REVIEW / SYNTHÈSE

Arbuscular mycorrhiza on root-organ cultures[1]

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Abstract: The study of arbuscular mycorrhizal (AM) fungi and the AM symbiosis formed with host plant roots is complicated by the biotrophic and hypogeous nature of the mycobionts involved. To overcome this, several attempts have been made during the last three decades to obtain this symbiosis in vitro. The use of root-organ cultures has proved particularly successful. In this review, we describe the method by which root-organ cultures (transformed and nontransformed) have been obtained, together with the choice of host species, inoculation techniques, and culture media. We also outline the potential use of continuous cultures and cryopreservation of in vitro produced spores for long-term germ plasm storage. Furthermore, this review highlights the considerable impact that in vitro root-organ cultures have had on studies of AM fungal morphology, taxonomy, and phylogeny and how they have improved our understanding of the processes leading to root colonization and development of the extraradical mycelium. This is supported by a summary of some of the most important findings, regarding this symbiosis, that have been made at the physiological, biochemical, and molecular levels. We also summarize results from studies between AM fungi and certain pathogenic and nonpathogenic soil microorganisms. We describe some of the limitations of this in vitro system and propose diverse avenues of AM research that can now be undertaken, including the potential use of a similar system for ectomycorrhizal research.

Key words: arbuscular mycorrhiza, root-organ cultures, Glomales, in vitro, root symbioses, source of inoculum, cryopreservation, intraradical and extraradical mycelium, mycorrhizosphere.

Résumé : La nature biotrophique des champignons endomycorhiziens arbusculaires rend leur étude difficile tout comme celle de leurs relations symbiotiques. Pour pallier à cette difficulté de taille, plusieurs tentatives ont été faites, au cours des trois dernières décennies, pour obtenir la formation de ces mycorhizes en conditions aseptiques. Parmi ces essais, l'utilisation de la culture in vitro de racines isolées, transformées ou non, s'est avérée la plus réussie. Dans cette optique, nous décrivons en détail la méthode de base pour obtenir la culture in vitro de racines isolées en ayant soin de préciser le choix des espèces hôtes, la nature de l'inoculum et la composition du milieu de culture. On a aussi défini les paramètres expérimentaux de la culture continue et de la cryopréservation des spores, de manière à s'assurer la préservation à long terme des souches endomycorhiziennes. On décrit ensuite l'impact important qu'apporte la culture in vitro des racines isolées sur les études morphologiques, taxonomiques et phylogéniques des champignons endomycorhiziens arbusculaires tout en cherchant à compléter nos connaissances sur les processus qui président à la colonisation des racines hôtes de même qu'au développement du mycélium extraradical. Cette revue vise également à montrer l'apport significatif des études réalisées in vitro pour la meilleure compréhension des symbioses endomycorhiziennes au niveau physiologique, biochimique et moléculaire. On fait aussi état de l'étude des interactions entre les champignons

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endomycorhiziens arbusculaires et quelques organismes du sol, pathogènes ou non-pathogènes. De plus, on discute des limitations imposées par cette méthodologie et on propose diverses avenues de recherche qu'il est désormais possible d'aborder pour l'étude des glomales et de leur symbiose, ainsi que pour la production des inoculums. Finalement on souligne l'intérêt d'utiliser des méthodes similaires pour l'étude des ectomycorhizes.

Mots clés : endomycorhizes arbusculaires, culture de racines, Glomales, in vitro, symbiose racinaire, source d'inoculum, cryopréservation, mycélium intraradical et extraradical, mycorhizosphère.

Introduction

The establishment of in vitro root-organ cultures has greatly influenced our understanding of the arbuscular mycorrhizal (AM) symbiosis. Because of its potential for research and inoculum production, we outline a full description of the culture methods and a summary of the important findings that have resulted from the use of this in vitro system. We also outline possible avenues of research that will help to further our understanding of this symbiosis. For a full description of the AM symbiosis the reader is referred to Smith and Read (1997).

Mycorrhizal root-organ culture methods

Host roots

Root-organ cultures were first developed by White and coworkers (White 1943; Butcher and Street 1964; Butcher 1980). These authors used excised roots on synthetic mineral media supplemented with vitamins and a carbohydrate source. However, profuse root growth, characterized by the formation of numerous lower order branches, has been obtained with relatively few plant species. The formation of lower order roots is essential for rapid increase in root biomass and the establishment of continuous cultures (see the section titled Continuous cultures).

Pioneering work by Mosse and Hepper (1975) used root cultures obtained from *Lycopersicum esculentum* Mill. (tomato) and *Trifolium pratense* L. (red clover) to establish in vitro mycorrhiza with *Glomus mosseae* Nicolson & Gerd. The authors demonstrated for the first time that spores of an AM fungus could be successfully used to colonize excised roots growing on a mineral-based medium. Later, Strullu and Romand (1986, 1987) showed that it was also possible to reestablish mycorrhiza on excised roots of *Fragaria* ×*Ananassa* Duchesne (strawberry), *Allium cepa* L. (onion), and tomato, using the intraradical phase (i.e., vesicles or entire mycorrhizal root pieces) of several species of *Glomus* as inoculum.

A natural genetic transformation of plants by the ubiquitous soil bacterium *Agrobacterium rhizogenes* Conn. (Riker et al. 1930) produces a condition known as hairy roots. This stable transformation (Tepfer 1989) produces Ri T-DNA transformed plant tissues that are morphogenetically programmed to develop as roots. Their modified hormonal balance makes them particularly vigorous and allows profuse growth on artificial media (Tepfer 1989).

Daucus carota L. (carrot) and *Convolvulus sepium* L. (bindweed) were among the earliest species to be transformed using *Agrobacterium rhizogenes* Conn. (Tepfer and Tempé 1981). These Ri T-DNA transformed roots have since served in a wide range of fundamental and applied studies. One of the most important of these has been the study of the AM symbiosis. The first culture of hairy roots colonized by an AM fungus was achieved by Mugnier and Mosse (1987). These authors successfully colonized *Convolvulus sepium* hairy roots using spores of *G. mosseae* but, as was the case with nontransformed clover root-organ cultures (Hepper and Mosse 1975), no sporulation occurred. The first in vitro sporulation of an AM fungus was obtained by Bécard and Fortin (1988) using carrot hairy roots colonized by *Glomus intraradices* Schenck & Smith. Spore production followed reductions in the concentration of certain nutrients in the culture medium (see Bécard and Piché 1992) that allowed mycorrhizal inhibition to be avoided, but did not affect root growth and development. This led to the production of reproducible monoxenic cultures of *G. intraradices* that were characterized by large quantities of mycelium and spores (Diop et al. 1992).

Low mineral media minimal (M) and modified Strullu-Romand (MSR) media (see Table 1) were also successfully used to obtain mycorrhiza and fungal sporulation using nontransformed tomato root cultures (Chabot et al. 1992*a*; Diop et al. 1994*a*, 1994*b*; Bago et al. 1996). Nevertheless, transformed roots have a greater growth potential, which makes them more adaptable to different experimental conditions, and they can be generated from most dicotyledonous plants (Tepfer 1989). However, rigorous comparisons between transformed and nontransformed root cultures have never been made. Such studies should ideally be done using roots from the same plant material.

Whichever type of root system is chosen, success in establishing a mycorrhizal culture depends on the physiological state of the host root. Roots from the same clone, grown under the same conditions, can behave differently (G. Bécard, unpublished data). Subculture frequency, explant selection, and orientation of the Petri dishes during incubation (e.g., horizontal, upside down, or vertical) are important culture parameters that must be optimized for each clone.

Bécard and Fortin (1988) showed that 13-day-old *D. carota* hairy roots, which more closely resemble a "normal" root system (i.e., vigorous taproot, long elongation zone, and a pyramidal pattern of lateral root development), were colonized more readily than 9-day-old roots. The reduced mycorrhizal receptivity of the latter was probably due to a transitional phase, where developmental organization and growth rates were suboptimal. Recently, similar observations were made during a study of mycorrhizal and *Rhizobium*–legume symbioses using hairy roots of *Medicago truncatula* Gaertn. (Boisson-Dernier et al. 2001).

Fungal inocula

In most cases, two types of fungal inoculum can be used to initiate monoxenic cultures: either extraradical spores or

Table 1. Comparative composition of minimal (M) and modified Strullu-Romand (MSR) media.

	M (μM)	MSR (μM)
$N(NO_3^-)$	3200	3800
$N(NH_4^+)$	—	180
P	30	30
K	1735	1650
Ca	1200	1520
Mg	3000	3000
S	3000	3013
Cl	870	870
Na	20	20
Fe	20	20
Mn	30	11
Zn	9	1
B	24	30
I	4.5	—
Mo	0.01	0.22
Cu	0.96	0.96
Panthotenate Ca	—	1.88
Biotin	—	0.004
Pyridoxine	0.49	4.38
Thiamine	0.3	2.96
Cyanocabalamine	—	0.29
Nicotinic acid	4	8.10
Glycine (mg/L)	3	—
Myo-inositol (mg/L)	50	—
Sucrose (g/L)	10	10
pH (before autoclave)	5.5	5.5
Gellan agent (g/L)	5	3

Note: Minimal medium from Bécard and Fortin (1988); Strullu-Romand medium from Declerck et al. (1998), as modified from the original medium of Strullu and Romand (1986).

propagules from the intraradical phase (i.e., mycorrhizal root fragments and isolated vesicles) of the fungus. However, cultures of AM fungal species that do not produce vesicles (e.g., *Scutellospora* and *Gigaspora* species) are systematically produced using spores, which are usually large and germinate vigorously. Recently, sporocarps of *G. mosseae* have also been used in an attempt to establish in vitro cultures (Budi et al. 1999).

Spores

Spores are usually collected from the field, or from pot cultures, by wet sieving. With small spore samples (tens or hundreds), spores can be chosen individually under a dissecting microscope using a micropipette or fine tweezers. However, with larger spore samples gradient centrifugation must be used to separate out spores. Several centrifugation methods, based on the use of various highly concentrated substances (e.g., sucrose, glycerol, Percoll, and Radiopoaque contrast media), have been successfully used (Mertz et al. 1979; Furlan et al. 1980; Hosny et al. 1996). It is important, however, that spores are not subjected to prolonged exposure to these substances.

Before being used as in vitro inoculum, spores must be surface sterilized (see Bécard and Piché 1992). This step is critical because success depends on the elimination of all contaminants. It should be noted, however, that in some cases spores may carry bacteria between wall layers, making disinfection difficult or even impossible (Walley and Germida 1996).

A solution containing the strong oxidizing agent, chloramine T, and a surfactant (e.g., Tween 20) is widely used to sterilize AM fungal spores. Although 20 min in a 2% solution usually gives satisfactory results, concentration and treatment duration can be adapted depending on contaminant levels and spore sensitivity. Ideally, spores should be gently agitated during sterilization, or a vacuum applied to degas the spore surface. Spores are subsequently rinsed in a streptomycin–gentamycin antibiotic solution (Bécard and Piché 1992). To maintain spore dormancy, all steps from spore isolation to rinsing should be done on ice. If spores are not to be used immediately, they should be stored at 4°C, either in distilled water, on water agar, or on 0.1% $MgSO_4 \cdot 7H_2O$ solidified with 0.4% gellan gum. To reduce the risk of contamination by bacteria or fungi that were not eliminated during the sterilization process, spore number should be limited in each Petri dish.

Generally, AM fungal spores do not need specific conditions or the presence of a host root to germinate. However, root exudates and 2% CO_2 can stimulate germination and (or) postgermination hyphal growth (Bécard and Piché 1989*a*; Poulin et al. 1993; Buée 2000). Recalcitrant spores can be placed alongside a growing root. If spores fail to germinate within 20 days, either the sterilization treatment was too strong or the spores were immature, dormant, or dead. It is well known that spores of some AM fungal species require cold stratification (4°C) prior to germination (Smith and Read 1997). This requirement can vary within a genus: *Gigaspora gigantea* (Nicolson & Gerd.) Gerd. & Trappe (Koske 1981) and *Gigaspora margarita* Becker & Hall require a cold treatment, whereas *Gigaspora rosea* Nicolson & Schenck (formally misidentified as *Gigaspora margarita* (Bago et al. 1999*e*)) does not (Bécard and Fortin 1988). The cold treatment (14–21 days) is best applied prior to spore isolation, when the spores are still attached to the extraradical mycelium.

Mycorrhizal root fragments

In general, mycorrhizal roots used to initiate monoxenic cultures come from trap plants grown in pot cultures, with field-collected soil or AM fungal propagules. Leek (*Allium porrum* L.) plants are widely used because of their high susceptibility to colonization. Young, healthy, translucent leek roots should be chosen for in vitro culture establishment, as sections of roots with vesicles can be easily located. The roots are then disinfected in an ultrasonic processor under a laminar-flow hood (Declerck et al. 1998). This method has been successfully used to isolate numerous AM fungal species (Declerck et al. 1996, 1998, 2000; Plenchette et al. 1996; Strullu et al. 1997; Dalpé 2001). Treatment duration and reagent concentrations can be adapted to specific situations (e.g., host plant, root age, and contamination level).

Disinfected roots are cut into 5- to 10-mm lengths and incubated on a synthetic medium. MSR medium has given the best results (Declerck et al. 1998), but M medium (Bécard and Fortin 1988) and water-agar (Diop et al. 1994*a*) are also effective. Petri dishes should be incubated in the dark at

27°C. Hyphal regrowth from root pieces is usually observed within 2–15 days.

As shown for spores (see earlier in the paper), there is no evidence that mycorrhizal root pieces need specific exogenous conditions or a host plant for hyphal regrowth (Diop et al. 1994*a*). However, root exudates might stimulate vesicle germination and hyphal growth. Strullu et al. (1997) proposed that growth-promoting substances derived from host cells might accumulate in the intraradical structures of the mycorrhizal root pieces.

Following incubation, mycorrhizal root pieces showing hyphal regrowth are transferred, using a cork borer, to a fresh Petri dish with an actively growing root (Declerck et al. 1998), or an actively growing root is transferred to the Petri dish containing the mycorrhizal root.

Although field-collected roots have never been directly used as starter inoculum for in vitro cultures, their use should not be excluded. Vesicles within roots may be less contaminated than the root surface, offering a better source of inoculum. The vesicle extraction method (Strullu and Romand 1987; Strullu and Plenchette 1991) could be useful for such inocula. Vesicles, enzymatically extracted from roots, have been used to establish cultures with *G. intraradices*, *Glomus versiforme* (Karsten) Berch, and *Glomus macrocarpum* Tulasne & Tulasne (Strullu and Romand 1986, 1987), but vesicles are rarely used for routine inoculation. Comparisons have been made between the use of in vitro produced spores and vesicles isolated from leek plants grown in pot culture (Nantais 1997). Briefly, for a given number of propagules, root colonization was more efficient when using spores than when using isolated vesicles.

Culture media

The ingredients of the two most widely used and equally successful media for in vitro mycorrhizal root cultures are listed in Table 1. The M medium (Bécard and Fortin 1988) is a modified White's medium initially developed for tomato root-organ cultures (Butcher 1980). The macroelement composition of White's medium is considerably lower than that of MS and B5 media, commonly used for in vitro plant cultures (Bécard and Piché 1992). However, this dilute medium is adequate for root growth. The composition of M medium is even poorer and was developed following a bioassay that compared the effects of different element concentrations on mycorrhiza formation (Bécard and Fortin 1988).

The MSR medium (Declerck et al. 1998) is a modified A medium, which was developed to optimize the growth of the intraradical phase of the fungus in vitro. The macroelement composition of MSR is similar to that of the M medium. Differences between the two media occur in oligoelement and vitamin concentrations: MSR medium lacks iodide, myo-inositol, and glycine, and M medium lacks panthotenate, biotin, and cyanocobalamine. These various components are perhaps not essential, since their absence in either medium has no apparent negative effect on the AM symbiosis.

Both media are adjusted to pH 5.5 before autoclaving and are solidified with gellan gum. Almost 30 AM fungal isolates from the Acaulosporaceae, Gigasporaceae, and Glomaceae are now successfully grown on these media (see Table 2). However, as the compositions of the M and MSR media were established empirically, they could probably be further optimized. It is conceivable, for example, that AM fungi isolated from acidic or alkaline soils require either lower or higher pH, respectively, in vitro.

Continuous cultures

The first continuous culture was achieved by Strullu and Romand (1986) and is now commonly used for a wide range of *Glomus* (Strullu et al. 1997; Declerck et al. 1998) and *Gigaspora* species (G. Bécard, unpublished data). Continuous cultures are obtained by transferring mycorrhizal roots to fresh medium either with spores (St-Arnaud et al. 1996) or without (Declerck et al. 1996). Following this transfer, the preexisting root–fungus association continues to proliferate. If using older mycorrhizal roots, it is preferable to transfer them to a Petri dish containing an actively growing root (Declerck et al. 1996, 1998; Plenchette et al. 1996; Strullu et al. 1997).

For those *Glomus* species that produce few spores and vesicles (e.g., *G. macrocarpum* and *Glomus caledonium* (Nicholson & Gerd.) Trappe & Gerd.), mycorrhizal roots and spores can be transferred concomitantly to increase the probability of success (S. Declerck, unpublished data). Several *Glomus* species seem not to require cold stratification or specific compounds for subculturing. However, the possible impact of such treatments cannot be excluded.

For AM fungal species that do not produce internal vesicles (*Gigaspora* and *Scutellospora* spp.), direct subculturing is possible but more difficult to achieve. The subcultured sample, a square (10–15 cm^2) of nondisturbed mycorrhizal roots and external mycelium, is transferred to a Petri dish containing fresh medium (M. Buée and G. Bécard, unpublished data). Alternatively, with older cultures, the in vitro produced spores can be used to inoculate new roots (Bécard and Fortin 1988). However, spores of some *Gigaspora* species may require cold treatment prior to germination (see the section titled Fungal inocula).

Dual- and multi-compartment systems

Although the in vitro system is artificial, it allows nondestructive, morphological and physiological investigations of the AM symbiosis. Moreover, it is possible to increase its similarity to a natural system by providing the extramatrical mycelium with a nutrient environment closer to that of the mineral soil. This technique was developed by St-Arnaud et al. (1996) using bicompartmental Petri dishes (Fig. 1). These authors placed an appropriate growth medium for the AM root-organ culture in the proximal compartment and a mineral-only medium in the distal compartment. During incubation, only the extramatrical mycelium was allowed to develop in the latter compartment.

The first striking observation using this technique was a dramatic stimulation of mycelial growth and a 10-fold increase in spore production in the distal compartment compared with the proximal compartment (Fig. 2). It appears that the aging root in the proximal compartment releases chemicals that limit fungal growth and spore production. However, it is also possible that these observations are the result of pH changes and (or) alterations in nutrient availability.

Bi-, tri-, and quadri-compartmental Petri dishes were used by Villegas et al. (1996) and Bago et al. (1996) to study nitrogen absorption and substrate pH modifications by the extramatrical mycelium of *G. intraradices*. They have also

Table 2. Species of Glomales cultivated on root-organ cultures.

Acaulosporaceae	
Acaulospora laevis Gerd.& Trappe[a]	S. Declerck, unpublished data
Acaulospora morrowae Spain & Schenck	C.G. Wu, personal communication
Acaulospora rehmii Sieverding & Toro	S. Declerck, unpublished data
Gigasporaceae	
Gigaspora albida Schenck & Smith	C.G. Wu, personal communication
Gigaspora gigantea (Nicolson & Gerd.) Gerd. & Trappe	Gadkar et al. 1997
Gigaspora margarita Becker & Hall	Miller-Wideman and Watrud 1984; Karandashov et al. 1999
Gigaspora rosea Nicolson & Schenck	Forbes et al. 1998
Scutellospora castanea Walker[a]	S. Declerck, unpublished data
Scutellospora nigra (Redhead) Walker & Sanders	C.G. Wu, personal communication
Scutellospora reticulata (Koske, Miller, Walker) Walker Sanders	F.A. De Souza, personal communication
Glomaceae	
Glomus aggregatum Schenck & Smith emend. Koske	P. Moutoglis, personal communication
Glomus caledonium (Nicolson & Gerd.) Trappe & Gerd.	Karandashov et al. 1999
Glomus cerebriforme McGee	Samson et al. 2000
Glomus clarum Nicolson & Schenck	F.A. De Souza and Berbara 1999; C.G. Wu, personal communication
Glomus constrictum Trappe	T.A. Diop, personal communication; Mathur and Vyas 1999
Glomus deserticola Trappe, Bloss & Menge	Mathur and Vyas 1995
Glomus diaphanum Morton & Walker?[b]	S. Declerck, unpublished data
Glomus etunicatum Becker & Gerd.	Schreiner and Koide 1993; Pawlowska et al. 1999; Karandashov et al. 1999
Glomus fasciculatum (Thaxter sensu Gerd.) Gerd. & Trappe emend. Walker & Koske	Declerck et al. 1998
Glomus fistulosum Skou & Jakobson[a]	Nuutila et al. 1995; Gryndler et al. 1998
Glomus intraradices Schenck & Smith	Chabot et al. 1992*a*; Karandashov et al. 1999
Glomus lamellosum Dalpé, Koske & Tews	Y. Dalpé, unpublished data
Glomus macrocarpum Tulasne & Tulasne	Declerck et al. 1998
Glomus mosseae (Nicolson & Gerd.) Gerd. & Trappe	Y. Dalpé, unpublished data
Glomus proliferum Dalpé & Declerck	Declerck et al. 2000
Glomus versiforme (Karsten) Berch	Diop et al. 1994*a*
Sclerocystis sinuosa (Gerd. & Bakshi) Almeida & Schenck	C.G. Wu, personal communication

[a]Species differentiating only vesicle-like spores.
[b]Identification to be confirmed.

been widely used to study physiological relationships and interactions with other organisms.

Cryopreservation of in vitro produced spores

As mentioned earlier, the continuous culture method allows AM fungi to be maintained monoxenically over long periods (Plenchette et al. 1996; Strullu et al. 1997; Declerck et al. 1998) and serves to produce, and store, AM fungal germ plasm. However, as with AM fungi in pot cultures, this in vitro method does not ensure genetic stability of the fungal material over generations. Several methods of long-term storage have been developed to overcome this problem.

One method consists of storing cultures at 4°C. Although information exists concerning the survival of pot-culture inoculum using this method (Douds and Schenck 1990), there are little or no data concerning monoxenically produced inocula. At 4°C, fungal and root metabolism is slowed, but not halted. Addy et al. (1998) were the first to demonstrate that monoxenically grown extraradical mycelium of *G. intraradices* was able to survive exposure to –12°C. These authors slowly cooled cultures before freezing. Recently, Declerck and Angelo-van Coppenolle (2000) developed a cryopreservation technique based on the entrapment of monoxenically produced spores of *G. intraradices* in alginate beads (~100/bead). The entrapped spores were incubated for 24 h in 0.5 M trehalose and cryopreserved at –100°C, following a two-step decrease in temperature: a slow decrease (1°C/min) from ambient temperature to –35°C, and a fast decrease (18°C/min) from –35°C to –100°C. After fast thawing in a water bath at ambient temperature, spores within beads were able to germinate, colonize host roots, and complete their life cycle in vitro. This experiment was done using spores from 5-month-old cultures, which corresponds to the plateau phase in the spore production in *G. intraradices*. Most spores could, therefore, be considered mature. This is likely to be an important criterion for success. As other species and genera exhibit different sporulation kinetics, this factor should be borne in mind and the cryopreservation method adapted. Although cryopreservation is a very promising long-term storage technique, only one species has been tested to date, and over a short time period. More work is required to validate this method over longer storage periods and with other fungal species.

Glomales in vitro collection

Since 1975, when Mosse and Hepper (1975) first grew the mycelium of *G. mosseae* using an in vitro system, at least 27 AM fungal species have been successfully cultivated on

Fig. 1. A 3-month-old culture of *Glomus intraradices* grown in a 9-cm bicompartmentalized Petri dish. The proximal symbiosis compartment (P) contains the fungus and a Ri T-DNA transformed carrot root, and the distal compartment (D) is root free.

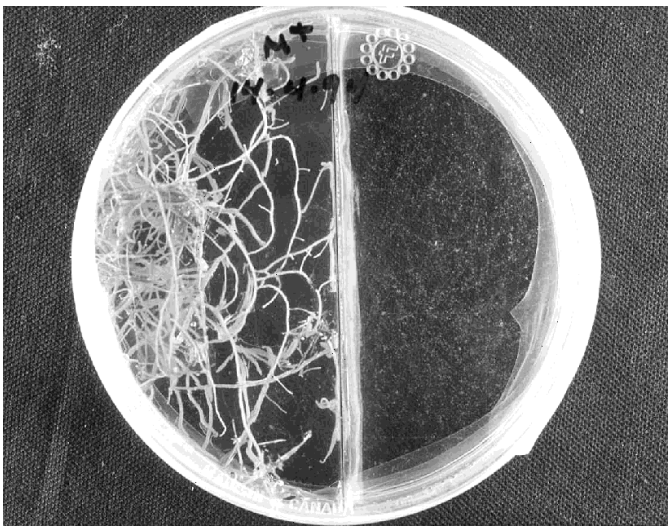
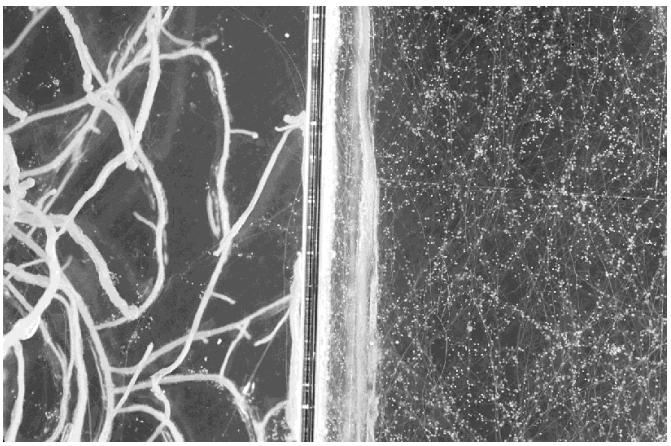


Fig. 2. Detail of part of Fig. 1, showing the difference in mycelial development and spore production between the proximal and root-free distal compartments.



root-organ cultures. The majority of these have been obtained during the last decade (Table 2). These species represent five genera within three of the Glomales families (Acaulosporaceae, Gigasporaceae, and Glomaceae). In addition to the identified species, several unidentified strains are also presently maintained on root-organ cultures. With the exception of *Acaulospora laevis* Gerd. & Trappe (S. Declerck, unpublished data), *Acaulospora rehmii* Sieverding & Toro (Y. Dalpé and S. Declerck, unpublished data), *Scutellospora castanea* Walker (S. Declerk, unpublished data), *Glomus fistulosum* Skou & Jacobsen (Nuutila et al. 1995), and some of the unidentified species, all strains have been maintained through several generations (Bécard and Fortin 1988; Chabot et al. 1992*a*; Plenchette et al. 1996; Strullu et al. 1997; Declerck et al. 1998; Gadkar et al. 1997; Pawlowska et al. 1999; De-Souza and Berbara 1999; Gadkar and Adholeya 2000; Karandashov et al. 2000). *Glomus mosseae*, which has been recalcitrant in accepting full in vitro cultivation (Mosse and Hepper 1975; Douds 1997), can now be replicated from mycorrhizal root segments (Y. Dalpé, unpublished data).

The increasing number of species of AM fungi cultivated in vitro, and the possibility of continuous cultivation and cryopreservation, has led to the development of an international collection of in vitro AM fungi: the Glomales in vitro collection (GINCO). This collection has resulted from a collaboration between the Mycothèque de l'Université Catholique de Louvain (MUCL, Belgium) and the Eastern Cereal and Oilseed Research Centre (ECORC, Agriculture and Agri-Food Canada), which is responsible for the Canadian Collection of Fungal Cultures (CCFC/DAOM, Canada). GINCO aims to conserve biodiversity and provide high-quality, contaminant-free AM fungal inocula for scientific research. GINCO, in collaboration with a team of scientists working on AM fungal physiology, biochemistry, taxonomy, and ecology, intends to increase the number of taxa available, offer specialized training, and develop an international network of collaborative research. Information concerning the collection's policies and species availability can be obtained via the Web (in Belgium: http://www.mbla.uclac.be/ginco-bel; in Canada: http://res2.agr.ca/ecorc/ginco-can/).

Root-organ cultures as a tool for AM fungal systematics

The taxonomy of AM fungi is based almost entirely on spore morphology, with description, identification, and classification to the species level being difficult. Isolation of AM fungi from pot cultures often produces spores lacking subtending hyphae and (or) with damaged spore wall layers (especially the outer evanescent layer). As a consequence, poor quality spore reference material has generated incomplete and sometimes unusable species descriptions. Moreover, the absence of living cultures of type specimens has dramatically reduced studies of spore ontogenesis. However, the root-organ culture system has renewed interest in AM fungal taxonomy. The contaminant-free cultures give constant access to clean fungal propagules, which can be observed and harvested at any stage during fungal development (Bécard and Piché 1992; Chabot et al. 1992*a*; Strullu et al. 1997; Dalpé 2001). This material is much more appropriate for morphological, ultrastructural, physiological, biochemical, and molecular studies than pot-cultured fungi. The in vitro grown AM fungi constitute a reliable material for species characterization and description (Declerck et al. 2001) and for evolutionary and interspecific studies. However, studies by Pawlowska et al. (1999) suggest that at least one species, *Glomus etunicatum* Becker & Gerd., grown using the in vitro system may produce slightly smaller spores than in pot culture.

Phylogenetic studies

Monoxenic spore production on root-organ cultures allowed the first amplification of ribosomal DNA (rDNA) from an AM fungus (Simon et al. 1993*a*). The use of this system gave the assurance that the sequences were not of homologous genes from non-AM fungi or other soil eukaryotes usually present in standard spore preparations. Following this breakthrough, Simon (1995) designed a spe-

cific rDNA primer (VANS1). All fungal samples sharing sites for this specific primer occur in a subgroup showing obvious associations with the Glomales (Simon et al. 1993*a*, 1993*b*). However, the annealing site of VANS1 might not be the only ancestral lineage of the Glomales (Clapp et al. 1999; Redecker et al. 1999; Schüssler et al. 2001).

Polymerase chain reaction (PCR) based molecular techniques and DNA sequencing are important tools for studying the genetic diversity and classification of AM fungi (Morton and Redecker 2001). However, the results should be interpreted cautiously, as AM fungal extracts often contain foreign prokaryotic (Bianciotto et al. 1996*a*) and eukaryotic genomes (Redecker et al. 1999; Schüssler et al. 2001).

Mycelial development and sporulation dynamics

As in vitro fungal colonies are directly observable under dissecting and inverted microscopes without disturbing or contaminating the system, several new developmental aspects have been observed, some of which are considered as specific taxonomic traits.

Developmental dynamics of the extraradical mycelium (Bago et al. 1998*a*, 1998*b*, 1998*c*; Dalpé 2001; Karandashov et al. 2000), and their relationship with spore maturation processes and root colonization (Pawlowska et al. 1999; De-Souza and Berbara 1999), may provide a new systematic approach complementing the present descriptive taxonomy. Experienced microscopists can distinguish between AM fungal isolates by comparing hyphal growth patterns and sporulation. For example, prior to establishment of the AM symbiosis, certain hyphae of *G. etunicatum* (Pawlowska et al. 1999) and *Glomus proliferum* Dalpé & Declerk (Declerck et al. 2000) swell, giving rise to small-diameter (20–45 μm), thin-walled, hyaline structures. These swellings are, in fact, juvenile spores that mature after establishment of the symbiosis. However, similar swellings in *G. mosseae* (Mosse and Hepper 1975), *G. caledonium* (Karandashov et al. 2000), *Glomus clarum* Nicolson & Schenck (De-Souza and Berbara 1999), and *Acaulospora rehmii* (Y. Dalpé and S. Declerck, unpublished data) do not develop further. These features are reminiscent of the vesicle-like structures of *Acaulospora appendicula* Spain, Sieverding & Schenck (Schenck et al. 1984), the extraradical vesicles observed in certain *Glomus* spp. (Wu and Sylvia 1993; Schenck et al. 1984), and the auxiliary cells of the Gigasporaceae (Adholeya et al. 1997; Séjalon-Delmas et al. 1998; Bi et al. 1999; Karandashov et al. 1999). In other species, hyphal swellings and spores share the same sporulating hyphae. Interestingly, cultures of species producing hyphal swellings only (i.e., without spore formation) can be used to replicate root-organ cultures.

From a taxonomist's viewpoint, the major advantage of AM root-organ cultures is the possibility of maintaining AM fungi under continuous culture and following spore ontogenesis from initiation to senescence. The sporulation dynamics of *Gi. rosea* (Bécard and Piché 1992), *G. versiforme* (Declerck et al. 1996), *G. etunicatum* (Pawlowska et al. 1999), *G. caledonium* (Karandashov et al. 2000), and *G. proliferum* (Declerck et al. 2000, 2001) have shown that spores of most *Glomus* species require only 2–3 days to achieve their mature size. Five days are necessary for spore wall differentiation in *Gi. rosea* and 8–9 days in *G. etunicatum* (Pawlowska et al. 1999).

The ultrastructural study of spore wall organization in in vitro produced spores of *G. intraradices* aged from 15 to 90 days allowed the first observations of the consecutive stages that give rise to the typical compact multilaminated spore walls (Y. Dalpé and S. Declerck, unpublished data). In vitro studies have also shown that the outer evanescent or mucilaginous spore wall layer originates from the wall of the initial hyphal swelling, which suggests that all soil-borne spores should have such a wall. This finding is of considerable taxonomic importance. Ontogenetic studies of spores of *A. rehmii* have also been done (unpublished data). Briefly, non-apical hyphal swelling was followed by the simultaneous differentiation of a sporiferous saccule and a spore, an ontogenesis partly contradicting the observations made using pot-culture material.

Intraspecific variability

The availability of several *G. intraradices* strains in vitro will allow in-depth investigations into the intraspecific variability of AM fungi without the risk of measuring artifacts due to growing conditions, contaminants, or host physiological status. Comparative analysis of spore ontogenesis, spore wall architecture, and ultrastructure, combined with biochemical analyses and molecular investigations, is now feasible. This multidisciplinary approach has been recently used for the description of the new species *G. proliferum* (Declerck et al. 2000).

AM root-organ cultures facilitate the study of environmental, nutritional, and physiological effects on fungal growth and spore morphology. A range of parameters related to sporulation, hyphal architecture, and spore ontogenesis can now be compared and priorized according to the systematic approaches used. The multiple characteristics (i.e., anatomical, ultrastructural, or biochemical) that can be generated using in vitro produced AM fungal colonies need to be classified according to their taxonomic significance, to discriminate between environmentally related and phylogenetically driven taxonomic parameters. For example, as previously mentioned, in vitro differentiated spores may be slightly smaller and are often less pigmented than soil-borne spores (Pawlowska et al. 1999; Dalpé 2001).

Fungal morphological features before and after root colonization

Previously, most structural studies concentrated on the intraradical plant–fungus interfaces and relatively few studies investigated the structural aspects of the precolonization and extraradical phases. However, in vitro cultivation of AM fungi using root-organ cultures opens new avenues for hyphal structural studies during spore germination, precolonization, and development of the extraradical mycelium.

In vitro germination of spores

The use of AM root-organ cultures allows the aseptic production of spores of various AM fungal species. Although it is well known that cold stratification is important to break the inherent dormancy-like stage found in certain AM fungal species, recent observations by Juge et al. (2001) showed that this treatment not only affects spore germinability but also has a dramatic effect on germ tube morphology. Cold

treatment applied for more than 14 days led to full germination with strong apical dominance and sparse branching as previously described by Mosse (1988). In the absence of a cold treatment, a unique germination pattern was observed: germ tubes were short with profuse branching, spiraling around and close to the spores.

Although AM fungi have the capacity for initial germination, germ tube elongation is fatally blocked in the absence of a host plant (Bonfante and Perotto 1995). Recently, in vitro studies using two-photon microscopy revealed autolytic zones in live but senescent germ tubes of *Gi. rosea* (Bago et al. 1998*d*). These areas coexisted with zones exhibiting complete cytoplasmic integrity. Cytological analyses suggested that portions of these coenocytic hyphae were undergoing cell death or apoptotic processes (programmed death). This precolonization senescence phenomenon is theoretically reversible. This is supported by Balaji et al. (1995), who showed that it was possible to inhibit or stimulate AM fungal germ tube growth by removing, or not, root exudates and volatiles under in vitro conditions.

In vitro development of the extraradical phase

The use of root-organ cultures in compartmentalized Petri dishes (St-Arnaud et al. 1996) also allows time-lapse studies of extraradical mycelial development in root-free compartments. When comparing ammonium and nitrate as nitrogen sources, Villegas (2001) showed that the presence of ammonium in the distal compartment drastically reduced spore production. The author also showed that in the presence of ammonium, the mycelium of the extraradical phase developed coiled hyphae and hyphal aggregations that were never observed in the presence of nitrate. This in vitro system also allowed Bago et al. (1996, 1998*a*, 1998*b*, 1998*c*) to observe the structural development of the extraradical phase of *G. intraradices*, which comprises an organized radial network of runner hyphae from which lower order branches (at a 45° angle) develop at regular intervals (between 25 and 300 μm). Some of these ramifications developed into new runner hyphae, and others bore arbuscule-like structures (ALS) and spores (Bago et al. 1998*a*). Ultrastructural investigations revealed that ALS (renamed branched absorbing structures or BAS; Bago et al. 1998*b*) are very similar to intraradical arbuscules and that, like arbuscules, they are sites of intense metabolic activity. Arbuscules and BAS are also similar in terms of their gross morphology (thinner diameter with increased dichotomous branching). The extent to which these structures are functionally comparable remains to be elucidated. However, prolific branching of the fungus to form BAS results in an important increase in surface area and so produces a structure better adapted for nutrient uptake. It has also been shown that increased acidification of the medium coincides with a higher production of spore-associated BAS. This change in pH could be a direct consequence of a greater phosphate uptake, to provide storage products for the spores (Bago et al. 1998*b*, 1998*c*). It also appears that inorganic nitrogen and phosphate absorption by extraradical mycelium is closely correlated with BAS development (Bago et al. 1998*b*).

BAS and arbuscules also have similar life-spans (approx. 7 days). However, the reason for such short life-spans and the evidence for possible host involvement are generally lacking (Smith and Read 1997). Apoptotic processes within these structures may explain the prompt degradation observed. This hypothesis is supported by the early events leading to nuclear degradation observed within lysed compartments in extraradical hyphae (Bago et al. 1999*a*).

Physiological aspects of host–fungal relationships

Physiological studies of the symbiotic relationships between plant roots and AM fungi are difficult to carry out because physiological events occur in a complex hypogeous environment. This is further complicated by the fact that one of the two partners is impossible to grow axenically. However, the use of AM root-organ cultures as a simplified model system has been particularly useful for the investigation of at least two physiological aspects: signaling between the symbiotic partners and metabolism of the fungus. This has been possible because most parameters (e.g., host root, fungal inoculum, and the physical, chemical, and microbiological environment) can be strictly controlled. Under these conditions, detailed nondestructive observations at the morphological and cellular levels can be made. The AM root-organ culture system also allows the production of pure fungal biomass, at various symbiotic stages and in sufficient quantities for further cytological or biochemical analyses. The most important physiological findings obtained using the root-organ culture system are outlined in the following sections.

Interpartner signalling

Spore germination does not generally require the presence of a host root (the non-symbiotic stage). However, for further growth and development, the AM fungus becomes dependent upon the presence, but without physical contact, of an adequate host (Mosse and Hepper 1975; Elias and Safir 1987; Bécard and Piché 1989*b*; Giovannetti et al. 1993, 1996). It was shown using *Gi. rosea* (Bécard and Piché 1989*a*; Chabot et al. 1992*b*; Poulin et al. 1993), and later confirmed with other *Gigaspora* spp. (M. Buée and G. Bécard, unpublished data), that this activated physiological stage (the pre-symbiotic stage) requires the simultaneous presence of root exudates and CO_2. Bécard and Piché (1989*a*) suggested that *Gi. rosea* was capable of fixing CO_2 as a mineral source of carbon. Recently, in vitro labelling with $^{13}CO_2$ and NMR spectroscopic analyses have confirmed that substantial dark fixation of CO_2 occurs in *G. intraradices* during spore germination (Bago et al. 1999*b*).

At this stage, fungal metabolism is predominantly dependent upon lipid catabolism for further biosynthesis. Lipid catabolism leads to formation of acetylCoA, a two C molecule, which is further oxidized in the TCA cycle and the net balance of C is theoretically zero. However, the glyoxylate cycle occurs in AM fungi (Bago et al. 1999*b*), and this cycle, unlike the TCA cycle, avoids the loss of the two CO_2 molecules. In addition to the glyoxylate cycle, CO_2 may represent an additional input of C and significantly activate fungal growth. Carbon dioxide can, therefore, be considered as the first nonspecific stimulatory compound emitted by the host root. Indeed, studies on more specific root stimulatory compounds (see later in the paper) are often conducted in a CO_2-enriched environment (i.e., incubators adjusted to 2% CO_2). Under these conditions fungal responsiveness is maximized and re-

sponse variations minimized (Bécard and Piché 1989*a*; Bécard et al. 1992, 1995; Chabot et al. 1992*b*; Poulin et al. 1993, 1997; Balaji et al. 1995; Buée et al. 2000). This is particularly true when single spores are used as the experimental unit, because they do not produce enough CO_2 to efficiently recycle it, even in closed Petri dishes.

Gemma and Koske (1988) proposed the implication of other root volatiles during pre-symbiotic fungal growth. Root chemoattraction of *Gi. gigantea* germ tubes was illustrated by using a whole-plant, in vitro culture system in which the attracted fungal hyphae were grown aeroponically. The stimulatory action of nonvolatile root exudates on auxiliary cell production and hyphal branching has been investigated using the in vitro system (unpublished data). Studies at the cellular level have revealed that plasmalemma H^+-ATPase activity, phosphate uptake, intracellular pH, and mitotic activity are correlated with the pre-symbiotic stimulation of the fungus (Lei et al. 1991; Jolicoeur et al. 1998; Buée 2000; Buée et al. 2000). The in vitro root-organ system will be indispensable for further investigations of the mechanisms by which the plant host triggers fungal growth and development at the cellular, biochemical, and molecular levels.

Very little is known about the chemical nature of the stimulatory compounds produced by roots. However, these substances are potentially produced by a wide range of plant species. By analogy with the *Rhizobium*–plant and *Agrobacterium*–plant interactions, phenolic compounds (e.g., various flavonoids and phenolic acids) have been tested on axenically germinating spores (Gianinazzi-Pearson et al. 1989; Tsai and Phillips 1991; Nair et al. 1991; Bécard et al. 1992; Chabot et al. 1992*b*; Poulin et al. 1993; Nagahashi et al. 1996; Douds et al. 1996). Some flavonoids are highly stimulatory (reviewed by Vierheilig et al. 1998). These results suggest a degree of chemical and stereo-specific activity which may vary between fungal species. For example, isoflavonoids such as biochanin A and formononetin stimulate some *Glomus* species (Nair et al. 1991; Poulin et al. 1997) but inhibit *G. etunicatum* (Tsai and Phillips 1991) and *Gi. rosea* (Bécard et al. 1992; Chabot et al. 1992*b*). Moreover, it has also been shown that flavonols such as quercetin, kaempferol, and myricetin strongly stimulate *Gigaspora* species (Bécard et al. 1992; Chabot et al. 1992*b*). Interestingly, flavonoids, which are known for their estrogenic activity in vertebrates, may attach to estrogen-like binding sites in AM fungi (Poulin et al. 1997).

Although the aforementioned chemicals are potentially present in root exudates of many plant species (Nair et al. 1991; Tsai and Phillips 1991; Bel-Rhlid et al. 1993), their role as essential plant signals for mycorrhiza establishment has been questioned (Bécard et al. 1995). In vitro and in vivo experiments showed that mycorrhiza formation, and normal symbiotic fungal growth and development, could be obtained in the absence of flavonoids. Recent in vitro investigations, carried out to isolate the active stimulatory root molecules, have shown that some purified fractions of root exudates have a strong effect on fungal branching (Nagahashi and Douds 1999) and cell proliferation (Buée et al. 2000). This activity was found in root exudates of all eight host plants tested, but not in the root exudates of four nonhost species.

Preliminary chemical analyses indicate that the molecules responsible are lipophilic, of low molecular weight, and thermoresistant, but that they are not flavonoids. Their presence at very low concentrations mimicked the presence of an intact root. Although their exact chemical nature has yet to be determined, plant mutants unable to produce these substances will have to be developed to obtain genetic evidence that the presence of these molecules in the rhizosphere is essential for mycorrhiza establishment.

Once fungal growth and branching have been stimulated by specific root compounds, the probability of a root–fungus contact increases. For root colonization to occur, there must be formation of an appressorium. Perception of specific topographical and (or) biochemical components of the root surface seems to be important for formation of this structure (Nagahashi and Douds 1997). This has been shown in vitro with germinating spores of *Gi. rosea* growing in the presence of large fragments of purified root cell wall, where fungal contact with host root epidermal cell wall fragments led to appressorium formation.

For further development of the symbiosis, the fungus must penetrate the root tissues to produce an intraradical mycelium with arbuscules, and in some cases vesicles. Since the discovery that certain pea mutants insensitive to nodulation (Nod^-) were also insensitive to colonization by AM fungi (Myc^-) (Duc et al. 1989), many other Nod^- legume mutants have been isolated. These mutants exhibit various Myc^- phenotypes (Harrison 1999; Peterson and Guinel 2000). In vitro studies using root-organ cultures of Myc^- mutants suggest that the plant genetic programs involved in both symbioses share some common genes (Balaji et al. 1994). These observations are further supported by other genetic-based studies (Gianinazzi-Pearson and Dénarié 1997; Hirsh and Kapulnik 1998; Catoira et al. 2000). Thus, these genetic programs may control recognition of the symbiotic partner as a nonpathogen and may be linked to certain plant defense mechanisms. They may also control interfacial transport of nutrients and the excessive development of the mycobiont. Therefore, a subtle molecular dialogue involving signals and specific responses from the two partners seems to occur at all stages of root colonization by the fungus. Although in vitro root-organ culture systems have rarely been used to investigate this molecular dialogue (Balaji et al. 1994, 1995; Boisson-Dernier et al. 2001), they are likely to be useful, particularly in the isolation and identification of the critical fungal signals involved.

Metabolism of the fungal partner

The compartmentalized Petri dish system (St-Arnaud et al. 1996) is particularly suitable for the studies of nutrient uptake and translocation in AM fungi under strictly controlled conditions. It also allows the differentiation between intraradical and extraradical fungal metabolism (Bago et al. 2000).

The carbon (C) metabolism of AM fungi has recently been examined by combining the in vitro compartmentalized system with ^{13}C-labelling and spectroscopic NMR analyses (Pfeffer et al. 1999; Bago et al. 2000). Various ^{13}C-labelled substrates were supplied either to the mycorrhizal root compartment or to the fungal compartment (extraradical hyphae). Incorporation of ^{13}C was analyzed in hydrophilic and lipophilic metabolites, extracted from the mycorrhizal roots and the extraradical mycelium. The results are reviewed in Pfeffer et al. (1998) and Bago et al. (2000). Previ-

ous data had shown, using in vivo NMR spectroscopic analyses of perfused mycorrhizal roots excised from leek (Shachar-Hill et al. 1995) and by respirometry experiments on mycorrhizal onion roots (Solaiman and Saito 1997), that glucose was the major source of C provided by the plant to the fungus. The ^{13}C-labelling experiments with the compartmentalized in vitro system (Pfeffer et al. 1999) confirmed that $^{13}C_1$-glucose and $^{13}C_1$-fructose, when supplied exogenously, were taken up by the fungus and rapidly converted to trehalose, glycogen, and lipids. The authors also showed that this sugar uptake occurred exclusively in the internal mycelium of the fungus. When the external mycelium was supplied with these labelled sugars, no labelling was found in the fungus. These data indicate that the role of the root is to trigger the pathways, allowing uptake and metabolism of simple sugars (glucose and fructose) by the AM fungus. These experiments suggest that the exogenous sucrose, routinely supplied to the mycorrhizal roots in vitro, is in fact used by the fungus as a carbon source. However, sucrose must be hydrolyzed by root invertase or sucrose synthase activity before being assimilated by the fungus. From these experiments it has been hypothesized that, in vivo, sucrose transported from the leaves to the roots is hydrolyzed in the root apoplast and that the products of this hydrolysis (glucose and fructose) represent the main C source for the fungus (Pfeffer et al. 1998, 1999).

Lipids represent about 50% of the dry biomass of AM fungi, and triacylglycerols are the major component (Sancholle et al. 1999). Therefore, lipid biosynthesis is an important activity of AM fungi (Sancholle et al. 2001). Heavy water (D_2O) labelling experiments showed that fatty acids (FA) are, for the most part, synthesized by the intraradical mycelium of *G. intraradices* (Pfeffer et al. 1999). Similar studies have also been carried out using ^{14}C-labelled acetate to investigate lipid metabolism in germinating spores (Beilby and Kidby 1982) and the extraradical mycelium (Fontaine 2001). In contrast with the previous studies using D_2O and ^{13}C-glucose labelling, lipid biosynthesis was detected in germinating spores and in extraradical mycelium, and mono-, di-, and triacylglycerols, phospholipids, and sterols were labelled. Only the ∂-amyrin, usually present in the fungus at low levels, was not synthesized by the fungus. However, it should be noted that the apparent contradictory results between ^{13}C and ^{14}C experiments are perhaps due to the greater sensitivity of ^{14}C techniques that allow the detection of even minimal amounts of label.

As a whole, these results indicate that the fungus is capable of a certain degree of lipid biosynthesis at all stages of its life cycle, even in germinating spores and extraradical hyphae. However, the D_2O experiment by Pfeffer et al. (1999) showed that quantitatively, at least for the FA, most biosynthetic activity occurs in the intraradical mycelium of the fungus.

These recent data shed new light on the obligately biotrophic nature of AM fungi. Rather than viewing these organisms as fundamentally impaired in their C metabolism, because of their long endosymbiotic history, it is possible to speculate that they possess all necessary genes for independent growth, but that some are regulated by the host plant. The putative plant regulated genes of AM fungi, especially those involved in C metabolism, are most likely to be expressed by the fungus in planta. Subtractive hybridization methods to isolate large numbers of differentially expressed genes are now available. Here again, the compartmentalized in vitro system will be particularly useful to compare gene expression between intraradical and extraradical mycelia.

The compartmentalized system has also been used by Joner et al. (2000) and Koide and Kabir (2000) to study P transport by the extraradical hyphae of *G. intraradices*. Radiotracing experiments showed that P in organic (^{32}P-AMP) and inorganic ($^{33}PO_4$) forms could be assimilated and transported by the fungus and utilized by the host root. These results indicate some mineralization capacity of the fungus (Joner et al. 2000). Similarly, Koide and Kabir showed that various organic P sources such as phytate, 5-bromo-4-chloro-3-indolyl phosphate, and phenolphtalein diphosphate could be hydrolyzed to serve as a P source for the host.

With regards to N nutrition, the compartmentalized in vitro system was used to show that the extraradical hyphae of *G. intraradices* have the capacity for nitrate (Bago et al. 1996) and ammonium absorption (Villegas et al. 1996; Villegas 2001). Nitrogen uptake was associated with a pH modification of the medium, suggesting the occurrence of an H^+-cotransport mechanism. Johansen et al. (1993) used an in vivo experimental system to show that nitrogen can be taken up from the soil by extraradical hyphae and transferred to the host. The compartmentalized in vitro system, coupled with long-term ^{15}N-labelling, was used by Pfeffer et al. (1998) to show that ammonium, nitrate, and even urea could be taken up, metabolized by the fungus, and subsequently used to label the host's amino acid pool. These in vitro experiments demonstrated that external hyphae of AM fungi can take up different forms of nitrogen and effectively transfer them to their host, but it remains to evaluate the quantitative or regulatory significance of this uptake for host plant nitrogen metabolism. Furthermore, in vitro studies of *Zea mays* (maize) colonized by *G. intraradices* showed expression and localization of nitrate reductase activity in both symbionts (Kaldorf et al. 1998).

Interactions with pathogenic and nonpathogenic rhizosphere microorganisms

Soil microorganisms, especially in the rhizosphere, are involved in most, if not all, plant–soil exchanges. It is likely that complex species associations play a major role in the stability of natural ecosystems. AM fungi, a dominant part of the rhizosphere microflora, form up to 25% of the total soil biomass (Hamel et al. 1991). They are of ecological importance, determining plant biodiversity, ecosystem variability, and productivity (van der Heijden et al. 1998). Therefore, understanding the complex relations between AM fungi, other components of the soil microbial biomass, and plants is a prerequisite for sustainable development.

Various approaches have been developed to study these interactions. Currently, the most promising tools for monitoring microbial population changes are fatty acids signatures (Olsson 1999), metabolic profiling (Howard 1999), immunodetection, and DNA-based assessments (van Elsas et al. 1998). Although field studies are needed to give a realistic picture of the complex interactions that occur in vivo,

they are difficult to achieve. Simple microcosms, in which each factor can be adjusted and closely controlled, are an attractive way to test hypotheses. The use of mycorrhizal root-organ cultures allows isolation of the organisms under study from other biotic and abiotic effects present in vivo. This approach also permits nondestructive, in situ observations of the interactions (St-Arnaud et al. 1995, 1996).

Effect of rhizosphere microorganisms on AM fungi

The first report of interactions between soil microbes and AM fungi under aseptic in vitro conditions was by Mosse (1962), who observed that root colonization could not be established without adding either a *Pseudomonas* suspension or various types of bacterial filtrates. Following this pioneering work, a wide range of soil bacteria and fungi have been shown to enhance in vitro germination of spores and hyphal growth of *G. mosseae* without direct contact between the organisms. These results suggest the involvement of volatile (e.g., CO_2) or highly diffusible substances (Azcón 1989; Azcón-Aguilar et al. 1986). Simultaneously, spore-associated bacteria have been identified to the genera *Pseudomonas* and *Corynebacterium* (Mayo et al. 1986), and cell-free fractions from rhizosphere bacteria cultures have the same stimulatory effect as complete bacterial cultures (Azcón 1987). These results reinforce the hypothesis for the existence of a diffusible factor excreted by bacteria. Other microorganisms within the rhizosphere have also been shown to have differential effects on AM fungi. Mugnier and Mosse (1987) and Tylka et al. (1991) showed that although spore germination of *G. mosseae* was stimulated by volatiles from several species of *Streptomyces*, germination of *Scutellospora heterogama* Walker was inhibited (see Table 3). In this case, the negative effect was attributed to a pH increase in the medium caused by bacterial growth.

Species of *Alternaria*, *Aspergillus*, *Fusarium*, *Gliocladium*, *Paedilomyces*, *Penicillium*, *Trichoderma*, and *Wardomyces*, isolated from rhizosphere soil or from *G. mosseae* sporocarps, were also tested for their effect on *G. mosseae* spore germination (Calvet et al. 1992; Fracchia et al. 1998; McAllister et al. 1994, 1996). Stimulatory, neutral, or inhibitory effects on germination and mycelium growth were observed. In certain cases spore germination was stimulated but hyphal growth inhibited (see Table 3). Here again, the role of soluble and volatile compounds on the interactions was suggested (Calvet et al. 1992; McAllister et al. 1994, 1996). It appears, therefore, that there is a differential effect of rhizosphere microorganisms on AM fungi that is controlled by microbial metabolite production. In all cases, however, the AM fungi were represented by germinating spores (i.e., at a pre-symbiotic stage and with a minimal biomass), which may not fully represent the interactions between a functional AM fungal mycelial network and other soil microbes present in vivo.

Only one published study has investigated the effect of a soil microbe on a functional symbiotic AM fungal mycelium in vitro (Rousseau et al. 1996). The compartmentalized system (St-Arnaud et al. 1995) was used to study the interaction between the biocontrol organism, *Trichoderma harzianum*, and *G. intraradices* growing on pea roots. Transmission electron microscope observations and gold labelling of cell wall constituents showed a marked antagonism of *T. harzianum* on *G. intraradices* spores and hyphae. The mycoparasite proliferated on the spore surface, penetrated the cell wall, and massively colonized the AM fungal hyphae, inducing disorganization, loss of protoplasm, hyphal bursting, and finally death of the *G. intraradices* hyphae. This study shows that the extraradical phase of AM fungi may be adversely affected by certain biocontrol microorganisms. The possibility for such interactions should be considered when developing biocontrol strategies. However, positive (Datnoff et al. 1995) and neutral (Fracchia et al. 1998), as well as negative (McGovern et al. 1992), interactions between *Trichoderma* species and AM fungal root colonization or biocontrol potential have also been reported in soil systems. Therefore, the strong antagonistic interaction described in vitro might not entirely reflect the in vivo situation and could have been emphasized by the aggressiveness of the biocontrol strain selected or by the in vitro growth conditions. This again highlights the complexity of microbial interactions within the rhizosphere community and emphasizes the need for additional research.

Effect of AM fungi on rhizosphere microorganisms

Chabot (1992) was the first to use root-organ cultures to test the effect of a symbiotic root system on soil microbes or pathogens. The author used plugs of gel containing exudates from the symbiotic association between *Gi. rosea* and Ri T-DNA transformed carrot roots to test for antibiosis-like effects on various fungal plant pathogens (Table 4). No growth inhibition or abnormal hyphal development was observed. However, while the gel plugs contained exudates of a functional extraradical mycelium and of the AM root system, they also contained sucrose, which may have masked any antibiosis effect. McAllister et al. (1994, 1996) also showed that germinated spores of *G. mosseae* had no effect on *Trichoderma koningii*, *Alternaria alternata*, and *Fusarium equiseti* growing on water agar (see Table 4).

The compartmentalized in vitro AM root-organ system, initially developed to study the interaction between a functional AM fungal extraradical network and specific soil microbes, was first used to study conidial germination of the root pathogen *Fusarium oxysporum* f.sp. *chrysanthemi* (St-Arnaud et al. 1995). Spore germination of the pathogen was strongly enhanced after 5 h of incubation with an active and symbiotic *G. intraradices* mycelium in monoxenic culture. However, significant negative correlations were found between conidia production and AM fungal hyphae or spore concentrations.

This was the first report of a direct effect of a pure, actively growing, symbiotic AM fungus on another microorganism, and it was suggested that AM fungal exudates might be involved. Using the same experimental approach, Filion et al. (1999) concentrated crude extracts from the growing medium of the extraradical mycelial compartment, presumably containing soluble biologically active substances, and tested them against soil bacteria and fungi. Species-differential responses were observed. Briefly, the growth of *Pseudomonas chlororaphis* and conidial germination of *T. harzianum* were stimulated, growth of *Clavibacter michiganensis* was unaffected, and conidial germination of *F. oxysporum* f.sp. *chrysanthemi* was decreased (see Ta-

Table 3. Effect of various rhizosphere microorganisms on AM fungi in vitro.

Microorganism	AM fungus	Effect on AM fungus	Reference
Bacteria			
Pseudomonas sp.	*Endogone* sp.	Enhanced colonization	Mosse 1962
Streptomyces orientalis	*Gigaspora margarita*	Increased spore germination	Mugnier and Mosse 1987; Tylka et al. 1991
Gliocladium roseum	*Glomus mosseae*	No effect on germination and growth	Fracchia et al. 1998
Streptomyces avermitilis; *S. griseus*; *S. orientalis*	*Glomus mosseae*	Increased spore germination	Tylka et al. 1991
Unidentified soil bacteria	*Glomus mosseae*	Increased spore germination, hyphal growth, and sporulation	Azcón 1987, 1989
Spore-associated bacteria including *Pseudomonas* sp. and *Corynebacterium* sp.	*Glomus versiforme*	Increased spore germination and hyphal growth	Mayo et al. 1986
Streptomyces avermitilis	*Scutellospora heterogama*	Suppression of spore germination in same growth compartment	Tylka et al. 1991
Streptomyces orientalis	*Streptomyces heterogama*	Suppression of spore germination in same growth compartment but increased germination in different compartments	Tylka et al. 1991
Fungi			
Trichoderma harzianum	*Glomus intraradices*	Spore and hyphae penetration, degradation, and killing	Rousseau et al. 1996
Alternaria alternata	*Glomus mosseae*	Inhibition of spore germination, no effect, or marked stimulation of hyphal growth	McAllister et al. 1996
Aspergillus fumigatus	*Glomus mosseae*	Inhibition of spore germination	Calvet et al. 1992
Fusarium equiseti	*Glomus mosseae*	Inhibition of spore germination, no effect, or marked stimulation of hyphal growth	McAllister et al. 1996
Fusarium solani	*Glomus mosseae*	No effect on spore germination; increased hyphal growth	McAllister et al. 1994
Paedilomyces farinosus	*Glomus mosseae*	Enhanced germination; no effect on growth	Fracchia et al. 1998
Penicillium decumbens	*Glomus mosseae*	Inhibition of spore germination	Calvet et al. 1992
Trichoderma aureoviride; *Trichoderma harzianum*	*Glomus mosseae*	Germination hastening; increased hyphal growth	Calvet et al. 1992
Trichoderma harzianum	*Glomus mosseae*	No effect on germination and growth	Fracchia et al. 1998
Trichoderma koningii	*Glomus mosseae*	Inhibition of spore germination; no effect on hyphal growth	McAllister et al. 1994
Trichoderma pseudokoningii	*Glomus mosseae*	No effect on germination and growth	Fracchia et al. 1998
Wardomyces inflatus	*Glomus mosseae*	Decreased germination and hyphal growth	Fracchia et al. 1998
Unidentified soil fungi	*Glomus mosseae*	Germination hastening; hyphal growth and sporulation enhancement	Azcón-Aguilar et al. 1986

Note: Species names are those used in the cited references.

ble 4). Differences in pH were noted between the extracts from AM and the non-AM control, but no significant influence of pH on growth or conidial germination was noted within the experimental pH range used. These results confirmed that substances released by the AM fungus in the growth medium were the main factor explaining differential growth of the organisms tested.

Benhamou et al. (1994) successfully used the in vitro system to study the effect of mycorrhizal colonization on plant disease processes. A strong stimulation of disease resistance was observed in mycorrhizal Ri T-DNA transformed carrot roots when challenged with a pathogen 7 days after AM inoculation. In this study, growth of the pathogen was restricted to the root epidermis and outer cortex. In contrast, extensive growth of the pathogen within the root and degradation of plant cells was observed in non-AM controls.

Recently, noncompartmentalized AM root-organ cultures were also used to study the interaction between AM fungi and nematodes (Elsen et al. 2001). Ri T-DNA transformed carrot roots colonized with *G. intraradices* were infected with the burrowing nematode *Radopholus similis*. Although the two organisms were able to complete their life cycle in co-culture, the nematode population density was reduced by 50% relative to that in non-mycorrhizal controls. However, the results were not significant for all developmental stages of the nematode, and the reduced population density was not correlated with AM fungal root colonization, or with mycelial or spore densities. Although the mechanisms involved in the nematode population reduction were not elucidated, this study supports the potential of the AM root-organ culture system for isolating the factors involved in the interaction between nematodes and AM fungi.

Table 4. Effect of AM fungi on various rhizosphere microorganisms and interaction with pathogens in vitro.

AM fungus	Microorganism	Effect on microorganism	Reference
Bacteria			
Gigaspora margarita	*Pseudomonas fluorescens*; *Rhizobium leguminosarum*	Bacteria adhere to and colonize the surface of germinating spores and growing hyphae	Bianciotto et al. 1996*b*
Glomus intraradices	*Clavibacter michiganensis* ssp. *michiganensis*	No effect of hyphosphere extract on bacteria growth	Filion et al. 1999
Glomus intraradices	*Pseudomonas aeruginosa*	Synergistic increased solubilization of insoluble P and decreased media pH	Villegas 2001
Glomus intraradices	*Pseudomonas chlororaphis*	Bacteria growth stimulation in hyphosphere extract	Filion et al. 1999
Glomus intraradices	*Pseudomonas putida*	Similar but weaker effect than that with *P. aeruginosa*	Villegas 2001
	Serratia plymutica	No effect on media pH and P availability	Villegas 2001
Fungi			
Gigaspora margarita[a]	*Bipolaris sorokiniana*, *Fusarium solani*, *Gaeumannomyces graminis*, *Ophiostoma ulmi*, *Phytophthora* sp., *Pythium ultimum*, *Pyrenochaeta terrestris*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Thielaviopsis basicola*, *Verticillium albo-atrum*, *Verticillium dahliae*	No effect on pathogens mycelia growth	Chabot 1992
Glomus intraradices	*Fusarium oxysporum* f.sp. *chrysanthemi*	Restriction of pathogen growth in AM carrot roots	Benhamou et al. 1994
Glomus intraradices	*Fusarium oxysporum* f.sp. *chrysanthemi*	Enhances conidia germination and hyphal growth; no effect on sporulation	St-Arnaud et al. 1995
Glomus intraradices	*Fusarium oxysporum* f.sp. *chrysanthemi*	Decrease conidial germination in hyphosphere extract	Filion et al. 1999
	Trichoderma harzianum	Stimulation of conidial germination by hyphosphere extract	Filion et al. 1999
Glomus mosseae	*Alternaria alternata*, *Fusarium equiseti*, *Trichoderma koningii*	No effect on hyphal growth	McAllister et al. 1996
Nematode			
Glomus intraradices	*Radopholus similis*	Reduction of the nematode population	Elsen et al. 2001

Note: Species names are those used in the cited references.
[a]This isolate is now recognized as *Gigaspora rosea* (Bago et al. 1998*b*).

Synergy between AM fungi and rhizosphere microorganisms

Recently, AM root-organ cultures were used to show a synergistic interaction between the extraradical mycelium of *G. intraradices* and soil bacteria in a study of rhizosphere nutritional dynamics (Villegas 2001). In this study, species-specific interactions were obtained between *G. intraradices* and *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Serratia plymutica*. Although the inherent ability of the fungus and the bacteria to solubilize a recalcitrant form of Ca–P was low, *P. aeruginosa* and *P. putida* interacting with the extraradical mycelium markedly increased P availability in the growth medium. This increase was dependent on the N source, which allowed a reduction of the pH (Villegas and Fortin 2001).

Limitations and potentials for using the mycorrhizal root-organ culture system

Although the use of mycorrhizal root-organ cultures has allowed the elucidation of many aspects of the AM symbiosis, the in vitro system has obvious limitations. Perhaps one of the most important of these is the fact that the plant host is replaced by a root organ. As a result, the symbiotic benefit to the plant is affected by the absence of photosynthetic tissues, a normal hormonal balance, and physiological source–sink relationships. Sucrose is added to the culture medium to compensate for the absence of photosynthates. Therefore, the root–fungus interface is bathed in a sugar solution, which does not occur in vivo. In this case, carbohydrates reach the cortex and the vascular system via the epidermis. It is possible that the presence of sugars at this interface modifies the biochemistry of the plant–fungal interaction. This might explain why arbuscules and vesicles are often scarce in Ri T-DNA transformed carrot roots, despite abundant intracortical mycelium. However, this hypothesis is not supported by recent work with *M. truncatula* hairy roots inoculated with *G. intraradices*, which exhibit colonization levels of up to 40%, this being mostly arbuscular (G. Bécard, unpublished data).

Despite the artificial nature of this in vitro system, there are several legitimate reasons for its continued use in the study of AM fungi. The fungus forms typical colonization

structures (i.e., appressoria, arbuscules, and vesicles) and produces profuse extraradical mycelium and spores. The production of spores, morphologically and structurally similar to those produced in pot cultures, and of intraradical structures capable of initiating new mycorrhizal symbiosis following subculturing indicates that the fungus is able to complete its life cycle. It can, therefore, be assumed that the mechanisms controlling the early colonization steps reflect those occurring in vivo.

This in vitro system has proved to be a useful tool for the cultivation and conservation of a large number of species and isolates of AM fungi (Table 2). It has also allowed many taxonomically important observations. It is likely that methodological improvements will help to establish cultures of some of the more recalcitrant Glomales species. To achieve this, media composition and growth conditions could be optimized. Studies suggest that mycorrhizal roots release compounds that are inhibitory for mycelial development and spore production (Fig. 2) (St-Arnaud et al. 1996). Although species such as *G. intraradices* can survive under the in vitro environment presently used, it is possible that some species or isolates cannot. Knowing the nature of these inhibitors would perhaps permit their elimination, removal, or sequestration, which might help the cultivation of more recalcitrant species.

The validity of the continued use of root-organ cultures for studying some of the most challenging questions regarding biochemical, genetical, and physiological relationships between AM fungi and their hosts is supported by the fact that these tissues show the same mycorrhizal characteristics as the plants from which they were developed. For example, hairy roots from non-host plants (e.g., *Beta vulgaris* L., *Brassica nigra* (L.) Koch, and *Brassica kaber* (DC) L.C. Wheeler (Bécard and Piché 1990; Schreiner and Koide 1993) and from Myc^- pea mutants (Balaji et al. 1994, 1995) still express a Myc^- phenotype. Furthermore, hairy roots and plants transformed with the ENOD 11-Gus gene (see later in the paper) show a similar gene expression pattern when mycorrhizal (Boisson-Dernier et al. 2001), and hairy roots developed from certain *Medicago sativa* L. (alfalfa) clones are, like the whole plants, resistant to *G. margarita* but receptive to *G. intraradices* (Douds et al. 1998). From these observations, one can postulate that the establishment of a mycorrhiza and the biotrophic mode of growth of AM fungi on root-organ cultures may be fundamentally similar to that occurring in vivo.

A particularly important field of study concerns the genetical and physiological basis behind the obligate biotrophic nature of AM fungi. In other words, what allows the fungus to complete its life cycle in the presence of a host root? The in vitro system, which allows control of most parameters and provides root and fungal material at various interactive stages, should permit more in-depth cellular, biochemical, and molecular investigations into this aspect. Since hairy roots from nonhost plants also exhibit a Myc^- phenotype (see earlier in the paper), this in vitro system could also be used to address the question as to why some plants are inherently non-mycotrophic.

To elucidate which fungal genes are specifically expressed in planta and which plant symbiotic genes are expressed in mycorrhizal roots requires sophisticated molecular analyses using PCR-based subtractive hybridization methods. For example, fungal genes specifically expressed in planta must be selected against the root genes and the extraradical fungal genes. This can only be achieved with a monoxenic system, such as that outlined earlier, which provides a means of harvesting sufficient quantities of mycorrhizal roots and isolated extraradical mycelium.

In addition to the transfer of T-DNA from the wild plasmid of *A. rhizogenes* to produce hairy roots, the bacterium can also be used as a vector to transfer other genes of interest (Boisson-Dernier et al. 2001). Studies on the role and the expression of putative symbiotic genes, using reporter genes, anti-sense, and the overexpression strategy, can, therefore, be carried out using transformed hairy roots. These can be obtained in 2–3 weeks (Boisson-Dernier et al. 2001). By contrast, the transformation and regeneration of intact plants usually takes 6 months.

The use of the AM root-organ culture technique has important implications for the production of AM inocula for research and commercial purposes. Although the results from most industry-based research are not generally publicly available, recently Moutoglis and Béland (2001) provided a brief insight into some of the potential techniques, and Jolicoeur et al. (1999) and Jolicoeur and Perrier (2001) proposed a bioreactor-based production technique using root-organ cultures. Although the nutritional parameters determining the productivity within these in vitro systems have been studied, further research is needed to optimize productivity and to develop low-cost techniques for the large-scale production of aseptic inocula.

The potential of the in vitro system for the study of interspecific AM fungal competition has not yet been investigated. Compatibility studies between AM fungi using root-organ cultures are possible and the results might suggest improvements that could be made to industrially produced inocula.

At the intraspecific level, in vitro studies (mycelial development and spore production) have highlighted the existence of phenotypic variations between different *G. intradices* isolates. The in vitro system could, therefore, be used to study mating types and the heredity nature of certain phenotypes within this species, which may also help improve commercial inocula.

The mycorrhizal root-organ culture has proven useful for taxonomists and physiologists, and potentially useful for geneticists. It is also promising for the study of interaction with root-born pathogens and other soil organisms. In its present state, however, the AM root-organ culture system is somewhat limiting because the root is bathed in a carbohydrate-rich solution. Studies of direct interactions between AM colonized roots and pathogens, and other soil organisms, could perhaps be achieved using an improved version of the root-hypocotyl system (Bunting and Horrocks 1964; Miller-Wideman and Watrud 1984), possibly adding auxins (Fortin and Piché 1979). Briefly, using this system, it would be possible to achieve a polarity whereby organic nutrients, which are absorbed by the aerial part of the plant, are translocated to the roots via the vascular system. Because the root system is exposed only to mineral nutrients, this in vitro system would allow interactions between soil organisms involved in polysaccharide decomposition, nitrogen fixation, nitrate reductase, ammonification, and phosphate solubilization to be investigated.

An in vitro tripartite culture system, consisting of the co-culture of in vitro cultivated strawberry plantlets, AM fungi (*G. intraradices* or *Gi. rosea*), and a carrot root-organ culture, has proved useful in highlighting the capacity of AM fungi to reduce water stress in micropropagated plantlets (Elmeskaoui et al. 1995). Using this tripartite system, Hernández-Sebastià et al. (1999) showed that colonization of in vitro cultivated strawberry plantlets by *G. intraradices* increased relative plant water content. This effect was related to enhanced water content of the mycorrhizal roots. However, root osmotic potential and dry weight did not significantly differ from that of non-mycorrhizal controls. In an attempt to explain this phenomenon, Hernández-Sebastià et al. (2000) proposed that mycorrhizal strawberry plantlets were able to change root cell amino-acid composition, and alter cell starch concentrations, to reduce water stress.

To limit the use of AM toxic compounds in the environment, Wan et al. (1998) and Wan and Rahe (1998) used in vitro AM root-organ cultures to study the sublethal toxicity of a range of pesticides (e.g., benomyl, glyphosate, dimethoate, and azadirachtin) on Glomales spp. This method could become a standard test for the regulation of pesticides.

The concept behind the use of excised roots for endomycorrhizal research stems from work on ectomycorrhiza. In return, the technique of in vitro culture on transformed roots developed for endomycorrhizal studies has recently been applied to ectomycorrhizal research. A model based on Ri T-DNA transformed roots of the Mediterranean shrub, *Cistus incanus*, has been developed for the inoculation of plants with the truffle-producing fungus, *Tuber melanosporum* (Wenkart et al. 2001). In the past, ectomycorrhizal studies have typically been carried out on fungi grown either saprophytically or with plant hosts but under nonsterile conditions (Read 1992). The transformed *Cistus* root model will allow the in vitro study of the effects of different edaphic factors on the growth and development of the extraradical phase of ectomycorrhizal fungi.

Initial experiments on fungal growth and development have shown that transformed *Cistus* roots can be used to form ectomycorrhizae with many species of ectomycorrhizal fungi and to dramatically increase fungal growth (Coughlan et al. 2001). As observed in studies on the endomycorrhizal symbiosis, the presence of transformed roots stimulates the production of highly branched structures absent on hyphae growing saprophytically (unpublished data). The production of these structures is likely to be important for nutrient uptake and formation of the symbiosis. Work is presently underway to characterize the exudates from these roots and to test their activity on endomycorrhizal fungi in an attempt to determine whether a common signalling pathway exists (G. Bécard, unpublished data).

In this review, we have attempted to cover all aspects of the mycorrhizal root-organ culture system and to outline the fact that the only real limit to its use is that of the user's imagination.

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