exudates is more involved in the stimulation of hyphal elongation (Carr et al., 1985; Elias and Safir, 1987), and also confirm some *in vitro* independent growth of the regenerated mycelium as already advanced (Strullu et al., 1991; Williams, 1992). In our experiments, *G. intraradices* produced spores in all media. *In vitro* sporulation of *G. versiforme* obviously required a supplemented medium or a living partner (Diop et al., 1994). We also hypothesized that most supplemented mineral elements in classical media could be toxic for VAM fungi. *G. versiforme* developed an extensive mycelium on both media and occasionally sporulated on water agar medium only. It would be interesting to test a large number of commercial agars on the behaviour of the extraradical phase from sheared mycorrhizal roots. The *in vitro* methods of mycorrhizal syntheses exploited the ability of germinating hyphae for independent growth (Strullu and Romand, 1987). This is supported by the extensive growth obtained from the same material associated with a root partner; both *G. intraradices* and *G. versiforme* produced more than 25% of hyphal growth during 7 days of dual culture (Diop et al., 1994).

In conclusion, the extensive mycelium and sporulation obtained from sheared VAM roots on poor media (water agar and M), show the importance of abiotic factors for the success in *in vitro* culture of the intraradical forms of obligate biotrophic microorganisms. This type of culture without supplemented living host partner allows an easy non-destructive observation of fungal development

and can be used to increase our knowledge of the biotrophic status of VAM fungi.

Acknowledgements

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