Lead Lecture

Advances in Arbuscular Mycorrhizal (AM) Biotechnology

K. M. Rodrigues and B. F. Rodrigues*

Prof. and Head, Department of Botany, Goa University, Goa 403 206 India
*E-mail: felinov@gmail.com

Introduction

Arbuscular mycorrhizal (AM) fungi from the phylum Glomeromycota are ubiquitous soil borne microbial symbionts forming mutualistic associations with a majority of terrestrial plants. They facilitate uptake of nutrients (mostly immobile P) through their extra-radical mycelial network and provide other benefits to their host plants. These benefits can be physiological, nutritional and ecological and therefore exploiting and managing AM fungi has important consequences for both agricultural and natural ecosystems. Nowadays, they are increasingly considered in agriculture, horticulture, and forestry programs, as well as for environmental reclamation, to increase crop yield and health and to limit the application of agrochemicals (Gianinazzi *et al.*, 2002; Johansson *et al.*, 2004). However, the obligate biotrophic nature of AM fungi has complicated the development of cost-efficient large-scale production methods to obtain high-quality AM fungal inoculum. This is one of the reasons why their commercial exploitation is still in its infancy (Ijdo *et al.*, 2011).

The inoculum production systems for AM fungi are classified into *viz.*, the classical sand/soil or more advanced substrate-based production systems and the *in vitro* cultivation systems, which are based either on excised roots i.e. root organ cultures (ROC) or on whole autotrophic plants (Ijdo *et al.*, 2011).

Am Fungal Inoculum Production and Multiplication:

2.1: Inoculum Production

AM fungal inoculum has been utilized in agriculture, horticulture, landscape restoration, and site remediation for almost two decades (Hamel, 1996). In the early 1990s, researchers described multiple ways in which AM species management would be useful for sustainable systems, including agro-systems and restoration (Bethlenfalvay and Linderman, 1992; Pfleger and Linderman, 1994). In a long-rm study comparing organic and conventional agriculture, laeder *et al.* (2002) found that AM were stimulated in ganic treatments, which was correlated to enhanced stem health (faunal diversity, soil stability, and microbial tivity) and to increased crop efficiency.

2.2: Sources of AM inoculum

AM fungi are obligate symbionts, growing only in association with a host plant. Current production systems therefore rely on soil-based systems (plots or pots), which are not sterile and are often contaminated with other AM species, and other microbes, including pathogens (Gianninazzi and Bosatka, 2004). Non-soil based approaches include *in vitro* systems involving the use of Ri T-DNA transformed plant root organs (genetically modified with *Agrobacterium rhizogens*) to grow on media under sterile conditions. These are much cleaner, but have a limited production capacity (Declerk *et al.*, 2005).

2.2.1: Soil based systems or pot cultures

Soil from the root zone of a plant hosting AM can be used as inoculum. Such inoculum is composed of dried root fragments or colonized root fragments, AM spores, sporocarps, and fragments of hyphae. Soil may not be a reliable inoculum unless one has some idea of the abundance, diversity, and activity of the indigenous AM species. Spores can be extracted from the soil and used as in-oculum but such spores tend to have very low viability or may be dead or parasitized. In such a case, soil sample can be taken to set up a 'trap culture' using a suitable host plant to boost the number of viable spore propagules for isolation, further multiplication and also to produce pure or monospecific cultures.

Pure cultures or monospecific cultures are obtained after a known isolate of AM and a suitable host are grown together in a me-dium (sterilized soil/sand) optimized for development of AM association and spore for-mation. It consists of spores, colonized root fragments, and AM hyphae.

2.2.2: Host plant species

The plant grown to host AM fungi in the inocu-lum production medium should be carefully selected. It should grow fast, be adapted to the prevailing grow-ing conditions, be readily colonized by AM, and pro-duce a large quantity of roots within a relatively short time (45–60 days). It should be resistant to any pests and diseases common in the inocula production environment.

Gilmore (1968) recommended strawberry (*Fragaria* sp.) for open pot culture propagation of AM fungi. The range of plant species used since then are too numerous to list. Some common temperate host plants included *Zea mays* (corn), *Allium cepa* (onion), and *Arachis hypogaea* (peanut). Widely-used tropical hosts included *Stylosanthes* spp., *Paspalum notatum* (bahia grass) and *Pueraria phaseoloides* (kudzu) (http://invam.wvu.edu/methods/cultures/host-plant-choices).

The host plant should also be fertilized by periodic additions of a nutrient solution such as Hoagland's solution (especially -P) so as to manage the chemical composition of the medium and to regulate the formation of AM association. To ensure that most of the spores in the inoculum are mature, it is essential to grow the host plant for 12–14 weeks. The medium is then allowed to dry slowly by reducing the frequency of watering over a week and then withdraw-ing water completely. The inoculum can then be further multiplied.

2.2.3: Advantages and disadvantages

Soil-based systems for cultivation of AM fungi (pots, bags, or beds) are the most widely adopted technique for AM fungal inoculum production. Soil-based production systems are the least artificial and support the production of a large set of AM fungal species (single or monospecific cultures). In general, they are considered as a convenient system for large-scale production that is able to reach inoculum densities set for mass production of 80–100 propagules per cubic centimeter (Feldmann and Grotkass, 2002). In soil-based production systems, the nutrient supplies to the AM fungus and plant can be monitored and regulated (Lee and George, 2005). More controlled culture conditions are an advantage as this can lead to insights on factors to optimize propagule production (Ijdo *et al.*, 2011).

A disadvantage of soil-based cultivations systems is that, in most cases, they cannot guarantee the absence of unwanted contaminants. Besides, these systems are often space consuming. The AM fungal propagules are isolated through wet sieving and decanting technique, which can be followed by sucrose density centrifugation. In addition to soil or sand as a substrate, technical adaptations such as the addition of glass beads, river sand, or vermiculite have been developed facilitating harvest of relatively clean AM fungal spores and colonized roots that can be chopped into pieces. The presence of a substrate, however, provides an inoculum which is not directly suitable for mechanical application, as is the case for soil-free production methods (Mohammad *et al.*, 2004).

2.3.1: *In vitro* systems or root organ cultures

Ri-plasmid transformed root cultures were pioneered by Mugnier and Mosse (1987). 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 transformation (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 *A. rhizogenes* Conn. (Tepfer and Tempé, 1981). For *in vitro* culture of AM fungi using Ri T-DNA roots, the disinfected AM fungal propagules (spores and colonized root fragments) are plated on to Modified Strullu Romand (MSR) media for germination after which the germinated propagules are associated with actively growing Ri T-DNA transformed roots for establishment of AM symbiosis (Bécard and Fortin, 1988).

2.3.2: Advantages and disadvantages

The most obvious advantage of *in vitro* cultivation systems is the absence of undesirable contaminants or microorganisms, which makes them more suitable for large-scale production of high-quality AM fungal inoculum. While cross-contaminations by other AM fungi are evidently excluded (if the starter inoculum is monospecific), the contamination by other microorganisms (endophytes, bacteria) may occur either at the establishment of the cultivation process or at later stages of culture. Therefore, it may be useful to control the cultures visually, by standard plate-counting techniques and by molecular techniques. The cultures may be placed in a growth chamber requiring minimal space for incubation with no light required. The possibility to follow sporulation dynamics during cultivation also provides a means to control the level of spore production and to determine the optimal harvesting time. Factors that influence optimal production (e.g., nutrient availability, presence of contaminants) can be more easily detected and controlled in (liquid) *in vitro* cultures. As a disadvantage, the diversity (in terms of genera) of AM fungi that have been grown *in vitro* is lower than under pot cultivation systems. Another disadvantage of *in vitro* production is the costs associated with the production systems, requiring skilled technicians and laboratory equipments such as sterile work flows, controlled incubators for ROC, and growth chambers for plant systems. Other advantages of the ROC systems are the low requirements in the follow-up of the cultures. Once successfully initiated, the cultures may be maintained for periods exceeding 6 to 12 months without intervention (Ijdo *et al.*, 2011). For harvesting of the *in vitro* produced AM fungal propagules, solubilization of the culture

nedium is carried out using citrate buffer. The application f sterile produced inoculum can be of great value for *in itro* propagation of high-value crops and ornamental plants Kapoor *et al.*, 2008). In addition, *in vitro* propagation in ssociation with AM fungi could reduce mortality rates and he transplantation shock of reintroduced endangered plant pecies. It could also be used to enhance the production of econdary metabolites used in the pharmaceutical industry Kapoor *et al.*, 2008). Although *in vitro* cultivation methods are currently still costly, it seems likely that the criteria for quality control of AM fungal inoculum will result in the utilization of techniques that are able to reduce contamination risks. Cultivation by *in vitro* methods may then become an important method to meet future quality standards for commercial mass production (Ijdo *et al.*, 2011).

Conclusion

Large-scale production of AM fungal bio-inoculum is not possible in the absence of a suitable host due to their obligate biotrophic nature, and it is not possible to identify AM species in their active live stages (growing mycelium). As a consequence, quality control is often a problem, and tracing the organisms into the field to strictly relate positive effects to the inoculated AM fungus is nearly impossible. In addition, no clear criteria have been set for the quality control of commercial inoculum, but most likely, the legislation dealing with the application of beneficial microorganisms will become more drastic in the coming decades (Ijdo *et al.*, 2011). The production of AM fungi on plants under *in vitro* conditions has been recently proposed (Voets *et al.*, 2005) and extended to hydroponic systems (Declerck *et al.*, 2009, WO/2009/090220). Following the pre-inoculation of a suitable autotrophic host plant in the system of Voets *et al.* (2009), a culture is transferred in a hydroponic cultivation system favoring the production of large quantities of propagules. Other *in vitro* methods might come up, which could involve spore production on callus or sporulation in sterile alginate beads or fully closed hydroponic plant cultivation suitable for the production of AM fungi (up-scaling the system of Dupré de Boulois *et al.* 2006). However, other relatively clean methods (e.g., in aeroponics) also have a strong developmental potential and could be further developed in the future. Biermann and Linderman (1983) already discussed that techniques such as sonication and gradient flotation as well as enzymatic methods could be developed to separate intra-radical spores and vesicles from roots. With an AM fungus as the only endophyte, such intra-radical propagules can serve as a high-quality inoculum.

The search for and the utilization of beneficial microbes for sustainable agro-ecosystems has created a potential for the exploitation of AM fungi and as such there is a necessity to accelerate their incorporation as biofertilizers in agricultural production systems. Therefore, continuous development of high-quality and low-cost AM fungal inoculum production methods is expected, which could lead to establishment of more advanced methods for large-scale production of AM bio-inoculum.

References

http://invam.wvu.edu/methods/cultures/host-plant-choices.

Bécard, G. and Fortin, J.A. 1988. Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytol* **108**:211-218.

Bethlenfalvay, G.J. and Linderman, R.G. 1992. Mycorrhizae in Sustainable Agriculture. Am Soc Agron Special Publication 54. American Society of Agronomy, Madison.

Biermann, B. and Linderman, R.G. 1983. Use of vesicular–arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. *New Phytol* **95**:97–105.

Declerck, S., Ijdo, M., Fernandez, K., Voets, L. and de la Providencia, I. 2009. Method and system for in vitro mass production of arbuscular mycorrhizal fungi. WO/2009/090220

Declerck, S., Strullu, D.G. and Fortin, J.A. (Eds). 2005. *In vitro* culture of Mycorrhizas. New York, NY: Springer Verlag Berlin Heidelberg, pp. 388.

Dupré de Boulois, H., Voets, L., Delvaux, B., Jakobsen, I. and Declerck, S. 2006. Transport of radiocaesium by arbuscular mycorrhizal ungi to Medicago truncatula under in vitro conditions. *Environ Microbiol* **8**:1926–1934.

Feldmann, F. and Grotkass, C. 2002. Directed inoculum production—shall we be able to design AMF populations to achieve predictable symbiotic effectiveness? In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (Eds) Mycorrhizal technology in agriculture: from genes to bioproducts. Birkhauser, Basel, pp 261–279.

Gianinazzi, S. and Bosatka, M. 2004. Inoculum of arbuscular mycorrhizal fungi for production systems: science meets business. *Can J Botany* **82**:1264–71.

Gianinazzi, S., Schüepp, H., Barea, J.M. and Haselwandter, K. 2002. Mycorrhizal technology in agriculture: from genes to bioproducts. Birkhauser, Basel.

Gilmore, A.E. 1968. Phycomycetous mycorrhizal organisms collected by open-pot culture methods. *Hilgardia* **39**:87–105.

Hamel, C. 1996. Prospects and problems pertaining to the management of arbuscular mycorrhizae in agriculture. *Agr Ecos Environ* **60**:197-210.

Ijdo, M., Cranenbrouck, S. and Declerck, S. 2011. Methods for large-scale production of AM fungi: past, present, and future. *Mycorrhiza* **21**:1 – 16.

Johansson, J.F., Paul, L.R. and Finlay, R.D. 2004. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. *FEMS Microbiol Ecol* **48**:1-13.

Kapoor, R., Sharma, D. and Bhatnagar, A.K. 2008. Arbuscular mycorrhizae in micropropagation systems and their potential applications. *Sci Hortic* **116**:227–239.

Lee, Y.J. and George, E. 2005. Development of a nutrient film technique culture system for arbuscular mycorrhizal plants. *Hort Science* **40**:378–380.

Maeder, P., Fleissbach, A., Dubois, D., *et al*. 2002. Soil fertility and biodiversity in organic farming. *Science* **296**:1694-97.

Mohammad, A., Mirta, B. and Khan, A.G. 2004. Effects of sheared-root inoculum of Glomus intraradices on wheat grown at different phosphorus levels in the field. *Agric Ecosyst Environ* **103**:245–249.

Mugnier, J. and Mosse, B. 1987. Vesicular–arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. *Phytopathology* **77**:1045–1050.

Pfleger, F.L. and Linderman, R.G. (Eds.) 1994. Mycorrhizae and plant health. The American Phytopathological Society St Paul, MN: APS Press.

Riker, A.J., Banfield, W.M., Wright, W.H., Keitt, G.W. and Sagen, H.E. 1930. Studies on infectious hairy root of nursery apple trees. *J Agric Res* **41**:507-540.

Tepfer, D. 1989. Ri T-DNA from *Agrobacterium rhizogenes*: A Source of Genes Having Applications in Rhizosphere Biology and Plant Development, Ecology and Evolution In: Kosuge T, Nester EW (Eds.), Plant Microbe interactions. McGraw-Hill Publishing, New York, pp. 294-342.

Tepfer, D.A. and Tempe, J. 1981. Production d'agropine par des racines formees sous l'action d' *Agrobacterium rhizogenes*, souche A4. *C R Acad Sci Paris* **292**:153-156.

Voets, L., de la Providencia, I.E., Fernandez, K., IJdo, M., Cranenbrouck, S. and Declerck, S. 2009. Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under in vitro conditions. *Mycorrhiza* **19**:347–356.

Voets, L., Dupré de Boulois, H., Renard, L., Strullu, D.G. and Declerck, S. 2005. Development of an autotrophic culture system for the in vitro mycorrhization of potato plantlets. *FEMS Microbiol Lett* **248**:111.