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In Vitro Culturing of Mycorrhiza and Mycorrhiza Like Fungi

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Arbuscular mycorrhizal (AM) fungi exist in rhizosphere of several vascular plants and have important roles in sustainable agriculture as well as agricultural ecosystems management. These fungi could be able to colonize host plants by their three sources including spores, mycorrhizal roots and extraradical mycelia. There are obvious differences among fungal families and genera in life cycle and ecology. Fungi in Glomeraceae and Acaulosporaceae families could be able to colonize host plants by 3 mentioned sources while in Gigasporaceae, the only inoculum sources are spores. Spore formation depended on different factors such as seasonality, nutrient levels as well as interaction with other soil microorganisms. There are so many efforts in order to get pure isolates of arbuscular mycorrhizal fungi but most of them faced to problems and failed due to biotrophic nature of these fungi. *In vitro* culturing of these fungi is very important especially for studying on host plant growth and taxonomic studies. First time, Mosse used toot tissue culture method for obtaining pure culture. Also, *in vitro* culturing of AM fungal species using carrot (*Daucus carota* L.) transformed hairy roots by Ri plasmid of *Agrobacterium rhizogenes* (Ricker) Conn. is also another method. Using this method, several fungal species could be propagated *in vitro* such as *Gigaspora margarita*, *G. gigantea*, *Rhizophagus fasciculatus*, *Rhizophagus intraradices*, *Diversispora versiformis* and *Funneliformis caledonium*. Using this method, it could be possible to study on molecular as well as biochemical aspects of arbuscular mycorrhizal symbiosis. Unfortunately, there have not been studies on *in vitro* culturing of these fungi isolated from rhizosphere. The main purpose of this study is possibility of *in vitro* culturing of most prevalent mycorrhiza and mycorrhiza like fungi using transformed hairy roots system as well as study on fungal life cycle under laboratory conditions.

Key words: Arbuscular mycorrhiza fungi, In vitro culturing, Monoxenic culture, Transformed roots

Introduction

The arbuscular mycorrhizal fungus plays crucial role to attain plant health and soil fertility. The plant root association with the mycorrhizal fungi overcome the challenges like water stress (Liu *et al.,* 2015), heavy metal resistance against aluminum and lead in *in vitro* (Gavito *et al.,* 2014) chromium

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immobilization (Wu *et al.,* 2016), mercury contaminated soils, copper stress (Almeida-Rodríguez *et al.,* 2015), arsenate stressed soils (Cattani *et al.,* 2015), against oxidative stress (Driai *et al.,* 2015). In association with soil microbial flora AMF supports plant systems on nutrient availability (Tekaya *et al.,* 2016), bioremediation (Ingrid *et al.,* 2016) and heavy metal resistance (Dhawi *et al.,* 2016). AMF association also gives resistance against many diseases like black sigatoka in *in vitro* banana (Anda *et al.,* 2015) and various soil borne plant pathogens (Tahat *et al.,* 2010). In sustainable agriculture, AMF has great potential in crop production and environment preservation and by using the AMF in farming as biofertilizer, can minimize the usage of toxic chemical pesticides and synthetic fertilizers (Oruru and Njeru, 2016). To achieve sustainable agriculture system, it is essential to re-establishing the mycorrhizal populations in the soil for uplifting the soil health, plant growth and yield (Wahbi *et al.,* 2015). But because of its obligate symbiotic nature of reproducibility and duration of its life cycle, makes major challenge for large scale production (Berruti *et al.,* 2016).The development of arbuscular mycorrhizal fungus (AMF) in *in vitro* root organ cultures has greatly influenced by the potential for research and large-scale production of uncontaminated inoculum production. *In vitro* root organ culture system was first reported by White (1943). AM fungal culture development under *in vitro* root organ culture process is the most promising way to obtain good amount of extraradical spores in a short span of time with contamination free inoculum (Binondo *et al.,* 2012). AMF cultivation techniques based on *Agrobacterium rhizogenes* transformed roots provide the development of the AMF inoculum *in vitro* (Bécard and Fortin, 1988; Adholeya *et al.,* 2005; Ijdo *et al.,* 2011; Schultze, 2013). After the development of monoxenic cultures of AM fungus through root organ culture made possible for the continuous observations of fungal colonization and mycelium development as well as the sporulation. Minimal mineral media (M) and modified Strullu-Romand (MSR) media were successfully used for AM fungal colonization on non-transformed tomato root cultures (Bago *et al.,* 1996). These root organ culture studies giving better knowledge in mycelium development (Bago *et al.,* 1998), functional aspects of the mycorrhizal root symbiosis (Debiane *et al.,* 2009), dynamics of sporulation in *in vitro* host roots (Declerck *et al.,* 2004; Voets *et al.,* 2009; Ijdo *et al.,* 2011), AMF spore ontogeny (De souza *et al.,* 2005), study of species level reproduction cycles and nutritional requirements (Labidi *et al.,* 2011) and large scale production of viable contamination free inoculum (Voets *et al.,* 2009; Ijdo *et al.,* 2011). Only a few species of AMF strains have been successfully established *in vitro* through root organ culture technique (Ijdo *et al.,* 2011). Although having the potential use of the *in vitro* root organ cultures, have obvious limitations in

many aspects the AM symbiosis. Importantly the plant host is replaced by a genetically transformed root organ, affected by the absence of regular photosynthetic organs and normal hormonal effects. Transformed root organs may not be active for too many subcultures for continuous production. Sucrose is added to the culture medium to compensate photosynthesis were affect the root-fungus interface. Sugars at this interface may modify the biochemistry of the plant fungal interaction. According to Fortin *et al.* (2002) these sugar concentrations might affect the arbuscules and vesicles development in *in vitro* transformed carrot roots.

This Paper is not aimed to propose a literature overview but rather to provide detailed protocols to succeed in the monoxenic culture of AM fungi. It is obvious that for every technique different protocols (disinfection process, growth medium, choice of propagule, etc.) have been published, and we cannot discuss these in detail. Therefore, we choose to describe the routine techniques.

Process Description

The process of obtaining and maintaining monoxenic cultures of AM fungi can be separated into four main steps:

1- Selection of the adequate AM fungal propagules
2- Sampling, disinfection and incubation of the propagules on a suitable growth medium
3- Association of the propagules with a suitable host root
4- Sub cultivation of the AM fungi

Prior to these four steps are the selection of the appropriate culture system, the preparation of the synthetic culture media and the management of the host root, i.e. transformation and sub cultivation.

Selection of the Culture System

Basically, two culture systems are used: the mono-compartmental system in square or round Petri plates, and the bi-compartmental system in round Petri plates. The first system consists of a mono-compartmental Petri plate filled with a growth medium, on which is placed a contaminant-free, actively growing excised root together with AM fungal propagules. This system was developed in the mid-1970s (Mosse and Hepper, 1975) and since then has been applied with success to numerous *Glomus* species. Recent reports have also demonstrated its applications to *Scutellospora reticulata* (De Souza and Declerck, 2003) and *Acaulospora rehmii* (Dalpé and Declerck, 2002). This system was slightly modified for the cultivation of *Gigaspora* species (*Gi.*

margarita, *Gi. roseae*, *Gi. gigantea*) by placing a Ri T-DNA transformed carrot root – having negative geotropism – in the upper part of a square Petri plate set vertically, with the germinated spore just below (Figure 1; Bécard and Piché, 1992; Diop *et al.*, 1992). Since the germ tube growth of these *Gigaspora* species is also negatively geotropic, the germinating hyphae are oriented towards the root, thus facilitating contact and colonization. The root growth is restricted to the upper part of the plate while after colonization the mycelium develops in the lower part. It was observed that the sporulation mainly occurs in the section with less root development.

The second system (also named split system) consists of a bi-compartmental Petri plate, with a proximal compartment in which the mycorrhizal root develops and containing a synthetic growth medium, and a distal compartment in which only the mycelium can grow on a similar synthetic medium, but lacking C source. Both compartments are physically separated by a plastic wall. Roots crossing the partition are trimmed at regular intervals. This system was developed by St-Arnaud *et al.* (1996; Figure 2). In the bi-compartmental system, the spore and mycelium density produced in the distal compartment is markedly higher in comparison to the proximal compartment, which is probably related to the absence of the root and the difference in availability of C (Fortin *et al.*, 2002), making this system more productive than the mono-compartmental system. It is particularly adapted to the culture of *Glomus* species.

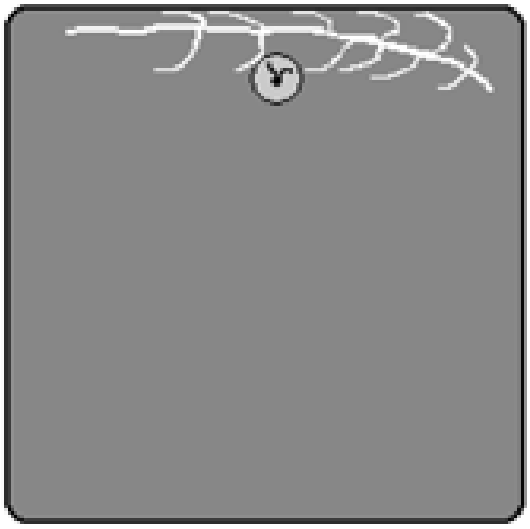

Figure 1- Placement of the propagule and the host root into a square Petri plate

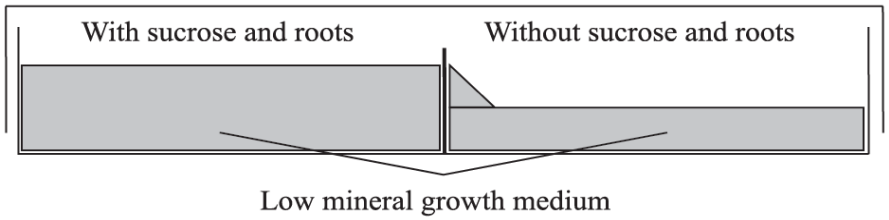

Figure 2- Diagram of the bi-compartmental system from St-Arnaud *et al.* (1996).

Culture Media Preparation

The most widely used mineral media for monoxenic cultivation of AM fungi are the Minimal (M) medium (Bécard and Fortin, 1988) and the Modified Strullu-Romand (MSR) medium (Declerck *et al.,* 1996). Both media result from the empiric modification of media usually used for in vitro plant culture and are equally successful for a range of AM fungi. The composition of M and MSR media is listed in Table 1. Differences between both media are discussed in Fortin *et al.* (2002). Similarly, a complete comparison of the successive media developed prior to the M and MSR media is given in Bécard and Piché (1992).

Choice of Host Root

Various plants have been used to establish monoxenic cultures with AM fungi. The first host roots used in monoxenic cultures were *Lycopersicon esculentum* Mill. (tomato) and *Trifolium pratense* L. (red clover) associated with *Glomus mosseae* Nicolson and Gerd. (Mosse and Hepper, 1975), and *Fragaria x ananassa* Duchesne. (strawberry) and *Alium cepa* L. (onion) associated with several *Glomus* species (Strullu and Romand, 1986). A natural genetic transformation of roots with the soil bacterium *Agrobacterium rhizogenes* Conn. was achieved decades ago (Riker *et al.,* 1930), but only applied in mycorrhizal research since the mid-1980s (Mugnier and Mosse, 1987). Since the development of the carrot hairy root line (*Daucus carota* L.), established by Bécard and Fortin (1988), this root has become the most widespread host for monoxenic cultivation of AM fungi. Transformed roots have several advantages over non-transformed roots for monoxenic cultivation. Their hormonal balance is modified, allowing profuse proliferation on synthetic media. It is generally accepted that this modification induces the production of growth hormones in the roots, thereby eliminating the necessity of incorporating of plant hormones into the culture medium. Stability of the

transformation over time is dependent on the host cultivar and bacterial strain combination (Labour *et al.*, 2003), but hairy roots used for further experiments have always tested positive. In association with AM fungi, Ri T-DNA transformed roots show greater AM intraradical colonization and sustain higher extraradical hyphal development than non-transformed roots, which is an advantage for fungal production (Fortin *et al.*, 2002). However, the pattern of colonization, distribution of vesicles, mycorrhizal spread and sporulation mechanisms – all the important growth stages of the fungus – can vary within different cultivars of the same host (Tiwari and Adholeya, 2003). Furthermore, transformation of the same host cultivar with a different bacterial strain results in a different mycorrhizal susceptibility (Labour *et al.*, 2003).

Host Root Transformation

Agrobacterium rhizogenes Conn., a gram-negative soil bacterium which induces hairy root disease of dicotyledonous plants, is used to induce hairy roots. In roots transformed with this bacterium, a segment of the bacterial DNA, named the region T (transferred DNA) of the plasmid Ri (root inducing) is incorporated into the host plant cells (Chilton *et al.*, 1982). Integration and expression of this DNA in the plant genome lead to the development of the hairy root phenotype and synthesis of novel low molecular weight compounds called opines (Tepfer and Tempé, 1981). Depending on the strain of *A. rhizogenes* used for the transformation, different principal opines can be found in the tissues of the hairy root: agropine, mannopine, cucumopine or mikimopine (Dessaux *et al.*, 1992). In addition, depending on the gene incorporated in the plant genome, the root can have a change of geotropism. Some hairy roots show a highly negative geotropic nature, some only have a slightly negative geotropic behavior while others keep their positive geotropism. This is due to a change of auxin sensitivity and the redistribution of this hormone after root transformation (Leguéet *al.*, 1996). Roots with a negative geotropism should be incubated in inverted position, to make the roots grow inside the medium.

Selection of AM Propagule

As described in Fortin *et al.* (2002), two types of propagule can be used to initiate monoxenic cultures: (1) spores or sporocarps, and (2) mycorrhizal root fragments containing vesicles or isolated vesicles. Depending on the AM fungi, often one type of propagule is better adapted to initiate a monoxenic culture. For *Glomus* species producing small spores (diameter < 100 μm), disinfection

of spores is often difficult without damage. The germination rate and hyphal re-growth are generally low or even absent. Species producing such spores habitually also produce a high number of vesicles within roots, i.e. *Rhizophagus intraradices*, *Rhizophagus prolifer*, which therefore should be considered the preferred propagules to initiate the monoxenic culture. Germination of vesicles either isolated or within the root segment is fast and efficient. In the case of mycorrhizal root segments, multiple germination arises from the root extremities (Declerck *et al.*, 1996), increasing the infective potential.

For species producing few (some Glomeraceae species, *Funneliformis caledonium*, *Funneliformis mosseae* etc.) or no vesicles (the Gigasporaceae) and/or big spores (diameter > 150 µm), spores should be used. When the fungus produces sporocarps, these are always the most appropriate propagules to initiate the monoxenic culture because of their multiple germination.

Table 1- Comparison of the composition of minimal (M) medium and modified Strullu-Romand (MSR) medium

	M medium	MSR medium
$N(NO_3^-)$, µM	3,200	3,800
$N(NH_4^+)$, µM	–	180
P (µM)	30	30
K (µM)	1,735	1,650
Ca (µM)	1,200	1,520
Mg (µM)	3,000	3,000
S (µM)	3,000	3,013
Cl (µM)	870	870
Na (µM)	20	20
Fe (µM)	20	20
Mn (µM)	30	11
Zn (µM)	9	1
B (µM)	24	30
I (µM)	4.5	–
Mo (µM)	0.01	0.22
Cu (µM)	0.96	0.96
Ca panthotenate (µM)	–	1.88
Biotin (µM)	–	0.004
Pyridoxine (µM)	0.49	4.38
Thiamine (µM)	0.3	2.96
Cyanocobalamine (µM)	–	0.29
Nicotinic acid (µM)	4	8.10
Glycine (mg/l)	3	–
Myo-inositol (mg/l)	50	–
Sucrose (g/l)	10	10
pH (before sterilization)	5.5	5.5
Gelling agent (g/l)	5	3

Disinfection Process

For a successful disinfection of spores and root pieces, a combination of antibiotic treatments should be applied on the extracted material (spores or root pieces). The successful combination of chloramine T and streptomycin was developed for *Glomus* species by Mosse (1962) and later a combination of chloramine T, streptomycin and gentamicin with the inclusion of the surfactant Tween 20 was reported by Mertz *et al.* (1979) for *Gigaspora* species. As an alternative, or as supplement to the use of antibiotics, sodium hypochlorite was used by Daniels and Menge (1981). Nowadays, sodium hypochlorite is replaced by calcium hypochlorite, since it is an instable product which degrades when remaining attached to the fungal propagules. The sodium hypochlorite solution, however, when not rinsed thoroughly, stays around the disinfected spores or root pieces where it can have a toxic effect. Besides this chemical treatment, Strullu and Romand (1986) adjusted a physical treatment, i.e. a treatment with sonications. With this treatment, even the microparticles which remain attached to the root pieces and spores are removed, assuring a thorough cleaning of the material (Figure 3).

Species type	Inoculum	The desinfection occurs in	Pretreatment 1	Pretreatment 2	Step1 Chloramine T treatment	Step2 Antibiotics treatments
Species producing large spores eg. Gigasporaceae – Glomaceae eg. *Glomus caledonium, mosseae*, etc.	Spore 	Vacuum filtration apparatus	None	None	10 min (2-10min)[1]	10 min (2-10 min)[1]
Species producing sporocarps : Glomaceae : *Glomus mosseae*	Sporocarp 	Vacuum filtration apparatus	Ultrasonic 1 min	None	10 min (2-10min)[1]	10 min (2-10min)[1]
Species with high root colonization and Vesicles production : Acaulosporacea-Glomaceae eg. *Glomus intraradices, fasciculatum, proliferum*, etc.	Root fragments 	(Ultrasonic bath (half of the time))[2]	(Ethanol 98 % 10s)[2]	Ca-hypochlorite 6% 2 min	10 min (2-10min)[1]	10 min (2-10min)[1]

Figure 3- Summary of the disinfection procedure. [1] Exposure time must be adapted to the contamination level of the starting material and the sensibility of the structures. [2] These steps can be suppressed if the root is not too contaminated and fragile.

Germination of Disinfected Propagules

The germination of spores and sporocarps often occurs in a period varying between 2 and 30 days after disinfection. In general, spores and sporocarps do not require any specific conditions for germination. However, it has been observed for some AM fungal strains that germination was stimulated by the presence of a root or CO2 (Bécard and Piché, 1989). Therefore, for some strains, the propagules are placed in the vicinity of a root. It was also demonstrated for some *Gigaspora* species (e.g. *Gi. margarita* and *Gi. rosea*) that cold treatment is always required before isolation and disinfection. For mycorrhizal root fragments, the hyphal re-growth from the extremities of mycorrhizal root segments is usually observed within 2–15 days. Germination of vesicles occurs 2–10 days after disinfection. For both inoculum types, spores/sporocarps and mycorrhizal root segments or isolated vesicles, it is important to associate the germinated propagules with a host root shortly after germination to avoid damage of the newly formed hyphae during the association process and to increase colonization aptitude.

Association Establishment

For a *Rhizophagus* species like *Rhizophagus intraradices*, a mycelium appears after a few days and new spores are produced very quickly thereafter. The mycelium grows extensively, rapidly invading the complete volume of the Petri plate. In the bi-compartmental system, the sporulation is higher in the distal compartment containing no carbon. The sporulation capacity of the fungal strain and the development of mycelium depend on the strain. After 3 months, a culture of *Rhizophagus intraradices* can produce more than 10,000 spores. For *Funneliformis* strains, for example, *Funneliformis caledonium*, which produces large spores and few vesicles, mycelium can grow rapidly and extensively but spores appear very slowly (after one or more months). For Gigasporaceae (*Gi. rosea*, *S. reticulata*, etc.), the production of mycelium is less profuse, auxiliary cells appear rapidly, but it takes several weeks for spores to be produced (Fortin *et al.*, 2002).

Continuous Culture

The first continuous culture of *Glomus* species was achieved by Strullu and Romand in 1986 using the intraradical form, and was thereafter extended to various AM fungi. The continuous culture is obtained by associating monoxenic mycorrhizal roots and/or spores (Chabot *et al.*, 1992), often

attached to extraradical mycelia, to a new, actively growing non-mycorrhizal host root, onto a fresh medium. This first method is largely used and is effective for a range of *Glomus* species (Strullu *et al.,* 1997; Declerck *et al.,* 1998, 2000) and for *Scutellospora reticulata* (De Souza and Declerck, 2003). A second method has been used by St-Arnaud *et al.* (1996) and is effective for *Glomus* species having a well-developed intraradical phase, such as *Rhizophagus intraradices*. In this method, apical segments of actively growing mycorrhizal roots with or without extraradical mycelium-supporting spores are transferred to a fresh medium. The root and associated fungus continue to proliferate across successive transfers onto fresh medium. This procedure requires the use of young, actively growing cultures, to allow the continuous growth of the host root. For *Glomus* species producing few spores and vesicles, continuous cultures are difficult to obtain. For *Gigaspora* species they were only reported as unpublished results in Fortin *et al.* (2002). Hence, for this latter species, it is necessary to periodically start new monoxenic cultures with disinfected spores from pot cultures.

Limitations and potentials

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 (Fortin *et al.,* 2002). 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 sub culturing 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. 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 (St-Arnaud *et al.,* 1996). Although species such as *Rhizophagus 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. 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. 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.The use of the AM root-organ culture technique has important implications to produce AM inocula for research and commercial purposes. Although the results from most industry-based research are not generally publicly available, recently Moutoglis and Bérand (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 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-borne 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. Briefly, using this system, it

would be possible to achieve a polarity whereby organic nutrient, 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. 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.

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