Scope and Limitations of AMF Biofertilizer Production

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Introduction

Arbuscular mycorrhizal (AM) fungi are soil-borne microbes belonging to phylum Glomeromycota that form a symbiotic association with roots of higher plants. Hyphae colonize their host roots and form a mycelial network in the rhizosphere to facilitate nutrient uptake, especially P (Rodrigues and Rodrigues 2014), and in turn acquire photosynthates from the host plant. Around 90% of vascular plants form AM association (Smith and Read 2008). Plant genes and signal molecules enable hyphal entry and development of the fungus in the plant (Parniske 2008). The extra-radical mycelium extends several centimetres beyond the depletion zone absorbing nutrients that are transported to host roots (Khan *et al.* 2000). These fungi play an important role in agriculture, forestry, and horticulture by increasing crop yield, health, and resistance to stress by reducing the cost of agrochemicals (Johansson *et al.* 2004). Occurrence of AM symbiosis is dated back to >460 million years ago (Read *et al.* 2000). Based on the spore morphology, approximately 240 AM fungal taxa belonging to order Glomales have been described (Schubler and Walker 2010; Kruger *et al.* 2012), although molecular analysis data shows that the actual number of AM fungal taxa can be much higher (Vandenkoornhuyse *et al.* 2002).

Culture Techniques for AM Fungal Inoculum

Various cultivation techniques of AM fungal inoculum production have been attempted in the last few decades. Sand/soil- and substrate-based production techniques, substrate-free culture techniques (hydroponics and aeroponics), and *in vitro* cultivation methods have been attempted in the large-scale production of AM fungi. Several parameters must be taken into consideration for the culture of AM fungi, such as controlled or semi-controlled conditions in greenhouses, AM fungal species, the host plant, substrate, and amendments.

Substrate-Based Production System

Conventional production of AM fungi is commonly achieved by the cultivation of host plants and their symbionts in a soil- or sand-based substrate (substrate-based production system). The inoculum to initiate production consists of dried root fragments or colonized root fragments, AM spores, sporocarps, and fragments of hyphae. When spores are extracted from the soil and used as inoculum directly they tend to have very low viability or may even be dead or parasitized. To overcome this, initially, the rhizosphere soil is used to prepare a 'trap culture' using a suitable host plant. This increases the number of viable spore propagules for further isolation, multiplication, and production of monospecific cultures. The pure culture inoculum thus produced consists of spores, colonized root fragments, and AM hyphae of a single species.

Selection of host plant is based on numerous criteria, such as plants exhibiting a short life cycle, rapid growth, adaptation to the prevailing growing conditions, and ready colonization by a range of AM fungal species. A large quantity of roots should also be produced in a relatively short period, and resistance to pest and diseases common to the inocula production environment.

A range of plant species, such as *Zea mays* (corn), *Allium cepa* (onion), *Arachis hypogaea* (peanut), *Paspalum notatum* (bahia grass), *Pueraria phaseoloides* (kudzu), coleus (*Plectranthus scutellarioides*), ragi (*Eleusine coracana*), etc., have been used as hosts with encouraging results.

Various substrates, such as soil, sand, peat, vermiculite, perlite, calcinated clay, and compost have been used to propagate AM fungi (Ijdo *et al.* 2011). Addition of different organic amendments also influences AM fungal colonization. Chitin and humic substances increase colonization levels (Gryndler *et al.* 2003; Gryndler *et al.* 2005). Manipulation of nutrient content has a further impact on AM fungal propagule production (Douds and Schenck 1990). The substrate-based culture technique is the most widely used method for AM fungal production as it requires a relatively little less technical support, is cheap, is the least artificial, and a large set of AM fungal species can be cultured (Ijdo *et al.* 2011). Conversely, the sand/soil-based systems have certain disadvantages such as the presence of unwanted contaminants, even with good phytosanitary care, fewer viable spores than *in vitro* system, and parasitized spores.

Substrate-Free Production System

Substrate-free cultivation systems, such as hydroponic and aeroponic have also been used for the

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multiplication of AM fungi wherein a continuous flow or mist of nutrient solution is provided for the plant and the symbionts. Although this system offers the advantage of providing inoculum which is free from attached substrate particles, a disadvantage has been that the nutrient solution is prone to microbial contamination and algal growth (Elmes and Mosse 1984).

Monoxenic Culture System

The first attempt to culture AM fungi monoxenically dates back to the late 1950s (Mosse 1959). Thereafter, tremendous progress has been made for the mass production of AM fungi using Ri T-DNA transformed roots (Mugnier and Mosse 1987). Different *in vitro* culture techniques have been derived such as the bicompartment system wherein AM fungal mycelia and spore are produced free from roots (St-Arnaud *et al.* 1996), and manipulation of culture medium to induce sporulation (Becard and Piche 1992). These developments have enabled studies in spore ontogeny (Pawlowska *et al.* 1999), sporulation dynamics (Declerck *et al.* 2001), response of AM fungi to cell wall-associated phenolics (Douds *et al.* 1996) and flavonoids (Morandi *et al.* 1992), lipid metabolism (Bago *et al.* 2002), transport of mineral nutrients to roots (Dupre de Boulois *et al.* 2005) and isolation of contaminant-free spores for molecular analysis (Pawlowska and Taylor 2004). A wide number of AM fungal species belonging to Glomeraceae and a few Gigasporaceae have been successfully cultured in the root organ culture (ROC) system.

Species, such as *Acaulospora rehmii* (Dalpe and Declerck 2002), *Gigaspora rosea* (Bago *et al.*1998c), *Gi. margarita* (Miller-Wideman and Watrud 1984; Diop *et al.* 1992; Gadkar and Adholeya 2000), *Gi. gigantea* (Gadkar *et al.* 1997), *Gi. decipiens* (Fernandez Bidondo *et al.* 2012), *Glomus etunicatum* (Schreiner and Koide 1993), *G. versiforme* (Diop *et al.* 1994; Declerck *et al.* 1996), *G. deserticola* (Mathur and Vyas 1995), *G. fistulosum* (Nuutila *et al.* 1995; Gryndler *et al.* 1998), *G. clarum* (De-Souza and Berbara 1999; Rodrigues and Rodrigues 2012), *Funneliformis caledonius* (Hepper 1981; Karandashov *et al.* 2000), *F. geosporus* (Declerck *et al.* 1998), *F. mosseae* (Douds 1997; Rodrigues and Rodrigues 2015), *Rhizophagus irregularis* (Chabot *et al.* 1992; St-Arnaud *et al.* 1996), *R. fasciculatus* (Declerck *et al.* 1998), *R. proliferus* (Declerck *et al.* 2000) and *Sclerocystis sinuosa* (Bi *et al.*2004) have been successfully cultured *in vitro*.

Culture media such as minimal (M) medium (Becard and Fortin 1988) and modified Strullu Romand (MSR) medium (Strullu and Romand 1986, modified by Declerck *et al.* 1998) are often used to culture AM fungi. The growth of germ tube is inhibited by the presence of sucrose in MSR medium. Healthy germination of AM fungal spores in MSR medium without sucrose was achieved by D'Souza *et al.* (2013). During the pre-symbiotic phase, AM spore use the reserve materials from propagules for the germination and growth of germ tubes (Clark 1997). Root Organ Culture (ROC) was first developed by White (1943), followed by development of further Ri T-DNA transformed roots of different plant species, *viz.*, clover (*Trifolium*) (Mosse and Hepper 1975), bindweed (*Convolvulus sepium*) (Tepfer and Tempe 1981), onion (*Allium cepa*), tomato (*Solanum lycopersicum*) (Strullu and Romand 1986, 1987), carrot (*Daucus carota*) (Mugnier and Mosse 1987), strawberry (*Fragaria x ananassa*), chicory (*Cichorium intybus*) (Boisson-Dernier *et al.* 2001), barrel medic (*Medicago truncatula*) (Fontaine *et al.* 2004) and linum (*Linum usitatissimum*) (Rodrigues and Rodrigues 2015).

Fungal inocula such as isolated spores or propagules from intra-radical phase (colonized root fragments and isolated vesicles) of AM fungi can be used to initiate monoxenic cultures (Rodrigues and Rodrigues 2015). The culture established needs to be maintained by continuous sub-culturing, transferring the mycorrhizal roots onto fresh medium (St-Arnaud *et al.* 1996). Under aseptic conditions, AM symbiosis with the transformed roots takes place by development of extra-radical mycelium which is often accompanied by formation of arbuscule-like structures (ALS) (Bago *et al.* 1998a) or branched absorbing structures (BAS) (Bago *et al.*1998b). These structures are probably nutrient-exchange sites between the fungus and its host (Diop 2003). Sporulation in AM fungi differs between species as well as between isolates of the single species and is related to spore size (Declerck *et al.* 2001).

The most important advantage offered by *in vitro* cultivation system is the absence of undesirable organisms. Contamination by other undesirable microorganisms can occur, however, during the establishment of culture process or during the later stages of culture maintenance. This type of system can be used for the large-scale production of AM fungi consisting of high-quality inoculum with minimum space. Also, the factors influencing optimum production can be easily detected and controlled, and harvesting time can be determined. The maintenance of a successfully established culture is easily achieved by sub-culture and maintaining the plates in dark condition. As a disadvantage, the *in vitro*-grown AM fungal diversity is lower than that under-pot culture system (Rodrigues and Rodrigues 2013). Furthermore, the *in vitro* production is expensive, requiring skilled technicians and sophisticated

laboratory equipment to carry out the whole process in sterile and controlled conditions (Ijdo *et al. 2011*). Further studies are in progress to identify and eliminate contaminants in established cultures.

AM Fungi as Biofertilizers

It has been observed that AM fungal inoculation provides beneficial results in plant growth both in controlled and open-field conditions. AM fungi have been confirmed to show better performance in terms of plant growth and yield characteristics. This would make the AM fungal technology more suitable to sustainable cropping systems (Berruti *et al.* 2016). Khan *et al.* (2008) reported that the inoculation of a single or dual AM fungi increased the growth and nutrient uptake of *Medicago sativa* which resulted in the increased dry weight of shoot and root. Bhat *et al.* (2010) studied the effect of AM fungi and *Rhizobium* on green gram (*Vigna radiata*) and reported a significant effect on nodulation, yield, crude protein content, and NPK content in grain. Various further studies have proved that AM fungi are an effective resource when used as biofertilizers with no adverse environmental effect.

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