Vesicular-Arbuscular Mycorrhizal Infection in Transformed Root-Inducing T-DNA Roots Grown Axenically

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The authors thank Dr. D. Tepfer for providing the *Convolvulus sepium* root culture and for valuable advice on the labelling experiments. We thank Dr. J. Serigny for electron microscope studies and acknowledge gratefully the help and suggestions of Dr. J. L. Prioul.
Accepted for publication 13 January, 1987.

ABSTRACT

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

Details are given of a technique for obtaining typical vesicular-arbuscular mycorrhizal infections in root organ cultures of *Convolvulus sepium* transformed by root-inducing T-DNA from *Agrobacterium rhizogenes*. A bicompartmental culture system was used. Roots, growing from a modified Murashige and Skoog nutrient medium and sugar into a compartment containing water agar and neutralized peat, were infected with germinated spores of *Glomus mosseae*. The uses of such a system for studies of nutrient exchange between plant and fungus are discussed.

Inability to culture the causal fungi of vesicular-arbuscular (VA) mycorrhiza has been and is a major difficulty in their study and practical exploitation. Typical VA infections can be produced in entire plants grown axenically in agar media (1,6,10), in sand culture (17), and in flowing solution culture (8). VA mycorrhiza have also been produced in clover root organ cultures grown on a modified White's (20) tissue culture medium containing sucrose (11). However, the medium had to be adjusted to the tolerance limits of the fungus, and, in this medium, the growth potential of the roots was rather small. The technique did not produce sufficient infected roots or external mycelium for sustained physiological studies. Neither have any of the axenic systems using whole plants lent themselves to mass production of inoculum or to extensive use for physiological studies.

The pathogenic condition known as "hairy root" is caused by *Agrobacterium rhizogenes* (Riker et al) Conn. (2). It is due to the transfer of root-inducing (Ri) plasmid genes from the bacterium to the plant (3,9,19). Tepfer and Tempé (18) described a technique for obtaining such transformed roots in an axenic state. Such roots have a remarkable growth potential (Fig. 1). For instance, transformed roots of *Convolvulus sepium* L. (bindweed) grown in 1 L of Murashige and Skoog (14) medium in a 2-L Biolafitte fermentor can produce 14 g fresh weight in 17 days. If mycorrhizal infection could be established in such transformed roots, they might provide ideal material for physiological studies and for inoculum of VA mycorrhiza.

This paper described experiments leading to the production of typical VA infections in transformed roots of *C. sepium* and other plants and some preliminary studies of nutrient transfer in such systems.

MATERIALS AND METHODS

Transformed root organ cultures. The original culture of *C. sepium* roots transformed by *A. rhizogenes* strain 8196 was provided by Tepfer (INRA Versailles, 78 000 France). Thereafter, the transformed roots were maintained in a liquid medium based on Murashige and Skoog (14) but containing 0.33 g L^{-1} of NH_4NO_3 and 0.38 g L^{-1} of KNO_3 (Fig. 1). For confirmatory studies we also transformed roots of carrot (*Daucus carota* L.), potato (*Solanum tuberosum* L.), and cowpea (*Vigna unguiculata* Walp.) using *A. rhizogenes* strain A_4, ATCC 31798, according to the procedure of Ark and Thompson (2) and Tepfer and Tempé (18).

Endophytes. *Glomus mosseae* Gerdemann & Trappe (ex Rothamsted Experiment Station) was used for all exploratory experiments. *Gigaspora margarita* Beker & Hall was also used in confirmatory experiments.

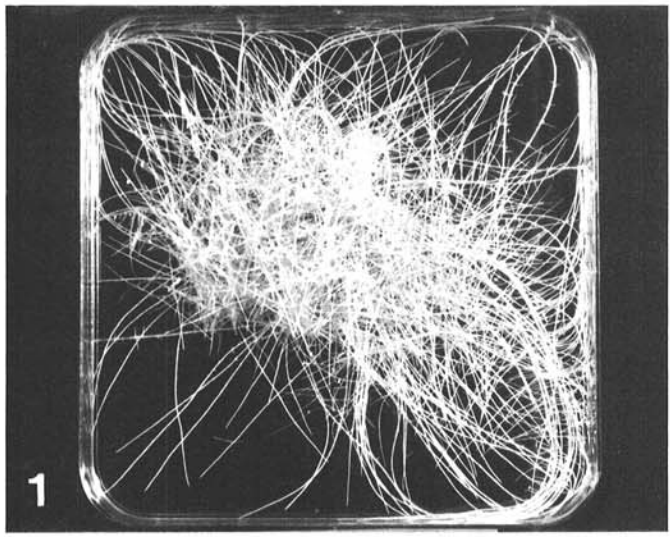
Glomus mosseae was maintained in pots in a greenhouse with *Allium cepa* L. as host, in a potting mixture containing 5:5:1 of perlite (Cecaperl), vermiculite, and soil of the following composition: N 0.046%, P 0.003%, K 0.006%, organic matter 1%, coarse sand 67%, fine sand 28%, silt 3%, and clay 2%. The potting mixture had a pH of 7.6 in water. The plants, watered as required and fed with NPK (10:1:10) solutions every 2 mo, produced 6,000 sporocarps per kilogram of potting compost after 6–8 mo.

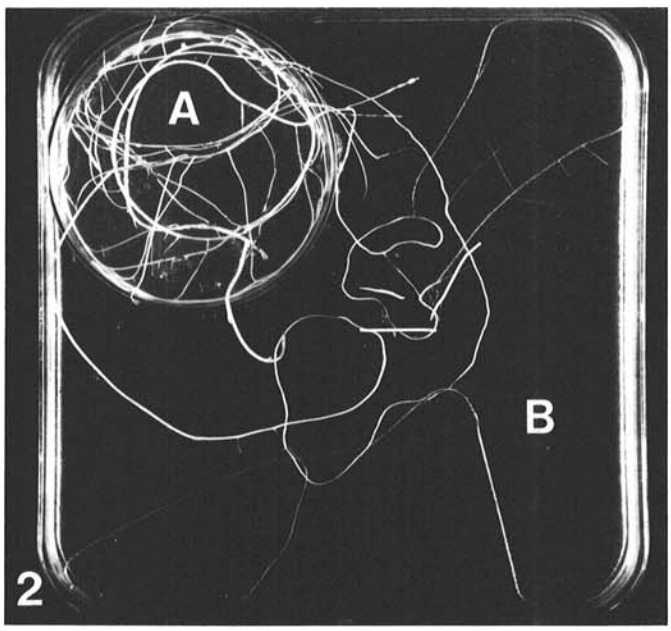
Spores were excised under a dissecting microscope. They were placed on a Millipore filter (Bedford, MA) in a Büchner funnel connected to a suction pump and sterilized for 15 min in 2% chloramine T and 200 μg ml^{-1} of streptomycin. After repeated rinsing in sterile distilled water, spores were placed in a petri dish containing a layer of 0.5% Difco agar (pH 7.0) overlying another layer of agar containing small, slow-growing colonies of an actinomycete, *Streptomyces orientalis* Centraalbureau voor Schimmelcultures, Baarn, Netherlands, number 35593. This technique (13) yielded 85–90% germinated spores after 8 days.

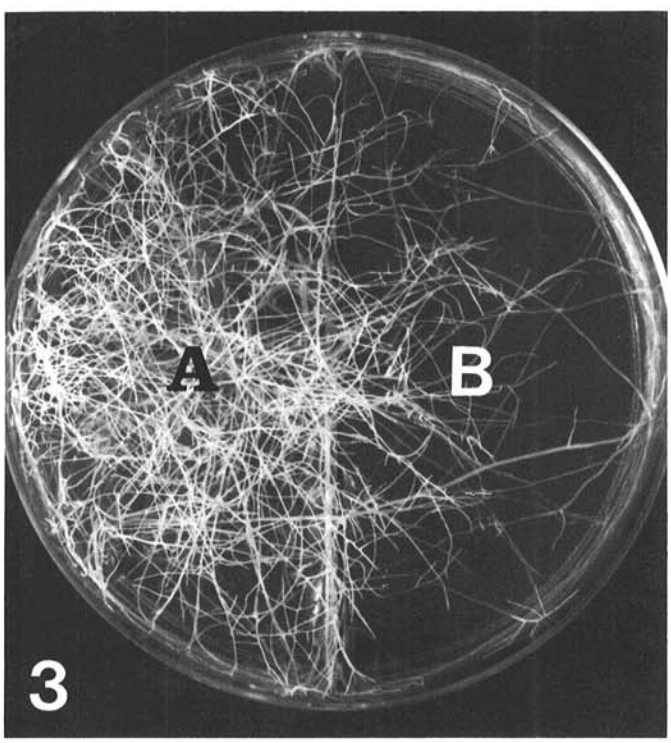
The germinated spores, together with a small amount of surrounding agar, were transferred to the roc^r organ cultures 1–2 days after germination, before the germ tubes had invaded the lower layer of agar containing the actinomycete.

Culture technique. A bicompartmental culture system was used, consisting either of a large petri dish containing another, smaller one inside it (Fig. 2) or of a divided petri dish (Fig. 3). Compartment A contained the nutrient medium needed to support root growth, and compartment B contained 1% water agar (Difco) and peat, prepared as described below. Approximately 0.5 g of young roots from the stock culture was washed in sterile distilled water and placed on the nutrient agar in compartment A with their growing tips pointed towards compartment B. They were incubated at 25 C in the dark. After 2–3 days new roots began to grow into compartment B and to form secondary branches. Pregerminated spores were then placed near the elongating zone of the secondary roots. The petri dish was sealed with tape and incubated at 25 C in the dark.

Nutrient medium. The final composition of the nutrient medium was, in milligrams per liter: $CaCl_2 \cdot 2H_2O$, 88; $MgSO_4 \cdot 7H_2O$, 74; KH_2PO_4, 34; Na_2EDTA, 7.5; $FeSO_4 \cdot 7H_2O$, 5.6; NH_4NO_3, 50; KNO_3, 50; $MnSO_4 \cdot 4H_{20}$, 0.04; $CuSO_4 \cdot 5H_{20}$, 0.025; $CoCl_2 \cdot 6H_{20}$, 0.025; $ZnSO \cdot 7H_{20}$, 0.22; H_3BO_3, 0.45; KI, 0.01; $NaMoO_4 \cdot 2H_2O$, 0.02; Ca pantothenate, 0.2; inositol, 1; biotin, 0.002; nicotinic acid, 0.2; pyridoxine, 0.2; thiamine, 0.2; saccharose, 12,000; agar Difco,







12,000. The vitamins were sterilized separately by filtration (0.22-μm filters). The pH was adjusted with 10 N NaOH to 6.9 after sterilization (120 C for 20 min).

Before normal mycorrhizal infections were established in the transformed roots, various modifications of this medium were tested, viz: nitrogen source: NH_4NO_3 and KNO_3 in various proportions at total N concentrations from 0.2 to 5 mM and NH_4SO_4 as sole nitrogen source; carbon source: sucrose from 29 mM (10 g L^{-1}), the minimum required for adequate root growth, to 87 mM (30 g L^{-1}); phosphorus source: KH_2PO_4 from 0.1 to 1.25 mM P; Na_2EDTA: from 5 to 100 mg L^{-1}; pH: from 5.0 to 7.2 in nutrient medium (A) and water agar (B) (adjusted after sterilization); peat: with and without in compartment B.

The progress of infection in the different media was followed in situ under a stereo dissecting microscope. Anatomical details were observed in roots cleared in KOH and stained in Trypan blue and lactophenol. Some transmission electron microscope observations of the infection were also made as described by Serigny (16).

Sterilization and neutralization of peat. The peat (sphagnum) was ground, passed through a 100-μm sieve, and wetted. The moist peat was then autoclaved twice at 120 C for 20 min. Thereafter all operations were carried out aseptically. The sterilized peat was placed on a Millipore filter and washed thoroughly with sterile, distilled water to remove soluble toxic substances. Washed peat (150–250 mg) was added to 1 L of 1% water agar at pH 6.8–7.2. The suspension was then cooled to 35 C before pouring into the petri dish, so that the peat particles remained suspended in the setting agar without sedimentation.

Experiments with radioactive isotopes. Some autoradiographs were obtained showing the movement of ^{32}P (orthophosphate), ^{14}C (sucrose), and ^{35}S (sulfate) from root to fungus. Details are given in Figures 11 and 12. The isotopes, in solution, were placed in compartment A (Fig. 2). After 2 or 10 days, root links between the two compartments were cut and the contents of compartment A were removed. The undisturbed contents of compartment B were dried, photographed to record the distribution of roots and hyphae, and covered with autoradiography stripping film. Roots were subsequently exposed to Kodak intensifying screens for 4–6 days at −80 C.

RESULTS

Stages in the infection process. Six distinct phases could be observed in the development of a successful VA infection in transformed roots (Figs. 4–10). They were:

1. The extension of germ tubes from pregerminated spores placed near growing roots (Fig. 4).

2. The attachment of germ tubes to the root surface (Fig. 5). Hyphae rarely became attached to roots with many root hairs. They grew across such roots without any change of direction or adverse effect on hyphal growth.

3. The formation, on the root surface, of a fan-like (15) growth of septate, much-branched hyphae (Fig. 6), resembling those of the sporocarp matrix. Occasionally such hyphae formed rhizoid-like structures in individual epidermal cells but did not spread further.

4. Formation of an appressorium by a single hypha, often arising from the center of the fan-like structure, and penetration into the subepidermal layer.

5. Intercellular spread of hyphae between the cortical cells, followed by intracellular penetration of cells (Fig. 7), formation of arbuscules and, eventually, vesicles. A transmission electron micrograph of a developing arbuscule (Fig. 10) shows the normal features of this very characteristic infection stage. Other electron

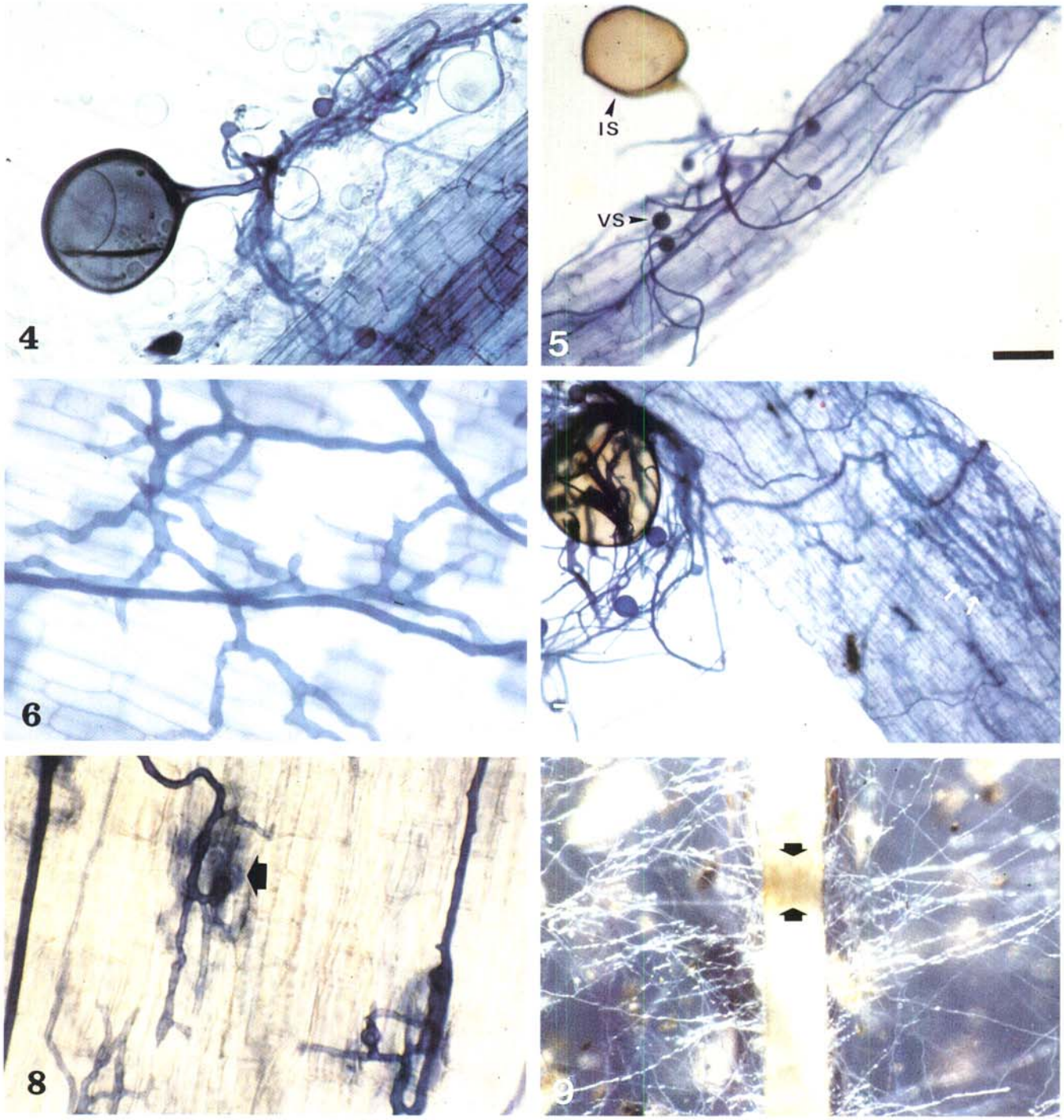
Figs. 1–3. 1, Ten days' growth of axenic transformed roots of morning glory on a modified Murashige and Skoog liquid medium. **2,** Ten days' growth of transformed roots of morning glory on a bicompartmental culture system. Petri dish A contains nutrient agar and sucrose, B contains water agar and neutralized peat. **3,** Ten days' growth of transformed roots of carrot on a bicompartmental culture system. Compartment A contains nutrient agar and sucrose, B contains water agar.

micrographs (16) show the normal features of arbuscule proliferation and the final stages of senescence, with typical aggregations of wall remnants remaining after the hyphae have collapsed inwards.

6. Spread of the infection. This occurred by proliferation of the external mycelium growing into the medium and encountering fresh roots, which became infected, or growing along the surface of the infected root and forming secondary infection points (Fig. 8). After 2–3 wk there was a very extensive system of external mycelium bearing many small (60 μm) vegetative spores. In older cultures, very heavily infected parts of the root system sometimes developed a brownish discoloration (Fig. 9). In spite of its vigorous hyphal growth in the external medium, the fungus could not be subcultured. It remained ultimately dependent on some substance or condition in the root.

These stages correspond closely to those occurring in the development of VA infections in whole plants. In the transformed roots, the infection process could be arrested at any of the above stages, which were controlled by the substances being present in the medium.

Effects of medium. The most important determining factor was the concentration of nitrogen in the medium. Above 2 mM total N,



Figs. 4–9. Infection stages of *Glomus mosseae* stained with Trypan blue in transformed roots of *Convolvulus sepium*. **4,** Stage 1. Extension of a germ tube from a pregerminated spore placed near a transformed root. Scale bar = 40 μm. **5,** Stage 2. Germ tube growth from inoculant spore (IS) along and around a root. New vegetative spores (VS) have grown from the germ tubes. Scale bar = 60 μm. **6,** Stage 3. Fan-shaped growth pattern of septate hyphae closely attached to the root surface and occasionally penetrating individual epidermal cells (stage 4) without further development. Scale bar = 160 μm. **7,** Stage 5. Development of a primary infection. Arrow points to cells with arbuscules. Scale bar = 40 μm. **8,** Stage 6. Secondary infections arising from external hyphae growing along the root surface after primary infection from germ tubes has taken place. Scale bar = 160 μm. **9,** Vigorous outgrowth of external hyphae from the root surface 10–20 days after infection (photo of a living root). Note the discoloration (arrowed) in a heavily infected part of the root. Scale bar = 600 μm.

germ tubes stopped growing (stage 1). In the range of 1 to 2 mM N, germ tubes developed, but passed across roots without becoming attached to the surface. When the nutrient medium contained less than 0.2 mM N, the fungus showed some directional growth towards the root, and the hyphae became attached (stage 2). They began to form the characteristic, strongly branched, septate fan-like structures on the root surface (stage 3) 3 days after inoculation. This was followed by formation of an appressorium and root penetration (stage 4) by day 5. The fungus spread intercellularly and arbuscules began to form (stage 5) by day 10. The proportions of NH_4NO_3:KNO_3 could vary from 0:1 to 5:1. When NH_4SO_4 was used as the only N source the roots died; most probably this was a pH effect.

Contrary to expectations (4), the phosphorus concentration in the nutrient medium had little effect on the infection process. This proceeded normally even with 170 mg L^{-1} of KH_2PO_4 (1.25 mM P).

With more than 20 g L^{-1} of sucrose in the nutrient medium hyphal growth was arrested in stage 1, i.e., there was no attachment to the root surface.

Without EDTA in the nutrient medium (compartment A) fungal development was arrested at stage 1, i.e., germ tubes stopped growing. EDTA is known to complex various cations, but its effect on root physiology and particularly root exudates is not well known.

When the pH of the nutrient medium (compartment A) was less than 6.0 at the beginning of the experiment, root growth in compartment B continued, but no infection occurred. When it was below pH 6.0 in the water agar (compartment B), germ tube growth was arrested. In soil, the Rothamsted isolate of *Glomus mosseae* is also known to grow badly or not at all below pH 6.0.

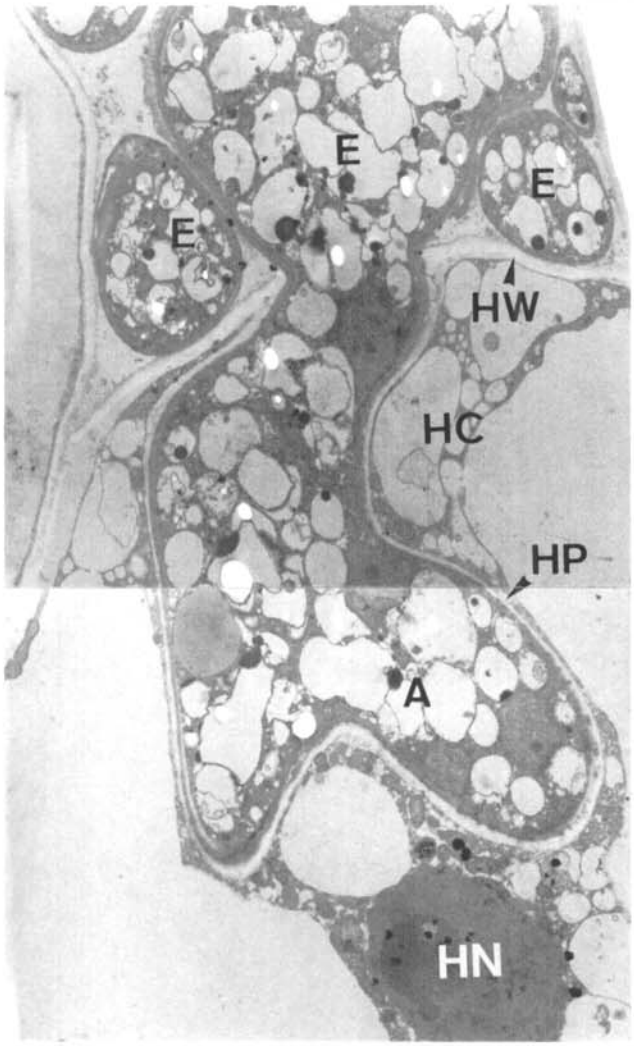


Fig. 10. Transmission electron micrograph (×6,400) of host cell penetration and early stages in the development of an arbuscule. HN = host nucleus, HP = host plasmalemma, HW = host cell wall, HC = host cytoplasm, E = endophyte hyphae (transverse section) in intercellular space, A = young arbuscule developing inside a host cell. Note normal, undisturbed structure in both host and fungal cytoplasm. (Courtesy J. Serigny)

The presence of neutralized peat in compartment B greatly improved fungal spread (stage 6). Without peat few secondary infections developed. The effect of the peat could be twofold. It could act as a buffer in the poorly buffered water agar, which quickly becomes acid as root exudates accumulate. The peat could also act as an adsorbent of staling substances that accumulate in the agar as the roots grow.

Effect of host and endophyte species. Surface sterilized spores of *Gigaspora margarita* germinated readily, 80% in 6 days, without extra stimulation. Using the same techniques as for *Glomus mosseae, Gigaspora margarita* produced typical infection in *C. sepium* (Fig. 12B). Typical VA infections were also produced in transformed roots of cowpea, potato, and carrot, the latter proving a very good host species.

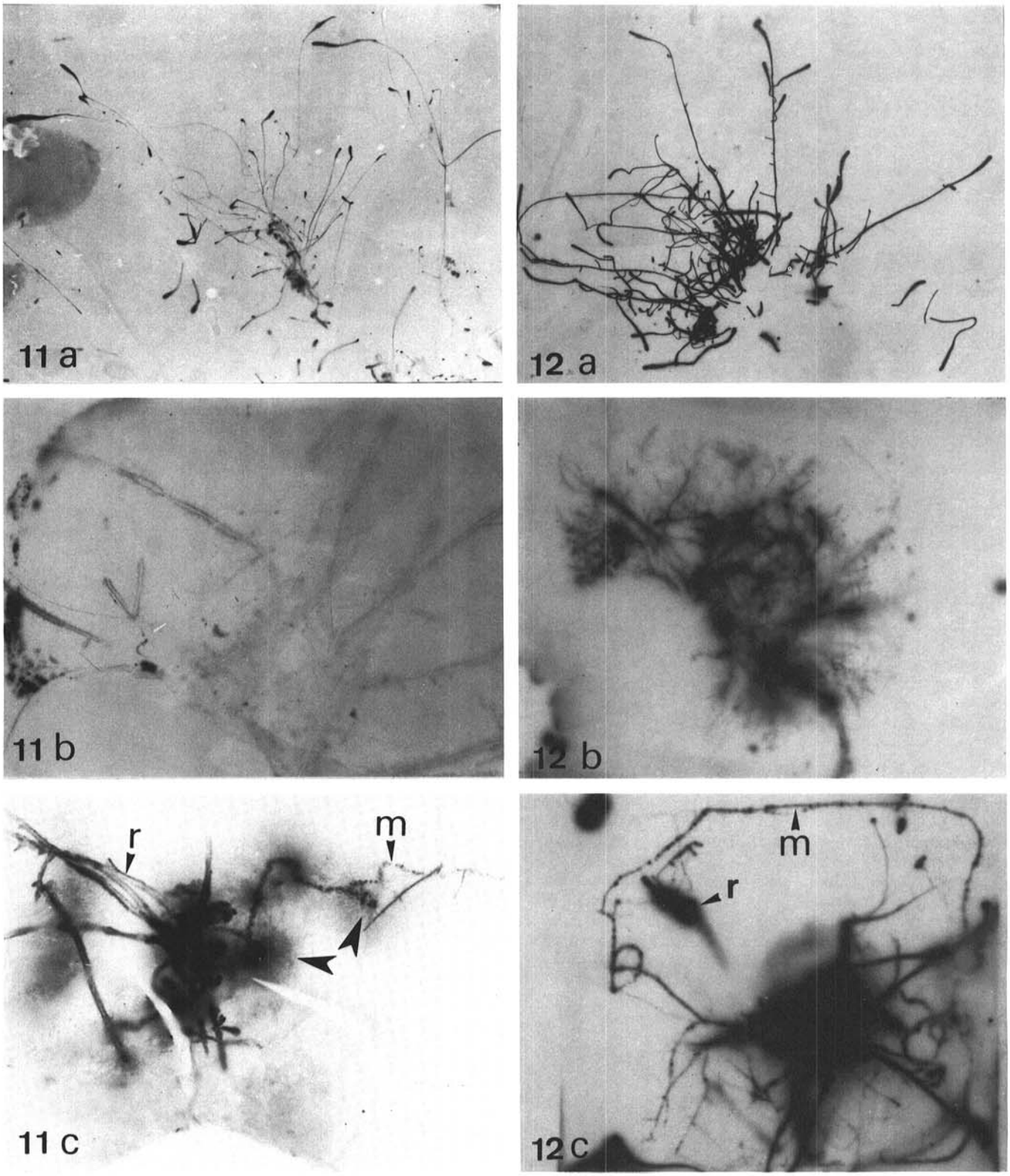
Labelling experiments. The results obtained on the movement of isotopes require confirmation. They are included here to illustrate the type of physiological information that can be obtained from the bicompartmental culture system. The cultures used for these experiments were well established; they had already passed the initial stage of rapid root growth and were well infected when the label was added. In all examples discussed here, ^{14}C sucrose, ^{32}P orthophosphate, or ^{35}S sulfur was added to the nutrient medium in compartment A, and movement of the isotope into uninoculated or inoculated (mycorrhizal) roots and attached external mycelium in compartment B was monitored. The contents of compartment A (roots and agar) were removed before autoradiography.

After 2 days, ^{14}C labelled substances metabolized by roots in compartment A had passed into the stele and growing tips of uninoculated roots in compartment B (Fig. 11A). After 10 days little label remained in the stele (Fig. 11B) but the root cortex and the surrounding agar contained labelled products, exudates, and/or substances lost from sloughed and dying cells. Figure 11C shows the distribution of radioactivity 10 days after labelling in mycorrhizal roots and external mycelium of *Glomus mosseae* in compartment B. Radioactivity in the external mycelium was concentrated in small granules, possibly lipids. This culture was contaminated during the adding label procedure by another, saprophytic fungus, and uptake of label into colonies of the contaminant can also be seen, but the labelling is weaker than that in the endophyte hyphae. As the contaminant was in discrete colonies, not on the root surface, it is assumed that it took up labelled substances that had diffused into the agar medium. The more strongly labelled endophyte mycelium probably obtained at least some of its labelled substances directly from the root. These interpretations agree with generally accepted views concerning changing patterns of carbon allocation after mycorrhizal infection (4). Autoradiography using roots in a bicompartmental system could provide relatively direct confirmation and increased understanding of such changes.

^{32}P added in compartment A was also translocated into uninoculated compartment B roots, but they appeared to retain the label. After 10 days there was no evidence that ^{32}P labelled substances had diffused out of the roots into the surrounding agar (Fig. 12A). Figure 12B and C shows clearly that, 10 days after labelling, ^{32}P containing substances, not necessarily orthophosphate, had been translocated into mycorrhizal roots in compartment B, and thence into external hyphae of the endophyte. Again there is evidence of accumulation of the labelled compounds in granules, particularly in *Gigaspora margarita* (Fig. 12B), less so in *G. mosseae* (Fig. 12C). It seems that some labelled phosphorus compounds moved directly out of the roots into the endophyte hyphae, indicating that movement of phosphorus, not necessary in the form of orthophosphate, can also occur from plant to fungus. The pattern of movement of ^{35}S was similar to that of ^{32}P.

The autoradiographs clearly show that under certain conditions both ^{14}C and ^{32}P labelled compounds can move from the roots into the external mycelium of the associated endophyte and tend to

accumulate there in discrete granules. The results also suggest that, in a nutrient medium conducive to the establishment of VA infection, uninfected roots essentially retain ^{32}P labelled compounds, whereas ^{14}C labelled compounds diffuse out. It would be possible and very interesting to observe if these patterns change when N and sugar levels in the nutrient medium are changed and how such changes might relate to the initiation of VA infection. Equally, the dependence of the endophyte on sulfur-containing compounds supplied through the host could be studied by this technique.



Figs. 11–12. 11A–C, Movement of ^{14}C labelled substances in transformed roots of *Convolvulus sepium*, fed proximally (in compartment A) with ^{14}C sucrose. Autoradiographs show the distribution of labelled substances in compartment B. **11A** and **B,** Uninoculated roots, 2 and 10 days, respectively, after adding label. **11C,** Mycorrhizal roots (r) and external mycelium (m) of *Glomus mosseae*, 10 days after adding label. Note also colonies of a contaminant fungus marked by large arrow. **12A–C,** Movement of ^{32}P labelled substances in transformed roots of *Convolvulus sepium*, fed proximally (compartment A) with ^{32}P labelled orthophosphate. Autoradiographs show the distribution of labelled substances in compartment B, 10 days after labelling. **12A,** Uninfected roots. **12B** and **C,** Mycorrhizal roots (r) and external mycelium (m) of *Glomus mosseae* and *Gigaspora margarita*, respectively.

DISCUSSION

The uptake and movement of mineral nutrients like phosphate, sulfate, and zinc into plants with VA mycorrhiza are well documented (5). Because these functions ultimately affect plant nutrient uptake in the field they have been studied extensively and are reasonably well understood. The converse movement of substances from the plant into the symbiont fungus is less well understood but has much relevance to the obligate nature of VA endophytes and attempts to establish them in monoxenic culture. The bicompartmental system using transformed roots has many advantages for studying this. The roots are easily accessible, allowing the direct introduction of isotopically labelled substances without the intervention of shoot metabolic processes, transpiration rates, light, or other factors of the shoot environment. Because root and fungus can be dried in situ without disorganization of their spatial relationships, or appreciable movement of water soluble substances, rates of movement and distribution of isotopically labelled substances can be followed very accurately and, if desired, related to infection phases.

Even quite large molecules such as antibiotics or biocides can be introduced into the root. The systemic, anti-oomycete fungicide fosetyl Al (Aliette) stimulates mycorrhizal development (7). It greatly increases soluble sugar levels in root exudates and total and free amino acids in root extracts. Various other studies have implicated root exudates and soluble sugar levels in the establishment of mycorrhizal infection (5). In entire plants, root exudates and sugar levels are difficult to control experimentally. They have been varied by different light regimes and by drastically changing nutrient balances, particularly N:P ratios, but such measures have other side effects, making it difficult to assign causal relationships. The growth potential of transformed roots yields sufficient biomass to study changes in root physiology, quantity, and composition of root exudates and pH changes in the root vicinity. Root organ cultures require an external carbon source and their carbon supply can therefore be more easily and accurately controlled than that of entire plants. Our tests indicated that carbon and nitrogen levels were critical for the establishment of VA infection in the root organ cultures. In the entire plant, phosphate supply could be one method of indirectly controlling exudates and soluble sugar levels, whereas they could be more directly controlled in the root organ cultures. That might explain why, in the entire plant, formation of VA mycorrhiza is usually sensitive to P levels, whereas these were not critical in the bicompartmental root organ cultures where sugar levels could be more directly controlled and nitrogen was minimal.

Transformed roots are being used also to study obligate root pathogens, e.g., *Polymyxa betae* Keskin (12). Contrary to VA endophytes, the obligate pathogens depend little on host nutrition, but they are much more dependent on host species, which has to be that of a susceptible plant. That emphasizes the possibly central role of plant nutrition in the development of the VAM symbiosis.

Many root symbionts are sensitive to sugars. They can react by becoming pathogenic, like some orchid rhizoctonias, by becoming noninvasive like rhizobia, or by arrested growth, like VA endophytes. A bicompartmental transformed root system can supply separate nutrition for host and microsymbiont, and can thus provide a relatively normal nutritional environment for both.

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