

# A PROTOCOL ON IN VITRO PROPAGATION OF ARBUSCULAR MYCORRHIZAL FUNGI USING ROOT ORGAN CULTURE TECHNIQUE

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## ABSTRACT

*The technique of in vitro propagation of Arbuscular mycorrhizal fungi has been developed over the past few decades and opens up areas of studying plant-fungi interactions. It is a scientific break through, especially for the study of the Arbuscular mycorrhizal fungi, since these obligate symbionts depend on host plant. The objective of this paper is to find out the in vitro culture of Arbuscular Mycorrhizal Fungi using Root Organ Culture technique. Ascertain of root colonization of these fungi could be affected in vitro without undertaking complex and complicated culture conditions. This could form an economically viable technique for root organ culture of Arbuscular mycorrhizal fungi.*

**Key words:** In-vitro, arbuscular mycorrhizal fungi, root culture.

## INTRODUCTION

A natural genetic transformation of roots with the soil bacterium and applied to excised roots for the cultivation of Arbuscular Mycorrhizal Fungi (AMF) of this technology is known as the root organ culture technique (ROC), where studying various aspects of the symbiosis. Among the several types of symbiotic associations, the mycorrhizal symbiosis involving plant root and soil fungi is immensely beneficial to plants. Although in AM symbiosis, there is no host specificity, there is host preference and hence mycorrhizal research assumes great significance.

Fungi break down dead organic matter or grow on other organisms to get nutrients for growth. Fungi are major player of Biotechnology. Fungi produce primary and secondary metabolites of industrial importance. Production often occurs after growth has stopped, when nutrients are limited, but excess carbon is available e.g. Penicillin, Cephalosporins, Griseofulvin, Cyclosporin, Ergot alkaloids. They are well developed and understood secretory machinery allowing simple purification of products from culture medium. Fungi are relatively easily grown in mass culture.

Mycorrhiza are mutually beneficial relationship between fungi and plant roots, specifically they are two types. One is ectomycorrhizal fungi, which forms sheaths of hyphae over a root and also grows into the

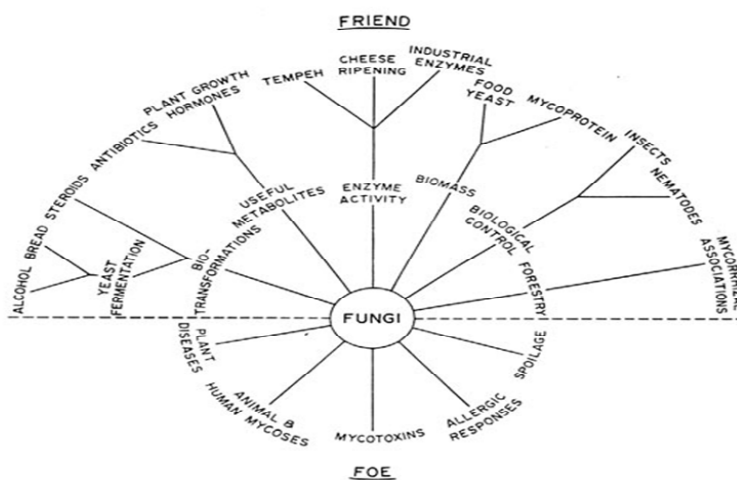
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extracellular spaces of the root cortex and another is *Arbuscular mycorrhizal* fungi (AMF), which extends hyphae through the cell walls of root cells and into tubes formed by invagination of the root cell membrane. AMF are symbiotic obligate associations between a group of fungi and the roots of higher plants (Krik, Cannon, Davi & Stalpers, 2001). AMF colonize approximately 80-90 percentages of terrestrial plants and their beneficial effects on the growth and health of plants have been recognized for some time. However, their obligate biotrophic nature has limited *in vitro* culture and large-scale production, regarding their potential use as inoculums in agricultural practices (Plenchette, Declerck, Diop & Strullu, 1996). The principle beneficiary being the host plant due to higher nutrient uptake from the soil and in exchange AMF receive a rhizospheric niche and organic nutrients for its growth and development. This protocol aimed to determine the *in vitro* propagation of AMF using ROC technique. Therefore, it becomes imperative that the fungi will predominantly flourish only under strict host rhizosphere.

## DESCRIPTION

Fungi not only directly produce substances that humans use as medicine, but they are also versatile tools in the vast field of medical research. Some fungi attack insects and therefore can be used as natural pesticides. Similarly, yeast has been used for thousands of year in the production of alcoholic beverage. The majority of grasses and trees require a mycorrhizal relationship with fungi to survive.



**Fig. 1:** Ray diagram showing the summary of the activities of fungi, relate to humans

**Source:** Retrieve 22.02.2017 from [www.mycorrhizea.com](http://www.mycorrhizea.com)

Mycorrhizal plants have many advantages over non-mycorrhizal plants, including better nutrient uptake especially Phosphorus (P), faster rehabilitation of degraded sites, greater tolerance to diseases and soil toxins, and greater survivability under stress conditions. Mycorrhizal research carried out over the years has confirmed their and nurseries and to prove the benefits of mycorrhiza in enhancing plant growth, thereby effectively reducing the costs incurred in the use of expensive phosphate fertilizers (Figure 1).

### **Significance of ROC technique**

Wide use of these fungi with or without or in combinations with other manure and synthetic fertilizers is coming up to drastically reduce use and subsequent deleterious effects of industrial fertilizers in the agro ecosystem (Cranenbrouck, Voets, Bivort, Renard, Strullu & Declerck., 2005; Ju, Kou, Christie, Dou & Zhang., 2007; Kong, Zhu, Fu, Han Zhang & He., 2008). In this regards, there have been several efforts by workers to have *in-vitro* cultures of AMF on specified culture media grown roots and then infected by AMF with colorizations in the rhizospheres (Gaur & Adholeya, 1994). After drying such roots, these could be utilized as biofertilizers. However, culturing conditions could involve substantial economical inputs.

### **Preliminary works**

Genetic transformation of roots with the soil bacterium *Agrobacterium rhizogenes* Conn. was achieved decades ago (Riker, Banfield, Wright, Keitt & Sagen., 1930; Ark & Thompson, 1961). *In-vitro* cultures of AMF are due to their rigid symbiotic obligate association which is further complicated because biotrophic and hypogenous nature of the micobionts involved. To overcome these debilitations several attempts have been made during the last few years to obtain symbiosis under *in-vitro* conditions. The use of root organ culture has proved successful. Lists of such *Glomalean* species are cultivated on root organ cultures (Fortin Becard, Deterck, Dalpe, St-Arnaud & Chughlan., 2002). Various plants such as *Allium cepa* L. (Liliaceae), *Cucumis sativus* L. (Cucurbitaceae), *Daucus carota* L. (Apiaceae), *Linum usitatissimum* L. (Linaceae), *Lycopersicon esculantum* Mill. (Solanaceae), *Medicago truncatula* Gaertn. (Leguminoceae), *Solanum tuberosum* L. (Solanaceae), *Tagetes pastula* L. (Asteraceae), *Trifolium pretense* L. ( Leguminoceae), *T. repens* L. (Leguminoceae) etc. have been used to establish monoxenic (i.e. root organ) cultures of AMF.

### **Status of *in vitro* propagation of AMF in the world**

*In vitro* propagation of AMF is categorized into two groups.

### **Establishment in trifolium pretense root**

Mosse and Hepper (1975) first successfully performed root organ culture using a system based on dual culture of *Glomus mosseae* Nicolson and Gerd. spores with excised roots of *T. pretense*. Due to their obligate symbiotic nature, less than 5 percent of AMF were successfully cultivated using the dual culture approach. Most data generated under monoxenic culture conditions have been obtained with *Glomus* and *Gigaspora* species. While *in situ* observations on *in vitro*-produced cultures of *Scutellospora* species, have been reported.

### **Establishment in Daucus carota root**

The monoxenic method involved growing sterile AMF spores on dual culture plates with transformed *D. carota* root. Depending on the strain of *A. rhizogenes* used for the transformation, different principals of opines (novel compound synthesized during transformation of bacterium into the host plant) can be found in the tissues of the hairy root such as agropine, mannopine, cucumopine or mikimopine (Dessaux, Petit & Tempe., 1992).

Root organ culture has obvious advantages over traditional system, permitting production of contaminant-free propagules. So far 25 AMF species have been successfully cultivated in monoxenic culture (Fortin, Becard, Declerck, Dalpe, St-Arnaud & Chughlan., 2002).

## **PROCEDURES AND MATERIALS**

### **Apparatus required**

Petri plates, potted plants, Beaker, 250 ml screw cap bottles, 250 ml conical flask, Knife, test-tubes are required to carry out the *in vitro* propagation of arbuscular mycorrhizal fungi using ROC Technique.

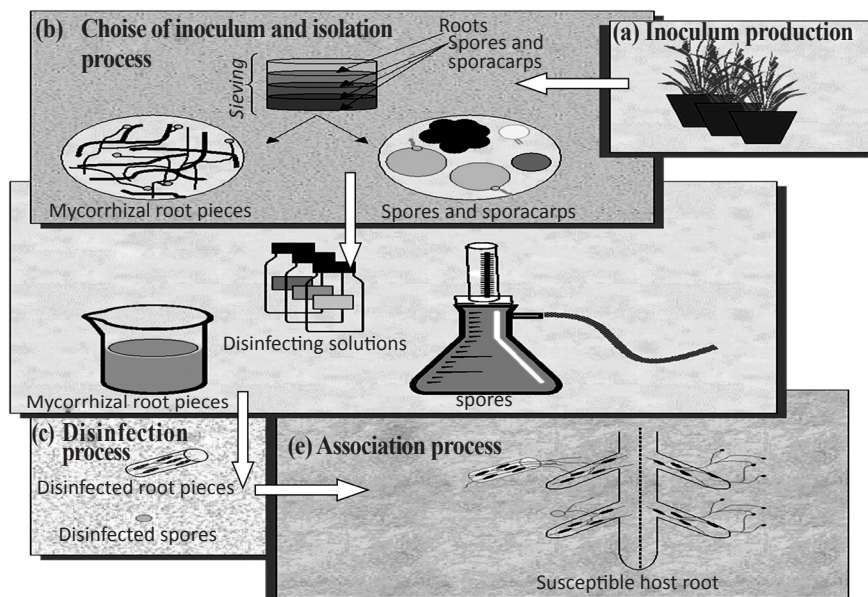
*A. rhizogenes* bacteria, spores of *Glomus*, Mycorrhizal root of *T. pretense*, Filter paper, Distilled water, Dissecting and compound microscope, Incubator, Laminar air flow; Autoclave, hot air oven etc. are the required for the *in vitro* culture.

### **Chemicals**

Ethanol/Sodium hypochloride, Tween 20 and antibiotics (streptomycin/gentamycin) are required.

### **Requirements *in vitro* establishment of mycorrhiza**

Following steps should be carried out for starting from pot cultures maintained under greenhouse (Figure 2):



**Fig. 2:** Protocol of Inoculums production, selection, isolation, disinfection of propagules, incubation and association to a susceptible host on the synthetic growth medium of AM fungi

**Source:** Retrived: 22.02.2017 from [www.Mycorrhizea.com](http://www.Mycorrhizea.com).

### Step first-inoculums production

Spores and mycorrhizal roots are subjects to the initiation for monoxenic cultures come from trap plants, grown under controlled greenhouse condition on a natural soil or in the presence of isolated AMF propagules (Figure 2a).

### Step second – Selection of starter inoculums and its isolation

- Spores and mycorrhizal roots are collected from the trap plants by wet sieving (Figure 2b).
- These are examined under dissecting microscope and recovered several isolations in water.
- Old spores and debris are eliminated, for this *Gigaspora* and *Scutellospora* species (which do not contain vesicles), are preferred.
- Practically *Glomus*, *Acaulospora* and *Entrophospora* species (which contain vesicles), are preferred for mycorrhizal roots having young, white and turgescent with no discernable surface contaminants and wounds are selected.

### **Step third - Subsequent disinfection of the Propagules**

- The disinfection process is the most critical step to achieve a successfully *in vitro* culture.
- Several disinfecting agents, (particularly) are used (for surface sterilization process): ethanol, calcium hypochlorite, chloramine T, added with few drops (approx. 1ml) of Tween 20 and antibiotics (streptomycin and gentamycin).
- Each step is separated by several rinsing in sterile water (121°C, for 15 min) (Figure 2c).

### **Step forth – Incubation of propagules**

- After disinfection, the propagules i.e. spore and mycorrhizal root pieces are incubated on a synthetic growth medium until hyphal re-growth is observed (Figure 2d)
- This step is conducted in order to select and eliminate the contaminated propagules.

### **Step fifth – Association to a susceptible host root on the synthetic growth medium**

- Germinating spores/mycorrhizal root pieces showing hyphal regrowth are reassociated with a susceptible host on the synthetic growth medium i.e. modified Strullu-Romand (MSR) media.
- In general the propagule is transferred into the Petriplate near an actively growing root or by transferring the actively growing host root in the vicinity of the propagules (Figure 2e).
- Sporulation is observed after several weeks or months depending on the strain consider.

### **Mechanisms**

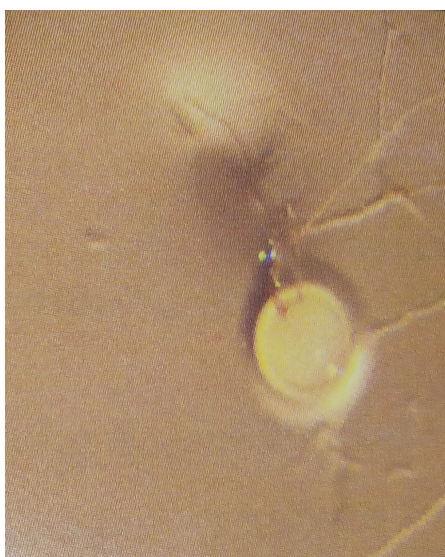
AMF are successfully propagates (cultivates) using the dual culture approach by using *Glomus mosseae* spores with excised roots of *T. pretense*.

- The monoxenic method involved growing sterile AMF spores on dual culture plates with transformed *D. carota* root.
- *A. rhizogenes*, (a gram –ve soil bacterium) which includes hairy root disease of dicotyledonous plants, is used to induce hairy roots.
- In roots transformer with this bacterium a segment of the bacterial DNA, the T (transformed DNA) of the root including plasmid (Ri), are incorporates into the host plant cells (Chilton, Tepfer, Petit, David, Casse-Delbert & Tempe, 1982). Integration and expression of this DNA



in the plant genome lead to the development of hairy root phenotype and synthesis of novel low molecular weight compounds called opines (Tepfer & Tempe, 1981).

- These adventitious roots are then culture *in vitro* on medium devoid of plant hormones, where they rapidly grow with a characteristic, highly branched and non-geotropic pattern.
- The combination of transformed carrot roots and sterile AMF spores can be used to produce "dual *in vitro* cultures" that provide an efficient method of producing abundant spores [ $>5000$ ] and mycelia on a 9 cm Petriplate] (Becard & Fortin, 1988) (Figure 3 & 4).



**Fig. 3:** *In vitro* spore germination



**Fig. 4:** *In vitro* sporulation

**Source:** Rodrigues, 2009.

### CONCLUSION

The success achieved by using root organ culture technique in cultivation of AMF *in vitro* is not only restricted to the study of the symbiotic interactions, but also permits the increase of knowledge in the morphology, taxonomy, phylogeny and biochemistry fields together with some aspects of their ecology. This protocol is an effort to compile widely used in AMF research. This protocol will serve as a useful guide for mycorrhizal researcher, teachers, and the student community.

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