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PRODUCTION OF AM FUNGI USING *IN VITRO* CULTURE TECHNIQUES

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ABSTRACT

The most abundant members of the soil organism that develop beneficial relationships with plants roots and contribute majorly to plant development are called mycorrhizal fungi. The most common well-known of these relations are the Vesicular Arbuscular Mycorrhizae (VAM).The obligate biotrophic nature of AM fungi has long been accepted. The helpful effects of AM Fungi on the growth and health of plants is recognizing VAM for some time, although their obligate biotrophic nature has limited. It's *in vitro* culture and large-scale production, reducing their potential for utilize as inocula in agricultural and horticultural practices has provided new tools for growing AMF under *in vitro* conditions, such as root organ culture and the monoxenic method involved growing sterile with AMF spores. Root organ culture has obvious advantages over conventional systems, permitting production of contaminant-free propagules. Many AMF species are successfully cultivated in monoxenic culture. *In vitro* culture of VAM fungi under axenic conditions continues to be one of the most challenging goals of modern biology. The *in vitro* systems have proved to be a helpful tool to study fundamental and practical aspects of AM symbiosis.

KEYWORDS: *In vitro*, **VAM, Propagules, Axenic Culture, Root-Organ culture, Monoxenic Culture**

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INTRODUCTION

The most abundant members of the soil organism that develop beneficial relationships with plants roots and provide majorly to plant growth are called mycorrhizal fungi. Arbuscular mycorrhiza (AM) is the commonest mycorrhizal type concerned in agricultural systems. Vesicular Arbuscular Mycorrhizas are aseptate mycelial fungi and are so-called because of the two characteristics structures-vesicles and arbuscules formed in roots.[1] The tremendous advances in research on mycorrhizal physiology and ecology more than the past 40 years have led to a greater thoughtful of the multiple roles of VAM in the ecosystem. VAM symbiosis is found in more than 80% of vascular plant families of today. The ecological and economic importance of this symbiosis has led to a vast interest in various aspects of AM fungal biology.[2] The beneficial effects of AMF on the growth and health of plants is recognizing VAM for some time, although their obligate biotrophic nature has restricted.[3] Research on AMF has increased exponentially since1970s and molecular biology has provided new tools for growing AMF under *in vitro* conditions, like root organ culture and monoxenic culture methods. Some relevant results point out efforts is not far from successful growth of AM fungi independent of a plant host. Axenic and monoxenic culture of AM fungi and achieve continuous culture of AM fungi without lost of infectivity. *In vitro* culture of AM fungi in axenic conditions continues to be one of the most challenging goals of modern biology. Since the mid-1970s, three major breakthroughs have been accomplished in the field of *in vitro* cultivation of AM fungi, which are of highest importance for culture collections. Mosse and Hepper [4] were the first to describe a simplified *in vitro* system for the study of VAM growth where in they used excised roots instead of whole plants. Mugnier and Mosse [5] modified the technique additional by using Ri T-DNA transformed hairy roots as the host tissue. The first subcultivation of an AM fungus in a system of consecutive isolations from mycorrhizal roots and re-associations with various root systems. It is not yet routine to cultivate all arbuscular mycorrhizal (AM) fungi *in vitro* on root organs. Because these fungi are not capable to complete their life cycle in the absence of a suitable host plant, and the mechanisms underlying the mandatory biography of the fungal partner are so far not fully understood. Although *in vitro* culture is a non-natural system, it may be a valuable tool to study fundamental and practical aspect of AM symbiosis, go together the experimental approaches. Expansion of extraradical mycelium under aseptic conditions is often accompanied by the production of so-called arbuscule like structures or branched absorbing structures. The produced propagules (spores, hyphae, infected roots) are able to germinate in new plants efficiently. Encapsulations stabilize the biological properties of mycorrhizal roots and isolated vesicles or spores. These immobilizations also conserve the infectivity of AM propagules under *in vitro* or *in vivo* assays.[6] The use of the AM root-organ methodology and the increasing number of fungal species cultivated *in vitro* offer many potential for the production of mycorrhizal inoculums for commercial purposes.[7] The step up of *in vitro* culture mycorrhizal systems has revolutionized the way we be aware of the arbuscular mycorrhizal (AM) symbiosis and provide a powerful model for the study of the relationship between the mycorrhizal partners.

MATERIALS AND METHODS

The AM inoculation protocols are divided into two categories, depending on whether axenic or non-axenic conditions.

A- Inoculation under axenic conditions

Manipulating in axenic environment requires the production of sterile fungal material and the advantage of avoiding contamination with other pathogens. This approach is of particular awareness since it is compatible with the use of Ri T-DNA transformed roots growing on sucrose-containing medium.[8] Sterile fungal material directly produces *in vitro* spores or mycorrhizal roots.

B - Inoculation under non-axenic conditions

Non-axenic inoculation, which can be used for well-developed plants, is suitable for high-through put experiments such as genetic screens and may be of particular interest for the large-scale invention of mycorrhizal roots. These environments can be used for either seedlings or composite plants.

Isolation of Roots and VAM Spores

The first stage in in-vitro cultivation of microorganism is isolation from the natural environment and the production of pure culture. Mycorrhizal fungi are, in general, difficult to isolate and culture in the laboratory. Some species are obligate symbionts and have not yet been cultured in the absence of host cells. VAM spores isolation mostly use wet sieving and decanting methods modified from Gerdemann and Nicolson.[9] The following Methods are:

- Processing a plant nearby soil for isolation of the roots and the VAM spores present in the soil- Remove the plant from the soil around the roots.
- Collection of intact roots- Collect the roots for VAM and use as an inoculums. Place the roots in a plastic carrier containing a small amount of water so they will not dry out. The roots may be fixed in Formaldehyde acetic acid (FAA) to be processes.
- Collection of additional roots and large pieces of organic debris (to be discard) on a 2 mm sieve.
- Collections of spores (propagules) of mycorrhizal fungi on a 38 µm sieve- Stir the soil-water mixture and decant part of this soil suspension onto the surface of the 250 µm sieve that stacked on top of the 38 µm sieve and only the fine soil particles along with the VAM spores collect on the 38 µm sieve.[10]

Sterilization and storage of spores

Sterilization

Concentrate the spores on the 38 µm sieve and then with a squeeze bottle wash down the spores into the sterilization solution.

Short term storage

The isolated, non-sterilized spores can be stored for 2 days in the refrigerator in a beaker of water sealed with

parafilm. For long term storage: Dry soil-sand and pour the sterilized and rinsed spore suspension over the sand, Allow the water to evaporate from the sand by leaving the cover off. Store the sealed and label dish at 4ºC until the spores are used.

MONOXENIC CULTURE

The present study describes the cultivation of AM fungi with Ri-T DNA transformed roots for the first time in monoxenic culture. The complete vegetative development of AM fungi in monoxenic culture is followed by transformed or non transformed roots.The monoxenic culture method involved growing sterile AMF spores on dual culture plates with transformed roots. Then roots are transformed by using a wild type strain of *Agrobacterium rhizogenes* containing a plasmid that induces a hairy root phenotype. These adventitious roots are then cultured *in vitro* on medium without of plant hormones, where they develop very rapidly, with a characteristic, highly branched and non-geotropic pattern.[11, 12]

Process of Monoxenic culture

The process of obtaining and maintaining monoxenic culture of AM fungi are separated into four main steps. These are the selection of the adequate AM fungal propagules, the sampling, disinfection and incubation of the propagules on a suitable host root and the subcultivation 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 subcultivation.

Culture System

Basically, two culture systems are used, first is 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, vigorously rising excised root together with AM fungal propagules

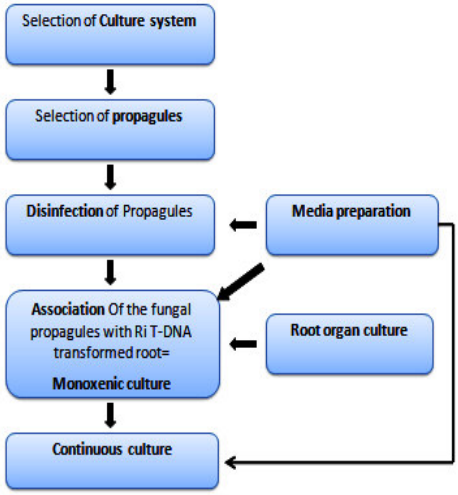

Figure 1
General scheme of the monoxenic culture process

The second system consists of a bi-compartmental Petri plate, with a proximal section in which the mycorrhizal root develops and containing a synthetic growth medium, and a distal compartment in which only the mycelium is allowed to grow on a similar synthetic medium. In the monoxenic culture two types of propagules can be used to initiate monoxenic cultures: (1) spores and (2) mycorrhizal root fragments containing vesicles. Depending on the AM fungi, often one type of propagule is better adapted to initiate a monoxenic culture. 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 are developed for *Glomus* species by Mosse.[13] The sodium hypochlorite solution, however, when not rinsed systematically, stays around the disinfected spores or root pieces where it can have a toxic effect.

Culture

The first continuous culture was achieved by Strullu and Romand in 1986 [14] using the intraradical form, and was there after comprehensive to various AM fungi. The continuous culture is obtained by associating monoxenic mycorrhizal roots or spores. A second method of continuous culture by St-Arnaud *et al* [15] is effective for *Glomus* species having a well-developed intraradical phase. In this method, apical segments of vigorously growing mycorrhizal roots with or without extraradical mycelium-supporting spores are transferred to a fresh medium. The root and correlated fungus continue to propagate across successive transfers in to fresh medium. This technique mostly use as an edge over other conformist modes of mass production, whereby a several-fold increase in spore and propagule production is achieved in less time and space. This technique offers pure, sterile, bulk, contaminant-free propagules.New approaches of AM fungi to growing in Ri T-DNA transformed roots cultures, in which some AM fungus species develop plentifully and form viable spores. Transformed roots are obtained by inoculation with *Agrobacterium rhizogenes* carrying the Ri T-plasmid. However, these techniques are labour consuming, are partial to few AM fungus species, and be likely to have a low rate of success.[16] The lifecycle of VAM fungi under

in vitro culture are describe morphological characteristics, including extraradical mycelium, intraradical mycelium structures and auxiliary cell formation.

Life Cycle of AM fungi

The proposed life cycles of AM Fungi are based on the germination potential of fungal propagules, linked to species description, nuclei mycelium behavior, and nutritive and environmental conditions. There are three growth phases in AM Fungi: Asymbiotic, Pre-symbiotic and Symbiotic phase.[17]

The life cycle of an AMF can be divided in these steps:-

(i) Establishment of the symbiosis- This involves propagule activation, host search, appressorium development, root penetration and arbuscule formation.

(ii) Vegetative growing phase- This involves intra and extraradical mycelium development, and an overall enhance of fungal biomass, formation of mycelia structures and expansion of the AM fungi colonization between plants.

(iii) Reproductive phase- This involves the development of reproductive structures. Latent spores are the major type of propagules.

(iv) The germination and hyphal growth from asexual spores in the soil is moved by signaling compounds released by roots (top right). These hyphae form infection structures (appresoria) on the outside of host roots the fungus grows into the root forming hyphae between cells and arbuscules that penetrate cell walls of plant. Hyphae also develop out into the soil forming a branched mycelium that functions to see the sights the soil and obtain mineral nutrients. Spores are formed by this external mycelium, completing the life cycle.

In Monoxenic culture, all phases of the fungal life cycle simultaneously exist and interact with each other.

Pre-Symbiotic Mycelium Stage

In this stage, germinated spores grow toward the host root by producing hyphal branches. This occurs before the formation of structures such as appressoria that occur on the host root epidermal cell walls. An appressorium is a term used to describe hyphal tip enlargement that attaches to the root surface of the host. This stage is referred to as presymbiotic because a one-on-one contact between the root and fungus is not required for stimulation of hyphal branches.[18]

Host Root Connecting Stage

The contact between the root and fungal hyphae may take one to several weeks to become established.[19] In the *in vivo* development conditions revealed that, once the root–fungi contact is established, the fungal morphology changes, with a reorientation of hyphal apical growth giving rise to either a direct entry point or to an exhaustive hyphal branching called “fan-like structure”.[20] Germinating hyphae from colonized root segments never differentiated fan-like structures but penetrated roots by single entry points. As recommended by Mosse [21], germ tubes issued from spores may need other signals for root colonization, different from those required by fungal hyphae and vesicles bounded by root tissues.

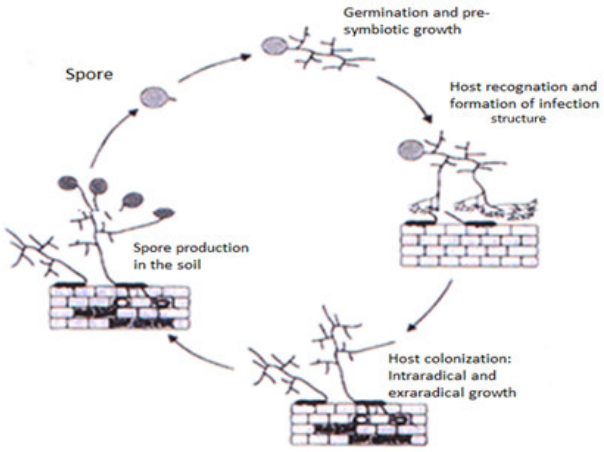

Figure 2

Life cycles of Arbuscular mycorrhizal fungi showing asymbiotic, presymbiotic and symbiotic stages of colonization (modified from Giovannetti).[17]

Symbiotic Stage

The symbiotic stage refers to the diffusion and development of the intraradical hyphae and the formation of arbuscules in the cortex of roots (Figure. 2). The extra radical hyphae growth arises after arbuscules formation and is characterize by the release of spores into the soil. Root colonization brings about the symbiotic interaction; but the benefits of root colonization are dependent on the survival of the AM fungal propagule particularly, the spores.[22] There are two types of AM colonization strategies are describe on base the structures of the intraradical mycelium and extraradical mycelium-

Intraradical Mycelium

Initiation the appressorium structure, one or more hyphae productively penetrate the cell wall by enzymatic and mechanical activities. Hyphal penetration after initial contact with the root. The intraradical mycelium pursues its progression, branching and anatomizing within the

intercellular root space channel of the root epidermal.

Extraradical Mycelium

The basic structure of the mycelium is made of large, straight-growing thick-walled hyphae, due to their capacity to extend rapidly, to colonize the substrates, and to begin root contact.[23] (Figure. 2). AM fungal species are non-specific in their relations with plants. Spore germination does not require external factors other than humidity and temperature to germinate.

Spores

Spore germination gives rise either to straight, thick-walled hyphae to stunted hyphae; depend on physiological condition the spore.[24] In addition to spores, several others AM fungi propagules are the potential to germinate. Spore germination through the lumen of their subtending hypha attachment, the germ tubes generating runner hyphae similar to those of AM fungi spore. New spores, called secondary spores, are characteristically synthesized outside of the plant root at the most important tip of individual fungal hyphae.[25] The AM fungi life cycle is completed after development of asexual spores on the external mycelium.

ROOT-ORGAN CULTURE

Root-organ cultures were first developed by White [26], the use of roots as host partner in AM symbiosis was first planned by Mosse and Hepper.[4] Roots can be propagated continuously in different solid and liquid media with high reproducibility. Initiation of isolate roots requires pre-germination of seeds earlier surface sterilized with traditional disinfectants (sodium hypochlorite, hydrogen peroxide), then thoroughly washed in sterile distilled water. Transformations of roots through the soil-borne microorganism *Agro-bacterium rhizogenes* provide a novel way to get hold of mass production of roots in a very short time. Therefore, transformed roots have a quick, energetic and homogenous growth in relative unfortunate substrates without supplementation of hormonal substances.[12]

Host Roots

A natural genetic transformation of plants by the everywhere soil bacterium *Agrobacterium rhizogenes* Conn. produces a condition known as hairy roots. This stable transformation produces Ri T-DNA transformed plant tissuesthat are morpho- genetically programmed to develop as roots. The transformed roots have a greater growth probable, which makes them more adaptable to different experimental situation and they can be generated from most dicotyledonous plants. The type of root system it chooses, success in establishing a mycorrhizal culture depends on the physiological condition of the host root.

Fungal Inocula

Mostly two types of fungal inoculums can be used to initiate root organ cultures: either extraradical spores or propagules from the intraradical phase (mycorrhizal root fragments and vesicles) of the fungus. In general, mycorrhizal roots used to begin root cultures come from trap plants grown in pot cultures, with field-collected soil or AM fungal propagules (roots). Young and well roots should be choosing for *in vitro* culture establishment. The Disinfected roots are cut into small pieces and incubated on a synthetic medium. Water-agar medium is also effective. Vesicles within roots may be a lesser amount of contaminated than the root surface, offering a better source of inoculums.[27] However, cultures of AM fungal species are systematically produced by spores, which are usually large and germinate vigorously. Spores are usually collected from the field, or from pot cultures, by wet sieving method. Before its use as an *in vitro* inoculum, spores must be surface sterilized. The surfactant (e.g., Tween 20) is widely used to sterilize AM fungal spores.[28] Generally, AM fungal spores don't need specific conditions or the presence of a host root to germinate.

Culture Media

The macro element composition of White's medium is significantly lower than that of MS and B5 media, generally used for *in vitro* plant cultures. The MSR medium is a modified medium, which was developed to optimize the growth of the intraradical phase of the fungus *in vitro.*[29,30] The macro element composition of MSR is similar to that of the M medium.

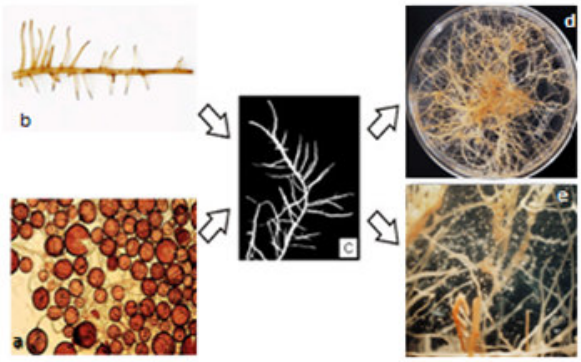

Figure 3

In vitro culture a. spores; b. germinating root segment; c. Root in culture; d. AM Fungi root-organ culture; e. closer view of an AMF root-organ culture

In vitro Germination of Spores

The utilization of AM root-organ cultures allows the aseptic production of spores of various AM fungal species. It is well known that cold stratification is significant to break the inherent dormancy-like stage found in AM fungal species.

In vitro Development of the Extraradical Phase

The uses of root-organ cultures in compartmentalize Petri dishes are extraradical mycelial development in root-free compartments.[31] The presence of ammonium in the distal section drastically reduced spore production. In this *in vitro* system observe the structural development of the extraradical phase.

Ri T-DNA Transformed Root Organs

Although Mosse and Hepper [4], working with AM fungi, were the first to obtain mycorrhiza using host-plant root organs, it was Mugnier and Mosse [5] who realized the potential value of Ri T-DNA transformed root organs for the study of mycorrhiza. Transformed root organs result from the natural incorporation into the plant's genome of the Ri T-DNA plasmid from the everywhere soil-borne bacterium *Agrobacterium rhizogenes*. This plasmid induces the production of growth hormones in the roots. The transformed roots exhibit increase growth rates and increased branching.

Optimum Growing Conditions

The mineral nutrient composition may affect fungal development. The optimum P levels vary with the host plant and cultivate fungal strains. Potassium, nitrogen, magnesium, and micro-elements ratios also affect inoculum development.[32, 33] Other edaphic factors such as pH, soil temperature, light intensity, relative humidity, and environment aeration must also be controlled to optimize AMF propagation.[34]

OTHER METHODS OF CULTURING AM FUNGI

Arbuscular mycorrhizal fungi are known as obligate symbionts to establish them in axenic cultures on a variety of media. A few reports claim the growth of Arbuscular mycorrhizal fungi on defined media, but not any of these withstand serious evaluation. The dixenic methods using transformed root organ cultures are successful. It is nevertheless possible to obtain cultures of AM Fungi open pot culture.[35] VAM Cultures may be produced by various methods, which are following-

Soil trap cultures

The simplest kind of culture to produce; soils are collected and mix with a disinfected substrate. The mixture is then put in a suitable container, which is not contaminated by AMF. Either seeds are seedlings (free of any existing mycorrhizae) plant in the mixture. The plants are then maintained for a period of time appropriate for the establishment of mycorrhizae. The usual result of soil trap cultures is a mixed AM species pot culture. Such a culture can then be used as base for further purification.

Pot substrate cultures

The use of substrate from obtainable pot cultures is another kind of `soil trap', though in this case, cultures would already be of only one species. In this type of culture, a small quantity of substrate from an existing pot culture is either mix through the disinfected substrate, or added in a depression into either a mycorrhiza-free plant is placed. The cultures usually establish quickly, and spores are normally produced within a month or two of the sub-culturing attempt.

Plant trap culture

Another method of producing culture is to lift small plants from the area of interest, carefully wash the roots to remove all traces of soil and external mycelium, and then plant them in a suitable sterile substrate. The result is usually a mixed culture, since plants may have more than one symbiont.

Aeroponic Cultures

An aeroponic system for the soil-less culture of Arbuscular mycorrhizal fungi and the production of sheared-root inoculum. The plants are suspended in a chamber in which a mist of a nutrient solution is create from an atomizing disk or pressurized spray. At harvest, roots are removed, washed over a coarse sieve to remove and separate spores. This material is collected on a fine sieve and used as "sheared-root" inoculums.

Multi and single spore cultures

In this culture firstly spores are extracting from soil. The necks of the funnel are filling with a substrate and the spores are place in the funnel base on the soil.[36] The funnel then fills and seeded or planted with a suitable host, after a sometime the symbiosis are establish. These are prepared in exactly the same way as multispore cultures, but, as their name implies, are produced from a single spore.

APPLICATIONS

Beneficial effects of AMF inoculation to micro propagate plantlets in their natural environment; plants are colonizing both by external and internal microorganisms. The VAM fungi, can improve plant performance under stress environments, and consequently enhance yield[37] - Development of a superior root system-Biopriming of micropropagate plantlets with AMF helps in the development of a superior and stronger root system by increasing the rooting strength and surface area of existing roots.[38] Increased water conducting capacity- The AM fungi symbiosis improves the hydraulic conductivity of the root at low soil water potential which finally influences the water potential, transpiration rate and leaf resistance. Enhanced nutrient uptake- AMF increase the fitness of the host plant by greater than ever the uptake of minerals such as P that are relatively immobile in soils and other mineral nutrients such as Ca, Cu, Mn and Zn.[39] Alleviate environmental stresses- AMF enable plants to stand a wide range of environmental stresses such as drought, toxic metals, saline soil, root pathogens, soil temperature and adverse pH[34, 40] Advances in biotechnology leading to the improved performance of microbial inocula are stimulated interest in the use of microorganisms to increase plant productivity.

CONCLUSIONS

The *in vitro* culture technique for VAM fungi are numerous advantages over traditional inoculums production systems. The system is also helpful for determining symbiotic factors provide by the root which govern fungal growth. The continuous culture of AM fungi would make it possible to maintain biodiversity through the creation of fungal libraries.

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