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T. A. Diop · C. Plenchette · D. G. Strullu

Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots

Abstract Surface-sterilized sheared-root inocula of two vesicular-arbuscular mycorrhizal (VAM) fungi (*Glomus intraradices* and *G. versiforme*) from pot cultures associated with excised tomato roots showed significant sporulation and the production of an extensive hyphal biomass. As many as 10^2–10^3 axenic mature spores were recovered in Petri dishes during 3 months incubation in the dark. Propagules of both species were able to complete their vegetative life cycle in vitro and efficiently colonize *Acacia albida* roots after 1 month under greenhouse conditions. The effectiveness of 0.5-cm pieces of VAM roots as starter inocula indicates the high inoculum potential of intravesicle propagules.

Key words Sheared-root inocula
Vesicular-arbuscular mycorrhizal (VAM) fungi
in vitro · Sporulation · Excised tomato root
Acacia albida

Introduction

The beneficial roles of vesicular-arbuscular mycorrhizal (VAM) fungi in the growth, nutrition and health of host plants have been amply demonstrated (Perrin 1991; Plenchette 1991). However, the large-scale use of vesicular-arbuscular endophytes still presents a great challenge. Despite the proliferation of cultivation systems, including various greenhouse and in vitro methods (Dehne and Backhaus 1986; Strullu and Romand 1986; Diop et al. 1992; Sylvia and Jarstfer 1992), the obligate biotrophic status of VAM fungi reduces the inoculum potential of these ubiquitous microorganisms.

Contaminant-free culture of VAM should lead to a better understanding of the complex biology of VAM symbiosis. Many axenic cultures of VAM fungi with living roots both attached to and separate from shoots have been applied in the last two decades to investigate functional aspects of the symbiosis or to produce inocula (Mosse and Hepper 1975; Mugnier and Mosse 1987; Bécard and Fortin 1988; Bécard and Piché 1989). Spores are the preferred starting material in most studies, even though these propagules require a lot of care to avoid contamination, dormancy and strain mutations under greenhouse conditions. The intraradical forms of VAM have been less used despite being potentially good inocula. Root pieces colonized by VAM fungi develop a high capacity for regrowth of hyphae (Magrou 1946; Strullu and Romand 1987) and establish typical mycorrhizae in association with host partners (Bierman and Linderman 1983; Strullu and Romand 1986). It has been proposed that the completion of the life cycle of VAM fungi is mainly achieved by natural destruction of infected root pieces, resulting in the liberation of intravesicles which then readily germinated (D. G. Strullu et al., unpublished work).

Recently Strullu and Romand (1986, 1987) described the reproducible in vitro production of VAM propagules using isolated vesicles, hyphae and endomycorrhizal roots as starting inoculum. The use of these inocula was facilitated by encapsulation of the intraradical forms of VAM fungi (Strullu et al. 1991).

The aims of the present study were: (1) to assess the inoculum potential of sheared-root inocula of VAM fungi in association with excised tomato roots; (2) to study the capacity of regeneration of newly formed propagules (i.e. spores, hyphae, colonized roots) in both in vitro and in vivo culture conditions; (3) to standardize

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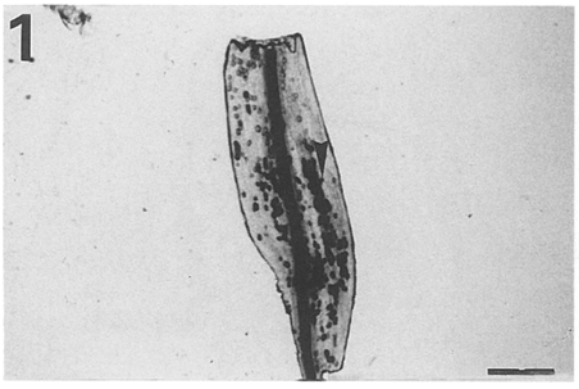


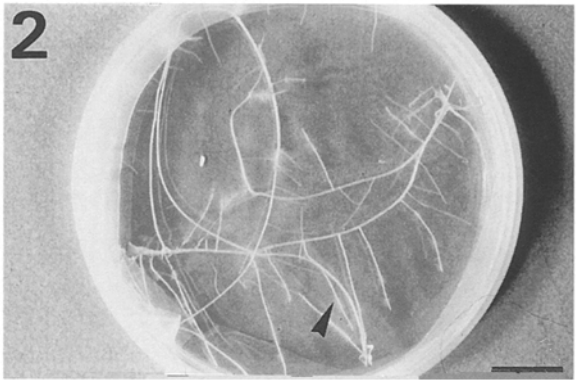


Fig. 1 Vesicular-arbuscular mycorrhizal (VAM) root piece showing vesicles (*arrow*) which forms an excellent starting inoculum for the establishment of VAM symbiosis; *bar* = 1 mm

Fig. 2 Culture of tomato roots in modified White's medium. Note the numerous fine lateral roots (*arrow*) which are potential host partners when the apical tips are transferred to suitable medium; *bar* = 15 mm

the in vitro cultivation system of Strullu and Romand (1987). Sheared leek roots colonized by *Glomus intraradices* (Schenck et Smith) and *G. versiforme* (Karten) Berch associated with excised tomato roots were cultivated in standard Petri dishes.

Materials and methods

Sheared-root inocula of VAM

G. intraradices and *G. versiforme* were routinely cultivated in association with *Allium porrum* under greenhouse conditions. After 4 months, heavily colonized leek roots freed of soil particles were carefully washed and nondestructively selected under a binocular microscope at ×160 magnification. Sheared roots of each inoculum were carefully washed prior to ultrasonic treatment (48 kHz) for 10 min (Strullu and Romand 1986). Surface sterilization was carried out under a laminar-flow hood by successive washes with 96% ethanol (10 s), 6% calcium hypochlorite (1 min), Chloramine T 2% plus two drops of Tween 20 (10 min), and rinsing for 10 min in an antibiotic solution containing 200 mg l^{-1} streptomycin sulfate and 100 mg l^{-1} gentamycin sulfate. Mycorrhizal roots were cut with scissors into 0.5-cm lengths and aseptically transferred to Petri dishes (9 cm in diameter) containing distilled water solidified with 0.8% Bacto agar. VAM root pieces contained 3–160 intravesicles (Fig. 1). The dishes were incubated in the dark at 27° C.

Root culture

Tomato seeds (*Solanum lycopersicon* Mill. var. Saint-Pierre) were surface disinfected in hydrogen peroxide (15%) for 3 min, then washed in sterile distilled water. The seeds germinated after 48 h at 27° C in the dark on water agar. The tips (2 cm) of emerged radicles were transferred to a modified White's medium (MW) described by Bécard and Fortin (1988). The composition of the medium in mg l^{-1} distilled water was as follow: $MgSO_4 \cdot 7H_2O$, 731; $Na_2SO_4 \cdot 10H_2O$, 453; KNO_3, 80; KCl, 65; $NaH_2PO_4 \cdot 2H_2O$, 21.5; $Ca(NO_3)_2 \cdot 4H_2O$, 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; pyridoxine, 0.1; nicotinic acid, 0.5; myo-inositol, 50; sucrose, 30000 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 repeated subculture in this medium.

Dual culture

The 0.5-cm lengths of VAM roots readily began to grow on agar medium in 2–3 days. Using a 13-mm cork borer, inoculum was delicately transferred to the vicinity of a 7-cm length of tomato root explant on minimal M medium (Bécard and Fortin 1988). This medium contains, in mg l^{-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; $ZnSO_3 \cdot 7H_2O$, 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; pyridoxine, 0.1; nicotinic acid, 0.5; myo-inositol, 50; sucrose, 10000 and Bacto agar 8000. The pH of the medium was adjusted to 5.5 before sterilization at 121° C for 15 min.

The experimental unit consisted of a standard Petri dish (∅ 9 cm) containing 40 ml M medium with one 0.5-cm VAM root piece and a 7-cm tomato root with a few branches. Eight replicates each of *G. intraradices* and *G. versiforme* in association with tomato roots were set up. The Petri dishes were incubated horizontally at 27° C in the dark.

Reinfection of VAM propagules produced

Newly formed VAM propagules, i.e. mature spores and isolated colonized roots, were re-associated with tomato root explants in axenic conditions. In addition, seeds of the leguminous tree *Acacia albida* were surface sterilized with sulfuric acid (30 min) and washed with sterile distilled water. After 48 h germination, *Acacia* seedlings were inoculated under greenhouse conditions with 50 in vitro-produced spores and a mixture of other propagules, i.e. axenic colonized tomato roots plus hyphae from each fungus. Five replicates were set up per treatment.

Assessment of variables

The establishment of VAM symbiosis was regularly checked over 3 months. Nondestructive observation of extraradical forms, development of hyphae and multigermination of spores was carried out under the binocular microscope at various magnifications. Viability and the ability of newly formed propagules to re-establish mycorrhizae were assessed: (i) by observing the germination and morphology of the extraradical phase after association with tomato roots in vitro; (ii) by evaluating the root colonization of *Acacia albida* seedlings in vivo.

The gridline-intersect method (Giovannetti and Mosse 1980) was used to follow the growth of hyphae of both fungi during the first week in dual culture. At the end of the experiments, the in-

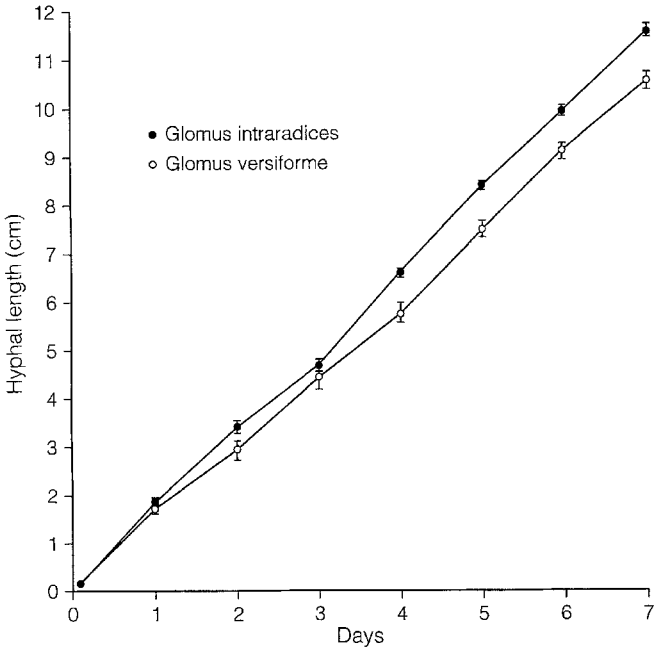


Fig. 3 Change in the length of hyphae from germinating sheared-root inocula of *Glomus* spp. associated with excised tomato roots during 7 days of dual culture. *Vertical lines* indicate standard deviations

tensity and the percentage of root colonization were evaluated by the same procedure in six randomly chosen Petri dishes (three for each treatment). The roots were cleared with 10% KOH for 1 h at 90° C and stained for 15 min with acid fuchsin (0.05% in lactoglycerol).

Results

Growth of tomato roots

Root apices from germinating tomato seeds grew vigorously on solidified MW medium and developed many thin lateral ramifications after 5 days of incubation (Fig. 2). Subculturing of vigorous tomato root apices on minimal M medium did not alter their morphology and after 7 days they were ready for the establishment of dual cultures with germinating, sheared endomycorrhizal leek roots.

Establishment of dual cultures

Both types of sheared endomycorrhizal leek roots regenerated vigorous hyphae. Hyphae grew well in all Petri dishes and reached more than 10 cm after 7 days (Fig. 3). Growth of *G. versiforme* was slightly faster than *G. intraradices*. Different germinating patterns were observed, with sheared-root inocula of *G. versiforme* often exhibiting more ramifications (Figs. 4–6). Hyphae emerged from the ends of the root cuttings or laterally when the root epidermis was damaged. Most of the hyphae arose from germinating intravesicles. The first contacts between hyphae and tomato roots occurred after 1–3 days of dual culture (Figs. 7, 8).

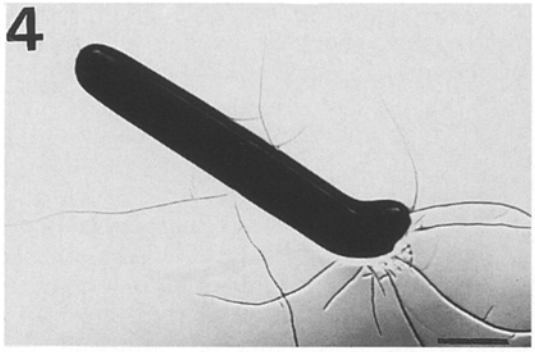
In vitro fungal development

The development of the extraradical mycelium was profuse and quickly covered the Petri dishes. Hyphae spread out in all directions and sometimes anastomosed into lateral branches. The main hyphae were often straight and bore numerous arbuscule-like branches. Sporulation of both fungi began 3 days after contact and spores were produced inside or outside the zone of tomato roots (Figs. 9, 10). No spores were formed inside the root organs. Mature and under developed spores were observed at all periods of the in vitro culture. New spores were uniform, globular and white, initially with many lipid droplets on maturation. Spores of *G. intraradices* were much bigger (∅ 80 μm) than those of *G. versiforme* (∅ 65 μm). However, the size of *G. intraradices* spores varied greatly at all periods of dual culture. An average of 893 mature spores of *G. intraradices* and 2065 mature spores of *G. versiforme* were recorded after 3 months incubation in the dark. All were formed terminally or were intercalary on hyphal branches. Spores were often grouped (Figs. 11, 12) and empty spores were sometimes detected, especially in cultures with sheared-root inocula of *G. versiforme* during aging of the dual culture.

At harvest, stained roots showed typical infections. Coiled hyphae and vesicles were observed inside roots (Fig. 13). However, these infection units, i.e. root segments with intravesicles, were slightly more numerous in roots inoculated with sheared-root inoculum of *G. versiforme*, while those inoculated with *G. intraradices* developed many penetration points. The percentage root colonization was only 24% in Petri dishes inoculated with sheared-root inoculum of *G. intraradices* and 35% for *G. versiforme*.

Viability and reinfection of VAM propagules

Both propagules, i.e. spores and fragments of infected roots, of each fungus germinated on minimal M medium without previous treatment. In association with isolated tomato roots, they were able to complete their life cycle. Inoculation of *Acacia albida* seedlings with newly formed propagules, spores and a mixture of hyphae and colonized tomato roots was successful. The spores produced by *G. intraradices* and mycelia plus infected roots of *G. versiforme* were the most efficient inocula after 1 month of greenhouse culture (Table 1).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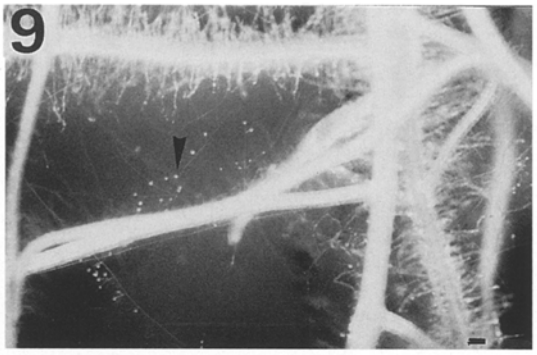
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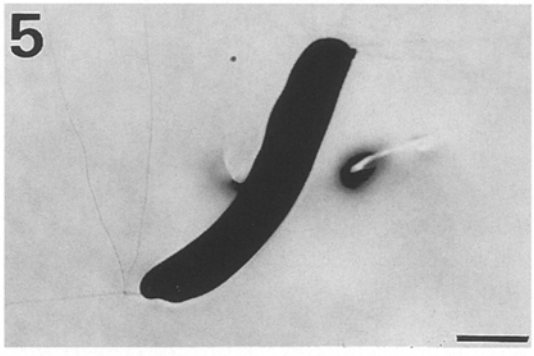
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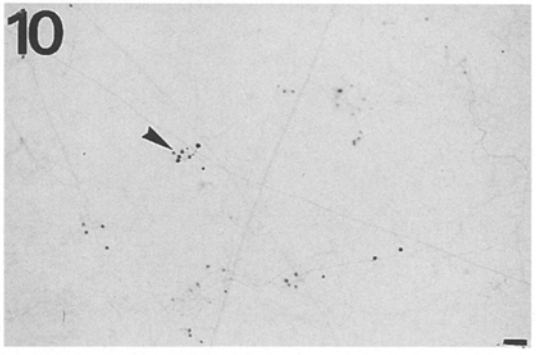
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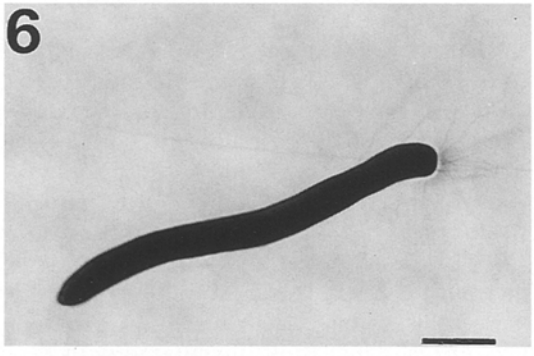
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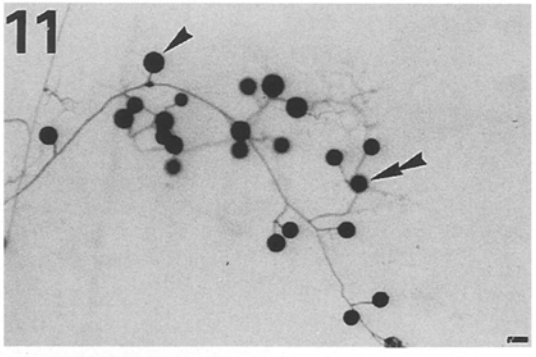
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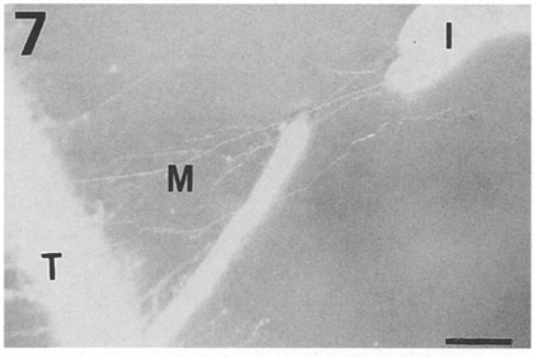
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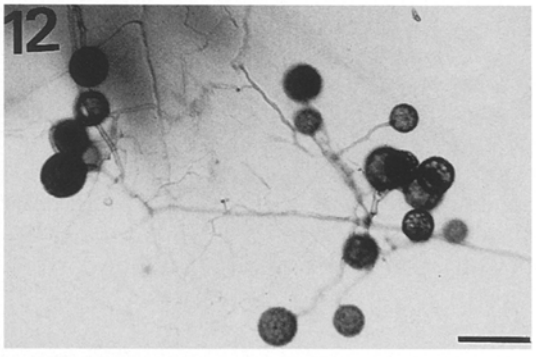
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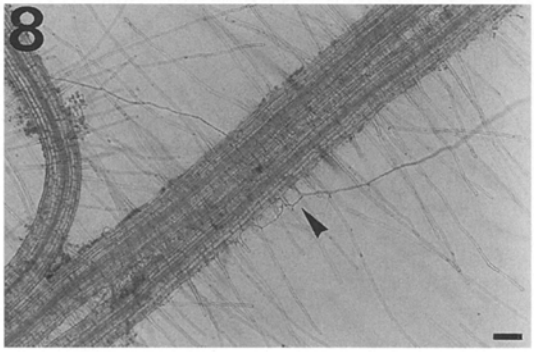
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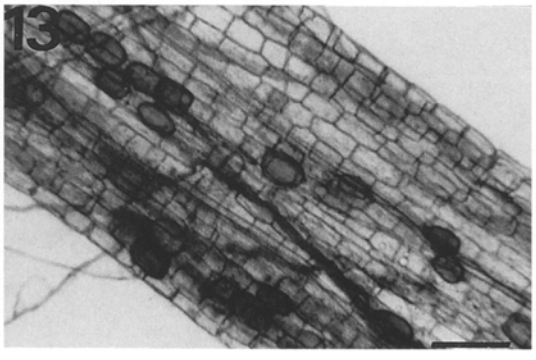
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Table 1 Reinfection of *Acacia albida* seedlings under greenhouse conditions by axenically produced propagules. Values in each column followed by the same letter are not significantly different ($P=0.05$, Newman-Keuls test)

Inocula	Root colonization (%)	Intensity of root colonization (%)
Spores of *Glomus intraradices*	80.8 a	53.0 a
Mycelia + infected roots by *Glomus intraradices*	41.6 b	17.8 b
Spores of *Glomus versiforme*	47.8 b	26.6 b
Mycelia + infected roots by *Glomus versiforme*	88.4 a	58.0 a

Discussion

Dual culture of tomato roots associated with sheared-root inocula of *G. versiforme* or *G. intraradices* gave excellent sporulation when compared to previous reports (Chabot et al. 1992; Diop et al. 1992). Both fungi developed extensive extraradical mycelium and massively sporulated in a relatively short period of time for obligate symbionts. Sporulation occurred after 3–4 days and the number of spores quickly increased to 893 and 2065, respectively, in plates inoculated with *G. intraradices* and *G. versiforme* in 3 incubation months. For *G. intraradices*, the results confirmed that sporulation of this fungus occurs outside roots as already described (Neiderhofer and Schenck 1987; Chabot et al. 1992). The same process of *G. intraradices* spore formation occurred under greenhouse conditions in association with *Allium porrum*. The minimal M medium previously used for establishment of mycorrhizae with spores of *Gigaspora margarita* (Bécard and Fortin 1988) was also suitable for the establishment of VAM symbiosis with excised colonized roots. In a further experiment (T. A. Diop et al., unpublished work), we noticed that isolated roots colonized by *G. versiforme* were unable to sporulate monoaxenically, despite the fact that they exhibited extensive mycelia. Thus, we think that the presence of root explant is a prerequisite for sporulation of sheared-root inocula of *G. versiforme*. Root exudations, especially CO_2 produced during root respiration, could explain the positive stimulation of extraradical mycelium from sheared-root inocula of vesicular-arbuscular fungi as observed for *Gigaspora margarita* (Bécard and Piché 1989). Moreover, nontransformed tomato roots, because of their convenient growth, appear to be more promising for the large-scale production of VAM inocula than Ri T-DNA-transformed carrot roots (Diop 1990).

As intravesicles in colonized roots act as reserves and propagules, they have a higher inoculum potential than other VAM propagules, i.e. spores and hyphae (Bierman and Linderman 1983; Strullu and Romand 1986, 1987; Mosse 1988). The multiple germinating hyphae observed from VAM sheared roots seemed to influence positively the physiological status of the VAM fungi by enhancing the number of penetration points with tomato roots and producing good sporulation. Considering the inability of some VAM fungi to produce spores (Johnson 1977; McGee 1989), the use of excised VAM roots as inocula offers an opportunity for taxonomic and genetic studies of these obligate biotrophic microorganisms. However, proper selection of sheared-root inocula is the key of the success of this cultivation system. Leek roots are highly mycotrophic (Plenchette et al. 1983) and provide an easy nondestructive way to observe intravesicles. In addition, Strullu and Romand's disinfection method leads to less contamination.

The ability of both propagules to complete their life cycle in vitro and to colonize *Acacia albida* roots in vivo enhances the interest of this cultivation system. The reproducibility of the system, and the obtaining of clean propagules rapidly in little space, provide a powerful tool for biological studies and practical applications. Based on earlier work (Strullu et al. 1991), the in vitro system also gives opportunities for automatic production because of the easy recovery of propagules by bubbling (Furlan and Fortin 1975) or by liquefying gel agar with cation chelators (Donner and Bécard 1991). Developing countries will benefit more from the VAM symbiosis in this culture system, which is cheaper than greenhouse culture. We are now assessing the effectivity of propagules obtained in vivo by the usual greenhouse methods and those produced in in vitro conditions.

Considering the high production of intravesicles (1 cm of heavily colonized leek roots sometimes contained more than 1500) and their richness in energy sources able to initiate sporulation, we recommend use

◀ **Figs. 4–13** Morphological features of sheared-root inocula of *Glomus* spp. in tomato root cultures. **Figs. 4, 5** Germinating hyphae of *G. intraradices* arising from root cut ends or the damaged root epidermis; *bar* = 1 mm. **Fig. 6** Sheared-root inocula of *G. versiforme* developed more profuse germinating hyphae than those of *G. intraradices*; *bar* = 1 mm. **Figs. 7, 8** Contacts between sheared-root inocula of VAM fungi and tomato root explants. Germ tubes spread out in all directions and contacts occurred within 3 days of dual culture. **Fig. 7** First events of inoculation of tomato root by leek root piece colonized by *G. intraradices*. *I* Inoculum, *M* mycelium, *T* tomato root; *bar* = 1 mm. **Fig. 8** Appressorium (*arrow*) after inoculation of excised tomato root by leek root piece colonized by *G. versiforme*; *bar* = 400 μm. **Fig. 9** Newly produced spores (*arrow*) of *G. versiforme* in a tomato root culture; *bar* = 200 μm. **Fig. 10** Extraradical phase of *G. versiforme* outside the zone of a tomato root showing dense mycelium and spores (*arrow*); *bar* = 200 μm. **Fig. 11** Arbuscule-like branches and cluster of spores of *G. intraradices*. Spores are often in terminal (*arrow*) and intercalary (*double arrow*) positions of the ramifications of main hyphae; *bar* = 100 μm. **Fig. 12** High magnification showing clusters of mature spores of *G. versiforme*. Note also the dense contents of the spores; *bar* = 200 μm. **Fig. 13** Tomato root stained with acid fuchsin showing typical infection of *G. versiforme*. Note the vesicles and hyphae inside the tomato root; *bar* = 200 μm

of these propagules as high-quality inoculum. Root cultures of tomato, surface sterilization of 0.5-cm sheared-root inocula of VAM fungi (Strullu and Romand 1986), and the minimal M medium (Bécard and Fortin 1988) could be the basis of the standardization of axenic culture of VAM fungi producing intravesicles.

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