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Arbuscular Mycorrhiza on Root-Organ Cultures

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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. 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.

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

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.

Mycorrhizal Root-organ Culture Methods

Host Roots

Root-organ cultures were first developed by White (1943), Butcher and Street (1964) and 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.

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 and 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 xAnanassa* 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

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transfonnation (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 Tempe 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), successfully colonized *Convolvulus sepium* hairy roots using spores of *G. mosseae* but, as was the case with nontransformed clover root-organ cultures (Mosse and Hepper, 1975), no sporulation occurred. Spore production followed reductions in the concentration of certain nutrients in the culture medium (Becard and Piche, 1990) 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. intra radices* 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 (Table 1) were also successfully used to obtain mycorrhiza and fungal sporulation using nontransformed tomato root cultures (Chabot *et al*., 1992; Diop *et al*., 1994a, b). 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. 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.

Fungal Inocula

In most cases, two types of fungal inoculum can be used to initiate monoxenic cultures: either extraradical spores or 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. 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 (Becard and Piche, 1990). 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).

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

Constituents	M (μM)	MSR (μM)
N (NO_3^-)	3200.00	3800.00
N (NH_4^+)	-	180.00
P	30.00	30.00
K	1735.00	1650.00
Ca	1200.00	1520.00
Mg	3000.00	3000.00
S	3000.00	3013.00
CI	870.00	870.00
Na	20.00	20.00
Fe	20.00	20.00
Mn	30.00	11.00
Zn	9.00	1.00
B	24.00	30.00
I	4.50	-
Mo	0.01	0.22
Cu	0.96	0.96
Panthotenate Ca	-	1.88
Biotin	-	0.004
Pyridoxine	0.49	4.38
Thiamine	0.30	2.96
Cyanocabalamine	-	0.29
Nicotinic acid	4.00	8.10
Glycine (mg L^{-1})	3.00	-
Myo-inositol (mg L^{-1})	50.00	-
Sucrose (g L^{-1})	10.00	10.00
pH (before autoclave)	5.50	5.50
Gellan agent (g L^{-1})	5.00	5.00

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 (Becard and Piche, 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% $MgSO_4.7H_2O$ 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 (Becard and Piche, 1989; Buee, 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 and Gerd.) Gerd. And Trappe (Koske, 1981) and *Gigaspora margarita* Becker and Hall require a cold treatment, whereas *Gigaspora rosea* Nicolson and Schenck (formally misidentified as *Gigaspora margarita* (Bago *et al*., 1998a). 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. 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 510 mm lengths and incubated on a synthetic medium. Water-agar medium is also effective (Diop *et al*., 1994a). 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 (mentioned earlier), there is no evidence that mycorrhizal root pieces need specific exogenous conditions or a host plant for hyphal regrowth (Diop *et al*., 1994a). Following incubation, mycorrhizal root pieces showing hyphal regrowth are transferred, using a cork borer, to a fresh Petri dish with an actively growing root 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 and 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 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 (Becard and Piche, 1990). 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.

The MSR medium 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 (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*.

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 root-organ cultures. The majority of these have been obtained during the last decade (Table 2).

Table 2: Species of *Glomales* cultivated on root-organ cultures

Gigasporaceae	
Gigaspora margarita Becker & Hall	Miller-Wideman and Watrud (1984); Karandashov *et al.* (1999)
Gigaspora rosea Nicolson & Schenck	Forbes *et al.* (1998)
Glomaceae	
Glomus caledonium (Nicolson & Gerd.) Trappe & Gerd.	Karandashov *et al.* (1999)
Glomus cerebriforme McGee	Samson *et al.* (2000)
Glomus constrictum Trappe	Mathur and Vyas (1999)
Glomus deserticola Trappe, Bloss & Menge	Mathur and Vyas (1995a)
Glomus etunicatum Becker & Gerd.	Schreiner and Koide (1993); Karandashov *et al.* (1999)
Glomus fasciculatum (Thaxter sensu Gerd.) Gerd. & Trappe emend. Walker & Koske	Nuutila *et al.* (1995); Gryndler *et al.* (1998)
Glomus intraradices Schenck & Smith	Chabot *et al.* (1992); Karandashov *et al.* (1999)
Glomus versiforme (Karsten) Berch	Diop *et al.* (1994a)

[a]Species differentiating only vesicle-like spores

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 Mycotheque de l'Universite 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.

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 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*., 1998b). 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.

***In vitro* Development of the Extraradical Phase**

The use of root-organ cultures in compartmentalized Petri dishes (St-Amaud *et al*., 1995) also allows time-lapse studies of extraradical mycelial development in root-free compartments. When

comparing ammonium and nitrate as nitrogen sources, it was found that the presence of ammonium in the distal compartment drastically reduced spore production. This also suggests 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 allowed 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. Ultrastructural investigations revealed that ALS (renamed branched absorbing structures or BAS) 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 in-creased 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. It also appears that inorganic nitrogen and phosphate absorption by extraradical mycelium is closely correlated with BAS development.

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).

MYCORRHIZATION OF THE MICRO PROPAGATED PLANTLETS-A CASE STUDY

Survival and Establishment of *in vitro* Raised *Ziziphus Nummularia* and *Ziziphus Mauritiana* by Application of Am Fungi

Glomus deserticola Trappe, Bloss and Menge and *Glomus constrictum* Trappe, are one of the most commonly occurring VAM fungi of arid and semiarid regions, were cultured and multiplied in root organ cultures of *Z. nummularia* and *Z. mauritiana* under *in vitro* conditions. The *in vitro* produced VAM fungi established efficient symbiosis with *in vitro* raised plantlets of *Z. nummularia* and *Ziziphus mauritiana*. This VAM strain improved the biomass production, nutrient uptake and acclimatization of the *in vitro* produced plantlets of *Z. nummularia* and *Ziziphus mauritiana* in pots under green house conditions.

Introduction

Z. nummularia and *Z. mauritiana* are an important multipurpose fruit plants of arid and semiarid regions. These plants are used as a source of fuel, fodder and food. The cultivation is done mostly in nutrient deficient sandy soils of drought prone areas. Rapid *in vitro* multiplication of this plant has successfully been done using tissue culture techniques (Mathur *et al*., 1993).

However the major handicap of tissue culturists in arid and semi-arid regions is survival and establishment of *in vitro* raised plantlets into fields (Mathur and Vyas, 1995a). Arid and semi-arid regions are characterised by high temperature, water scarcity and nutrient deficient sandy soils. All of these factors combined contribute towards harsh environmental conditions, due to the fact that the primary stresses imposed on vegetation by arid environments are lack of water and mineral nutrients (Fisher and Turner, 1978). Therefore, any factor which enhances nutrient uptake and water transport is likely to contribute to the success of desert vegetation. VAM symbiosis may be of particular significance in coping with P-deficiency stress in natural ecosystems (Mc Arthur and Knowles, 1993).

Due to its beneficial effect VA mycorrhizae are receiving considerable attention in agriculture and forestry (Peterson *et al*., 1984). *In vitro* production of different species of *Glomus,* viz., *G. fasciculatum* (Allen *et al*., 1982). *G. intraradices* (Chabot *et al*., 1992), *G. mosseae* (Nopamornbodi *et al*., 1988), either axenically or in root organ culture using various host plants has been reported. However, there are no reports about the culturing of *G. deserticola* and *G. constrictum* (most commonly occurring VAM species of arid and semiarid regions) under *in vitro* conditions. Hence, the present investigation was undertaken to produce *G. deserticola* and *G. constrictum* under *in vitro* conditions in association with *Z. nummularia* and *Z. mauritiana*.

MATERIALS AND METHODS

The plantlets of *Z. nummularia* and *Z. mauritiana* were raised from different explant source under *in vitro* conditions on modified MS medium containing various concentrations and combinations of auxins and cytokinins (Mathur *et al*., 1993). VA mycorrhizal fungi *G. deserticola* Trappe, Bloss and Menge and *G. constrictum* Trappe, collected from rhizosphere soils of *Z. nummularia* and *Z. mauritiana*, were cultured on *Cenchrus ciliaris* for establishing pot cultures following Mathur and Vyas (1996). Roots of *C. ciliaris* from these pot cultures were regularly examined to test purity of culture. The pure pot culture of *C. ciliaris* containing spores of only *G. deserticola* were used for inoculating *Z. nummularia* and only *G. constrictum* were used for inoculating *Z. mauritiana.* The spores of *G. deserticola* and *G. constrictum* along with the infected root segments of *C. ciliaris* were isolated from soils of the pot cultures using the wet sieving and decanting technique (Gerdemann and Nicolson, 1963). The spores were then purified from root pieces and debris by sucrose gradient centrifugation (Jenkins, 1964). The collected spores were washed repeatedly with sterilized distilled water to remove sucrose. These purified spores along with the infected root segments were surface sterilized with sodium hypochlorite (2.5% v/v) for 20 min and washed repeatedly with sterilized distilled water to remove traces of sodium hypochlorite. Because the infected root segments were fully colonized by active spores of *G. deserticola* and *G. constrictum*, respectively, these root segments were also used for inoculation. Hence, the spores of *G. deserticola* and *G. constrictum* along with *C. ciliaris* root segments were used to inoculate *in vitro* raised plantlets of *Z. nummularia* and *Z. mauritiana*, respectively.

At the time of rooting of *In vitro* raised plantlets, 10-15 spores of *G. deserticola* and *G. constrictum* along with infected root segments of *C. ciliaris* were inoculated onto roots of *Z. nummularia* and *Z. mauritiana* respectively in 250 mL flask. At the same time a control was maintained by inoculating roots of *Z. nummularia* and *Z. mauritiana* with sterilized root segments of *C. ciliaris* without any VAM spores. These flasks were kept under controlled conditions having 60% humidity and 20-25°C temperature. After 40 days the plantlets were transferred into pots of 18 cm diameter containing sterilized sand:vermiculite in 3:1 ratio (Mathur *et al*., 1993) and were kept under green house conditions. The samples were harvested 90, 120, 150 and 180 days after inoculation. Plant dry weight were recorded after drying them in a hot air oven at 60°C for 48 h. Root samples collected from the pots were gently washed under tap water and suitably processed (Phillips and Hayman, 1970) to calculate the percentage of root colonization (Giovannetti and Mosse, 1980). Phosphorus in plant material was estimated by Vanadomolybdate method (Jackson, 1973) and nitrogen in plant material was estimated by micro-Kjeldhal method. There were twenty-five replicates for each treatment. The control plants were not supplied with VAM inoculum.

RESULTS AND DISCUSSION

The inoculation of *Z. nummularia* plantlets with spores of *G. deserticola* and infected root segments of *C. ciliaris* resulted in establishment of a symbiotic relationship. The VAM fungi

proliferated vigorously under *in vitro* conditions and produced all the structures of VAM infection, viz. external and internal hyphae, vesicles and arbuscules. The influence of *in vitro* produced VAM fungi was evaluated for nutrient uptake and biomass production in the *in vitro* produced plantlets of *Z. nummularia* (Table 3). Overall growth of mycorrhizal *in vitro* raised plantlets was higher than non-mycorrhizal plantlets after all harvest. However, the samples of last harvest showed more than 2-fold increase in plant height and almost 3-fold increase in plant dry weight when inoculated with VAM fungi. Similar trend was also noticed in effect of VAM fungi on nutrient uptake in *in vitro* raised plantlets of *Z. nummularia*. Addition of VA mycorrhiza resulted in more than 140% increase in uptake of both the nutrients P and N, compared with the control (Table 4). This result likely is due to the vigorous proliferation and efficient colonization of the roots *in vitro* raised plantlets of *Z. nummularia* by *G. deserticola* (Table 3).

The present investigation reveals successful symbiosis of VA mycorrhizal fungus *Glomus deserticola* in association with *Z. nummularia* under *in vitro* conditions. Mosse and Hepper (1975) were first to produce a simplified *in vitro* system for the study of VAM. Strullu and Romand (1986) reported regeneration of vigorous hyphae of three *Glomus* species from sterilized root fragments. During the present investigation, culturing of VAM fungi was achieved on modified MS medium, which is also the best medium for micropropagation of the host plant *Z. nummularia* (Mathur *et al*., 1993). This is of importance because both the plantlets as well as the VAM fungi can be propagated simultaneously on the same medium under *in vitro* conditions.

Table 3: VAM spore population and percentage of root colonization in rhizosphere of *in vitro* raised *Z. nummularia* plantlets at different harvests

	VAM spores 100 mL^{-1} soil		Percentage root colonization	
Plant age (days after inoculation)	M	NM	M	NM
90	280	0	58.00	0
120	352	0	67.00	0
150	436	0	78.00	0
180	575	0	86.00	0
L.S.D. at $p \leq 0.05$	71.5	0	4.10	0

M = Mycorrhizal inoculated, NM = Non-mycorrhizal inoculated

Table 4: Biomass production and nutrient uptake *in vitro* raised plantlets of *Z. nummularia* by *in vitro* produced *G. deserticola* at different harvests

	Plant height (cm $plant^{-1}$)		Plant dry weight (g $plant^{-1}$)	
Plant age (days after tinoculation)	M	NM	M	NM
90	30.1±2581*	15.2±1.49	07.04±1.231*	5.42±0.6619
120	60.2±3.464*	30.1±2.409	16.00±1.825*	7.68±0.3793
150	75.3±1.874*	39.0±1.758	23.10±1.837*	9.90±0.2347
180	90.0±4.242*	42.8±1.873	33.00±1.712*	11.04±0.1344
LSD	3.49	3.02	8.2	1.56

M = Mycorrhizal inoculated, NM = Non-mycorrhizal inoculated, ANOVA test * Significant at 0.1% level (at each harvest) LSD at $p \leq 0.5$, comparing M vs NM

Table 4: Continued

	Total P (mg g^{-1} dry weight)		Total N (mg L^{-1} dry weight)	
Plant age (days after inoculation)	M	NM	M	NM
90	1.56±0.6469*	0.81±0.4450	1.70±0.3489*	1.40±0.4568
120	1.93±0.7282*	1.01±0.4194	2.31±0.8569*	1.53±0.7638
150	2.40±0.3394*	1.20±1.238	3.16±0.5896*	1.63±0.7423
180	3.18±0.5329*	1.70±0.4388	3.40±0.6266*	1.73±0.5964
LSD	0.19	0.43	0.38	0.20

M = Mycorrhizal inoculated, NM = Non-mycorrhizal inoculated, ANOVA test * Significant at 0.1% level (at each harvest) LSD at $p \leq 0.5$, comparing M vs NM

The *in vitro* produced VAM fungi also were found to be significantly beneficial for biomass production and nutrient uptake of *in vitro* raised *Z. nummularia* plantlets in pots. The increased nutrient uptake by VAM endophytes has been well recognised (Koide end Schriener, 1992; McArthur and Knowles, 1993). Thus, the increased plant biomass which can be attained by increased nutrient levels was observed during the present investigation.

The inoculation of *in vitro* produced *Z. mauritiana* plant- lets with spores and infected root segments of *G. constrictum* resulted in establishment of symbiotic relationship. The VAM fungi proliferated vigrously under *in vitro* conditions by germination of spores. These germinating spores then penetrated roots of *Z. mauritiana* and proliferated vigrously in cortical region and formed vesicles and arbuscules. The potentiality of *in vitro* produced VAM fungi was evaluated towards nutrient uptake, biomass production and establishment of *in vitro* produced plantlets of *Z. mauritiana.* Table 5 represents observations regarding plant biomass production and nutrient uptake in mycorrhizal and non-mycorrhizal *Z. mauritiana* plantlets. Overall growth of mycorrhizal *in vitro* raised plantlets was higher than non-mycorrhizal ones at all of the harvests. However, the samples of the last harvest showed a more than two-fold increase in plant height and plant dry weight when inoculated with VAM fungi. A similar trend was also noticed as an effect of VAM fungi on nutrient uptake *in vitro* raised plantlets of *Z. mauritiana.* Addition of VAM increased more than two-fold the uptake of both of the nutrients, i.e. N and P (Table 5), by vigrous proliferation and efficiently colonizing the roots of *in vitro* raised plantlets at a greater rate (Table 6), ultimately leading to better survival of plantlets into the field (Table 7). The present investigation reveals successful sporulation and proliferation of *G. constrictum*

Table 5: Biomass production and nutrient uptake in *in vitro* raised mycorrhizal plantlets and non-mycorrhizal plantlets of *Z. mauritiana*

Plant age (days after inoculation)	Plant height (cm plant^{-1})		Plant dry weight (g plant^{-1})		Total P (mg g^{-1} dry weight)		Total N (mg g^{-1} dry weight)	
	M	NM	M	NM	M	NM	M	NM
90	39.8*	14.80	6.5*	5.00	11.3*	5.42	8.0*	4.22
120	51.3*	17.10	16.2*	12.00	15.8*	7.68	9.0*	6.10
150	60.3*	26.60	23.4*	15.00	24.5*	9.90	14.0*	7.17
180	71.5*	35.50	33.4*	18.00	26.5*	11.10	18.0*	7.50
C. D. at 5% level	2.5	4.75	9.0	3.49	1.5	1.56	6.04	0.04

ANOVA test * Significant at 0.1% level (at each harvest); M = Mycorrhizal, NM = Non-mycorrhizal

Table 6: VAM spore population and percentage of root colonization *in vitro* raised mycorrhizal and non-mycorrhizal of *Z. mauritiana* plantlets

Plant age (days after inoculation)	VAM spores 100 mL^{-1} soil		Percentage root colonization	
	M	NM	M	NM
90	290	0	55	0
120	362	0	64	0
150	446	0	75	0
180	585	0	83	0
C. D. at 5% level	71.5	0	4.10	0

M = Mycorrhizal inoculated, NM = Non-mycorrhizal inoculated

Table 7: Rate of survival and RFMD of *in vitro* raised *Z. mauritiana* plantlets into the field

Days after inoculation	Rate of survival (Percent)		RFMD (%)
	M	NM	
90	100	100	23.0
120	100	80	25.9
150	95	60	35.8
180	92	45	46.0

M = Mycorrhizal, NM = Non-mycorrhizal. RFMD = Relative Field Mycorrhizal Dependency

under *in vitro* conditions in association with *Z. mauritiana* on modified MS medium. First contact between the root and fungus occurred within 2 days, possibly due to the use of VAM root segments in addition to spores as source of inoculum. After 15 days of growth, the medium surface was covered with a network of hyphae of VA mycorrhiza. Many stages of the vegetative life cycle of *G. constrictum,* including numerous arbuscules and vesicles and extramatrical mycelium, were easily observed in this *in vitro* dual culture system.

The *in vitro* sporulated *G. constrictum* significantly increased p-levels in *Z. mauritiana* plantlets by efficiently colonizing the roots. The increased p-uptake by VAM endophytes has been well recognized (Koide and Schriener, 1992; Ruiz Lozano *et al*., 1995). An improved net photosynthetic rate of *Z. mauritiana* by V A mycorrhizae has been reported under *Ex vitro* conditions (Mathur and Vyas, 1995b). Hence, improved biomass production of *Z. mauritiana* during the present study was observed, which could be attributed to improved nutrient uptake and possibly improved photosynthetic rate. The increased nitrogen content in mycorrhizal plants could be due to increased nitrate reductase activity which was attributed to improved p-nutrition provided by a VAM symbiosis (Cliquet and Stewart, 1993; Mathur and Vyas, 1995c). The present study revealed direct correlation between VAM spore populations and percentage of root colonization. These strains that efficiently colonized roots were found to produce a maximum number of spores.

Both the mycorrhizal and non-mycorrhizal plants survived in the field when transferred. However, rate of survival was quite different. During the first harvest 100% survival was observed for both the mycorrhizal and non-mycorrhizal plants, while the samples of the last harvest showed only 45% survival of non-mycorrhizal plants and 92% survival of mycorrhizal plants (Table 7). One of the major impediments to the success of micro-propagation is the very high mortality rate of tissue culture plantlets either during the acclimatization phase or during transfer to field conditions.

This is attributed mainly to certain aberrant features, characteristic of *in vitro* derived plantlets, which often lead to very high mortality due to desiccation and microbial infection. VA mycorrhizae are important in sustainable agriculture because they improve plant water relations and thus increase drought resistance of the host plant (Mathur and Vyas, 2000), improve disease control (Azcon-Augilar *et al*., 2002) and increase mineral uptake. All of these benefits provided by the VAM endophyte could be leading towards significantly improved survival of *in vitro* raised plantlets of *Z. mauritiana* into the field. Inoculation of micro-propagated plantlets of *Z. mauritiana* with active cultures of VAM appears to be critical for the survival and growth of *in vitro* plantlets; this avoids transient transplant shock and stunted growth upon transfer to the field (Lovato *et al*., 1995).

Hence the present investigation clearly suggests *In vitro* multiplication of VAM fungi *G. constrictum* in association with *Z. mauritiana.* The investigation further proves potentiality of this *in vitro* proliferated VAM strain on biomass production, nutrient uptake and survival of *in vitro* raised *Z. mauritiana* plantlets into the field. This work can be of importance in acclimatizing the *in vitro* raised plantlets of arid and semi-arid regions into the field, which may help in over-coming the major handicap of tissue culturists of arid areas.

Biological studies of VAM fungi are made difficult by the obligately biotropic relationship they form with vascular plants. The root organ culture technique for VAM fungi has obvious advantages over traditional systems permitting the observation of fungal morphology and development *in vitro*. Because the spores produced in this culture system are viable and able to colonize new roots, it also permits the propagation of VAM fungi monoaxenically. Thus, the present investigation may be of significance for acclimatizing *in vitro* raised plantlets of arid and semiarid regions into the field.

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.

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 com-pounds that are inhibitory for mycelial development and spore production (St-Amaud *et al*., 1995). Although species such as *G. intra radices* 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 (Becard and Piche, 1990; Schreiner and Koide, 1993). Furthermore, hairy roots and plants transformed with the ENOD 11-Gus gene show a similar gene expression pattern when mycorrhizal 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, 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. 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. 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 Beland (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 (Fig. 1).

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 Piche, 1979). Briefly, using

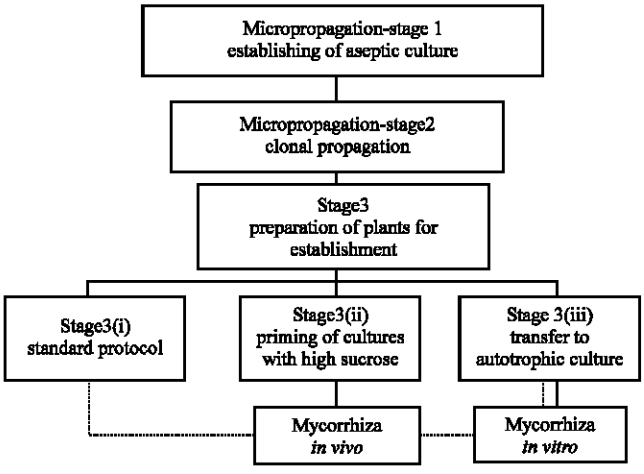

Fig. 1: Flow diagram showing alternative strategies for mycorrhization of micro-plants in *in vitro* and *in vivo*

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, HerninIndez-Sebastia *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, HerninIndez-Sebastia *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* sp. 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 non-sterile 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 signaling pathway exists.

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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