Chapter 18
Mycorrhizal Inoculants: Progress in Inoculant Production Technology

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Abstract Of the seven types of mycorrhizae, the symbiotic association of plants with arbuscular mycorrhizae (AM) and ectomycorrhiza (ECM) is the most abundant and widespread. Mycorrhizal inoculant technology, especially of AM and ECM, appears to be a promising avenue for sustainable agriculture and forestry because of their extensive and productive association with plants. Production of mycorrhizal inocula is a complex procedure that requires commercial enterprises to develop the necessary biotechnological skill and ability to respond to legal, ethical, educational, and commercial requirements. At present, commercial mycorrhizal inocula are produced in pots, nursery plots, containers with different substrates and plants, and aeroponic systems, and by nutrient film technique, or in vitro. Different formulated products are now marketed, which creates the need for the establishment of standards for widely accepted quality control. Generally, preparation and formulation of mycorrhizal inocula are carried out by applying polymer materials with well-established characteristics and which are useful for agriculture and forestry. The most commonly used methods involve entrapment of fungal materials in natural polysaccharide gels, which includes immobilization of mycorrhizal root pieces, vesicles, and spores, in some cases coentrapped with other plant-beneficial microorganisms. Efforts should be devoted toward registration procedures of mycorrhizal inoculants to stimulate the development of mycorrhizal products industry. Biotechnology research and development in such activities must be encouraged, particularly with regard to interactions of mycorrhizal fungi with other rhizosphere microbes, and selection of new plant varieties with enhanced mycorrhizal traits to provide maximum benefits to agriculture and forestry.

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18.1 Introduction

Mycorrhizal fungi form symbiotic relationships with plant roots in a fashion similar to that of root-nodule bacteria of legumes. Of the seven types of mycorrhizae documented (arbuscular, ecto-, ectendo-, arbutoid, monotropoid, ericoid, and orchidaceous mycorrhizae), arbuscular mycorrhizae and ectomycorrhizae are the most abundant and widespread (Smith and Read 1997; Allen et al. 2003). Arbuscular mycorrhizal (AM) fungi comprise the most common mycorrhizal association and form mutualistic relationships with over 80% of all vascular plants (Brundrett 2002). Ectomycorrhizal (ECM) fungi are also widespread in their distribution but are associated with only 3% of vascular plant families (Smith and Read 1997). These two groups of mycorrhizal fungi play an important role in sustainable agriculture and forestry (Siddiqui and Mahmood 1995; Akhtar and Siddiqui 2008; Futai et al. 2008; Siddiqui and Pichtel 2008; Akhtar et al. 2011). The production of commercial inocula of these fungi has been increasing, particularly in the last few years, due to the following:

1. Their positive impact on plant health and development, land reclamation, phytoremediation, and disease management,
2. Increased awareness about biodiversity, concerns about soil microbial communities, and acceptance of mycorrhizal inoculants as an alternative to agrochemicals, and
3. Greater emphasis by society toward sustainable agriculture and forestry.

Production of mycorrhizal inoculants is a complex process that requires development of the necessary biotechnological expertise along with related legal, ethical, educational, and commercial requirements.

18.2 Inocula Production of AM Fungi

AM inoculants are marketed today in varied formulations. Some companies market a single strain of mycorrhiza mixed with a carrier. Others sell liquids, powders, and tablets, and most sell cocktails containing a variety of organisms. AM in spore form alone is a poor inoculant; for improved results, AM fungi containing spores, root fragments, and hyphae are superior to those containing only spores. Of greater importance for mycorrhizal inoculants is the degree of infectivity present. Effectivity of an inoculant depends on how rapidly it can colonize the root system. The species used in the inoculant should be effective over a wide range of plant species, pH levels, and soil types. The main obstacle to producing substantial quantities of AM inocula is their obligate nature; this continues to be a major limitation. Two major systems for AM inocula production are (1) soil-based systems and (2) soil-less techniques.

18.2.1 Soil-Based Systems

The inoculum propagation process entails the following stages: (1) isolation of AMF pure culture strain, (2) choice of host plant, and (3) optimization of growing conditions. The soil-based system has been adapted to reproduce different AM strains for increasing propagule numbers in situ (Menge 1984). AM development and its influence on the host are at least partially under genetic control (Gianinazzi-Pearson et al. 1996). Mycorrhizal development is affected by nutrient availability in soil and the inoculum potential of AM fungus. For propagation of AM fungi using the soil-based system, starting fungal inocula usually composed of spores and colonized root segments are incorporated into a growing substrate for plant seedling production (Brundrett et al. 1996). The fungi become established and spread within the substrate and colonize the root seedlings. Both colonized substrates and roots then serve as mycorrhizal inocula. Bagyaraj (1992) found that a mixture of perlite and Soilrite mix (1:1 v/v) was the optimal substrate and *Chloris gayana* (Rhodes grass) the optimal host for mass propagation of mycorrhizal inocula. In addition, pesticides captan and Furadan added to the pot cultures at half the recommended level checked other microbial contaminants with no effect on the mycorrhizal fungi. This technique is very useful for the production of "clean" mycorrhizal inoculum (without other microbial contaminants) with high potentiality in a short span of time.

Douds et al. (2010) have suggested on-farm production of AM fungus to benefit vegetable farmers. perlite-, vermiculite-, and peat-based potting media were tested as diluents of yard clipping compost for media in which the inoculum was produced on *Paspalum notatum* Flugge. All substrates produced satisfactory numbers of AM fungus propagules, though vermiculite proved superior to other potting media (89 vs. 25 propagules cm^{-3}, respectively). Adoption of on-farm production of AM fungal inoculum by growers requires a greater degree of flexibility than that present in the method described earlier (Douds et al. 2006). The original method requires that compost be diluted with vermiculite and that the starter inoculum be in the form of purchased *P. notatum* seedlings colonized by specific isolates of AM fungi. These characteristics are restrictive, particularly the latter. Experiments with perlite-, vermiculite-, and peat-based potting media demonstrated that these restrictions are readily overcome (Douds et al. 2010).

The trap plants commonly used for pot culture of AM fungi are *Sorghum halepense*, *Paspalum notatum*, *Panicum maximum*, *Cenchrus ciliaris*, *Zea mays*, *Trifolium subterraneum*, *Allium cepa*, and *Chloris gayana* (Chellappan et al. 2001; Bagyaraj 1992). The inoculum consists of spores, hyphal segments, and infected root pieces and generally takes 3–4 months to produce on host plants. The practice of pot culture has certain drawbacks that include limited quantities of inocula, bulky nature of inocula, transport problems, risk of contamination, presence of impurities, and lack of genetic stability of inocula (Abdul-Khaliq et al. 2001). Large-scale production of AMF inoculum requires control and optimization of both

host growth and fungal development. The microscopic size of AMF together with complex identification processes contributes to the pitfalls of inoculum propagation.

18.2.2 Soil-Less Techniques

18.2.2.1 Aeroponic Culture

Soil-less culture systems such as aeroponic cultures enable production of spores with limited contamination and facilitate uniform nutrition of colonized plants (Jarstfer and Sylvia 1999). In aeroponic cultures, pure and viable spores of a selected fungus are used to inoculate the cultured plants, which are later transferred into a controlled aeroponic chamber (Singh and Tilak 2001). A fine mist of a well-defined nutrient solution is applied to the roots of the host plant in aeroponic culture. Mycorrhizal cultures have been established successfully using this system (Weathers and Zobel 1992; Mohammad et al. 2000). Three basic methods for producing atomized nutrient solution are as follows:

1. An impeller system making use of an atomizing disk (Zobel et al. 1976),
2. Pressurized spray through nozzles, and
3. Ultrasonically generated fog (Weathers and Zobel 1992).

The fine mist of nutrient solution is required for successful aeroponic culture. Standardization of droplet size is needed so that drops attach to the root system for an adequate time period. Generally, a 45-mm droplet size is optimum; modified Hoagland solution (Epstein 1972) has been used for cultivation of Bahia grass and sweet potato (Wu et al. 1995; Hung and Sylvia 1988). Lack of substrate ensures extensive root growth, colonization and sporulation of the fungus and makes it an ideal system for obtaining sufficient amounts of clean AM fungus propagules (Abdul-Khaliq et al. 2001).

18.2.2.2 Monoxenic Culture

The successful propagation of some AM fungal strains on root organ culture has allowed the cultivation of monoxenic strains that can be used either directly as inoculum or as starting inoculum for large-scale production (Fortin et al. 2002). In vitro bulk production of AMF inoculum is promising, offering clean, viable, contamination-free fungi. The cost of in vitro inoculum may appear prohibitive compared to the cost of greenhouse-propagated inoculum, but its use is a warranty of purity. In vitro production provides research and industry scientists with pure and reliable material for starting inoculum production for both fundamental research and applied technologies (Dalpe 2004). Mass production of AM fungi has

been achieved with several species with increased spore production on monoxenic cultivation. Chabot et al. (1992) produced 25 spores/ml during a 4-month incubation time. St-Arnaud et al. (1996) produced 1,000 spores/ml in 3–4 months. Similarly, Douds (2002) produced 3,250 spores/ml in 7 months, while Adholeya (2003) produced 3,000 spores/ml in 3 months through monoxenic-based inoculum production.

Agrobacterium rhizogenes, a Gram-negative soil bacterium, produces hairy roots and allows roots to grow rapidly on artificial media (Abdul-Khaliq et al. 2001). Once the hairy roots are ready, the collected AM inoculum is surface-sterilized using a suitable surfactant solution. Generally, Tween 20 and a solution choramine T are used for sterilization of AM spores (Fortin et al. 2002). The spores are subsequently rinsed in streptomycin–gentamycin solution (Becard and Piche 1992). The rinsed spores should be stored at 4°C in distilled water or water agar, or on 0.1% $MgSO_4$ $7H_2O$ solidified with gellan gum (Fortin et al. 2002). The nutrient media should be carefully selected to allow growth of the host as well as the fungus. Since roots require rich nutrient medium for growth, AM fungi require a relatively poor nutrient medium (Abdul-Khaliq et al. 2001). Generally, Murashige and Skoog's medium (1962) and White's medium are used for dual culture of host root and AM fungus. Regardless of the high technological investment and high cost, not all AM fungi are successfully culturable in this system. Additionally, the suitability of inoculum produced in vitro, in particular its competitive ability toward other microbes in field soil, has yet to be tested.

18.2.2.3 Nutrient Film Technique

Nutrient film technique (NFT) is a specialized technique developed for commercial production of crops that entails continuous recycling of a large volume of nutrient liquid over a film, which flows over plant roots. The major concern in NFT is the concentration of nutrients. The requirements of nutrient elements vary from one particular mycorrhizal system to another depending upon the size, physiological requirements, and other features of the plants (Sharma et al. 2000). It is necessary to maintain the nutrient solution in the form of a thin film (5 mm to 1 cm). Chemical forms of nutrient elements also affect mycorrhizal infection. Therefore, it is desirable to use a balanced and proper composition.

Low sporulation can be obtained compared to soil-based systems. Problems of contamination by undesirable organisms like rotifers, protozoans, and eelworms are expected because of the common nutrient solution used. The inoculum produced by the NFT method is ideal for the production of easily harvestable solid mats of roots with more concentrated and less bulky forms of inoculum than that produced by plants grown in soil-based or other solid media (Abdul-Khaliq et al. 2001; Chellappan et al. 2001).

18.2.2.4 Polymer-Based Inoculum

It is desirable to apply inoculants to soil with a carrier that can provide physical protection and nutrients for microbial cells (Gentry et al. 2004). For preparation of microbial inoculants, the key issues include microbial selection and characterization, mass production of target microorganisms, selection of carrier material, microbial behavior after formulation, and effectiveness and competitiveness after application (Vassilev et al. 2005).

The simplest method of applying polymer materials is based on the use of hydrogels. Several hydrogels have been used as carriers of AM fungi (Johnson and Hummel 1985; Nemec and Ferguson 1985); however, pH extremes of gel materials have imparted adverse effects on spore germination and root colonization (Vassilev et al. 2005). Entrapment or encapsulation of microbial cells in polymer materials is a highly successful method of immobilization. This method involves entrapment of cells or spores within porous structures, which are formed in situ around the biological material. The carrier should be relatively economical and compatible with the materials that are used for the production of product. The preferred carrier materials include natural polysaccharides and various hydrophilic hydrogels. Various combinations of natural, semisynthetic, and synthetic polymers are available, but the majority incorporates natural polysaccharides including kappa carrageenan, agar, and alginates. Calcium alginates are the most widely used carrier of about 1,350 combinations of carriers in use (Vassilev et al. 2005). The encapsulation of AM fungi produced monoxenically in alginate beads offers the possibility to diversify the inoculation process (Diop 2003). It would be useful to incorporate flavonoids into the capsules (Bécard and Piché 1989; Gianinazzi-Pearson et al. 1989). Some commercially prepared AM inoculants are listed in Table 18.1.

18.2.2.5 Integrated Method

One of the reasons for lower survival and establishment of micropropagated plants during transplantation is the absence of natural associates (Varma and Schuepp 1995). Use of mycorrhizae helper bacteria (MHB) promotes AM symbiosis in various crop plants (Von 1998). The role of MHBs in growth and development of different AM fungi was reported by several workers (Siddiqui and Mahmood 1998; Vosatka et al. 1999). Combined and judicious use of AM fungi and plant-growth-promoting rhizobacteria (PGPR) can provide proper establishment of in vitro propagated plantlets under field conditions. Bhowmik and Singh (2004) reported that PGPR considerably enhanced mycorrhizal colonization and can be used in mass production of AM fungal cultures. da Silva et al. (2007) observed production and infectivity of inoculum of AM fungi multiplied in substrate supplemented with Tris–HCl buffer. Sporulation of AM fungi was also improved in solution with buffer. Large-scale production of inoculum can be obtained by addition of Tris–HCl buffer in nutrient solution and storage at 4°C (da Silva et al. 2007).

Interactions of nitrogen fixers and P-solubilizers with AM fungi have been suggested as one reason for improved growth of many plant species (Turk et al. 2006),

Table 18.1 Commercial AM fungi inoculants produced by different companies

Product	Type of mycorrhiza	Web address for detailed information
AgBio-Endos	Endomycorrhizal inoculant	http://www.agbio-inc.com/agbio-endos.html
Rhizanova™	Endomycorrhizae	http://www.arthurclesen.com/resources/Rhizanova%20Overview%20Sheet.pdf
Bio/Organics	Endomycorrhizal inoculant	http://www.biconet.com/soil/BOmycorrhizae.html
Endorize	Mycorrhizal product	http://www.agron.co.il/en/Endorize.aspx
BuRize	VAM inoculant	http://www.biosci.com/brochure/BRZBro.pdf
Cerakinkong	VA mycorrhizal fungi	http://www.cgc-jp.com/products/microbial/
MYCOgold	AM fungi	http://www.alibaba.com/product/my100200874-100160217-0/Mycogold_Crop_Enhancer_Bio_Fertilizer_.html
BIOGROW Hydo-sol	Endomycorrhizae	http://www.hollandsgiants.com/soil.html
Mycor	Endo/ectomycorrhizae	http://www.planthealthcare.co.uk/pdfs/mycorflyer.pdf
PRO-MIX 'BX'	Endomycorrhizal fungi	http://www.premierhort.com/eProMix/Horticulture/TechnicalData/pdf/TD2-PRO-MIXBX-MYCORISE.pdf
AM 120	Microbial inoculant	http://www.ssseeds.com/other_products.html
BioVAm	Mycorrhizal powder	http://www.harbergraphics.com/Biovam/index.html
Diehard™	Endodrench	http://www.horticulturalalliance.com/DIEHARD_Endo_Drench.asp
MYCOSYM	Mycorrhiza Vitalizer	http://www.mycosym.com/Documents/Flyer%20Olive%20and%20Verticilosis%20WEB.pdf

and these associations are useful in improving survival rates of micropropagated plants (Webster et al. 1995). Microorganisms such as *Frankia*, *Rhizobium,* and *Bradyrhizobium* improve soil-binding capacity, stability, and properties making soil conducive for the establishment of micropropagated plantlets as that of mycorrhiza (Varma and Schuepp 1995).

18.3 Storage of AM Inocula

Propagules of AM fungi must be used immediately once they are extracted or produced. Propagules obtained from soil-less propagation generally have the same requirements for immediate use as those produced in soil-based media. Factors that predispose propagules to higher mortality are harvesting pots, when they are moist, and chopping roots. Chopping roots and mixing contents should be carried out only

just prior to inoculum usage. Conditions for successful long-term storage of AM propagules remain vaguely defined. Spores are generally air-dried and then stored at 4°C. Temperate isolates can be stored at 4–10°C, whereas tropical isolates should be stored at 20–25°C. Feldmann and Idczak (1992) observed that the infectivity of *Glomus etunicatum* stored at 20–23°C and 30–50% relative humidity for 3 years was reduced by only 10–15%.

Fungal viability and mycorrhizal efficiency can be maintained for several months at room temperature (20–25°C), especially when semidry inocula are stored in plastic containers or packaging. Long-term storage (up to 1–2 years) may be conducted at 5°C. More sophisticated and expensive preservation techniques are performed by research institutions. These include the maintenance of inocula on a living plant host grown on sterile growth substrate with regular checks for monospecificity of the cultivated strains, storage in liquid nitrogen (Douds and Schenck 1990), and freeze-drying under vacuum. Kim et al. (2002) reported that cold storage of mixed inoculum enhanced colonization and growth-promoting activity of *G. intraradices* compared to freshly prepared inoculum.

18.4 Inocula Production of Ectomycorrhizal Fungi

The successful application of ECM fungi in plantation forestry depends on the availability of a range of fungi capable of improving the economics of tree production in various environments, and the ability to supply the fungi as inocula (Kuek et al. 1992). Inocula of ECM fungi are usually composed of biomass and carrier material. Many existing or advocated types of inocula only partially satisfy these criteria (Kuek et al. 1992). Three main types of ectomycorrhizal inoculants have been used in nurseries during the last few decades: soil, fungal spores, and vegetative mycelia. Fungal spores obtained from fruiting bodies harvested in natural forests, old nurseries, or established plantations have been used in many parts of the world (Theodorou 1971). They are easy to obtain and apply to plants. Effect of *Scleroderma* on colonization and growth of exotic *Eucalyptus globulus*, *E. urophylla*, *Pinus elliottii*, and *Pinus radiata* was studied (Chen et al. 2006). The results suggest that there is a need to source *Scleroderma* from outside China for inoculating eucalypts in Chinese nurseries, whereas Chinese collections of *Scleroderma* could be used in pine nurseries (Chen et al. 2006).

On the other hand, Lamb and Richards (1974a, b) demonstrated that chlamydospores were less effective than basidiospores as inoculum, and there were significant differences in yield by different fungal species at high inoculum densities and in the presence of added phosphate. Generally, fungal spores are small (ca. 10 μm in length; Clémençon et al. 2004) and are usually produced in large amounts (e.g., 1×10^8 – 1×10^9 spores per sporocarp in *Suillus bovinus*; Dahlberg and Stenlid 1994), enabling long-distance (e.g., intercontinental; Nagarajan and Singh 1990) dispersal by wind or animals (Allen 1991; Ishida et al. 2008). However, basidiospores of most ECM require special environmental conditions for germination,

which are still unknown for many species. Spores of only a few species have been germinated under controlled conditions, a necessary prerequisite to obtain monosporus mycelia to perform mating tests (Martín and Gràcia 2000).

Among ectomycorrhizal basidiomycetes, three main types of germination activators have been reported (Fries 1987): (1) nonectomycorrhizal microorganisms such as colonies of the yeast *Rhodotorula glutinis* (Fries 1976, 1978), the filamentous fungus *Ceratocystis fagacearum* (Oort 1974), and some bacterial isolates obtained from sporophores, mycorrhizae, or soil (Ali and Jackson 1988), (2) a mycelium of the same species as the spores (Fries 1978; Iwase 1992), and (3) roots of higher plants (Melin 1962; Kope and Fortin 1990). Generally, germination activation is caused by some stimulation, such as those from exudates from microorganisms or root exudates. These exudates presumably contain compounds possessing the capacity to trigger spore germination (Kikuchi et al. 2007). Kikuchi et al. (2006) showed that spores of the ectomycorrhizal fungus *Suillus bovinus* germinated through the combination of activated charcoal treatment of media and coculture with seedlings of *Pinus densiflora*. Moreover, they showed that flavonoids play a role as signaling molecules in symbiotic relationships between woody plants and ectomycorrhizal fungi (Kikuchi et al. 2007).

Submerged cultivation of ectomycorrhizal fungi is a convenient technique that has many advantages in relation to solid-state fermentation, viz., a higher viability and biomass productivity, smaller volumes of inoculants, and lower cost compared with other cultivation methods. Inoculant production may be achieved using small bioreactors, and bioreactor cost may be minimized by the adoption of pneumatic reactors such as airlift systems, whose construction and maintenance are less expensive than those of conventional stirred-tank bioreactors. The mycelia produced in submerged culture should be immobilized in alginate gel or other polymeric carriers to maintain viability during storage and after inoculation in the nursery. The application of such alginate-immobilized inoculant is easy and inexpensive.

In order to achieve optimum performance of large-scale bioreactors for inoculant production, it is essential to undertake biochemical and physiological studies of the growth and nutrition of the fungi involved. Only then is it possible to obtain ectomycorrhizal fungal inoculants of high quality at an acceptably low cost and in quantities sufficient to meet the needs of the forest industry (Rossi et al. 2007).

In the production of vegetative ectomycorrhizal inoculants, the selection of mycorrhizal fungi and suitable carrier is important as is the survival and development of inoculant ectomycorrhizal fungi on roots. Techniques for inoculation with pure cultures of selected mycorrhizal fungi have been developed for quasi-operational use by many investigators. Unfortunately, it is the common experience of mycorrhiza researchers worldwide that many mycorrhizal fungi grow poorly or not at all in the pure culture methods attempted thus far. Thus, the practical use of mycelial culture inoculum is limited at present. Fortunately, some of the fungi that grow well in culture have also proven highly beneficial to survival and growth in outplanted stock (Trappe 1977). The ectomycorrhizal fungal genus *Lactarius* has

been intensively marketed in Europe, Asia, and northern Africa, especially the choice edible species *Lactarius deliciosus* and *Lactarius sanguifluus*. *Lactarius* forms ectomycorrhizae with a variety of host plants (Trappe 1962; Hutchison 1999). Some pure culture inoculation studies demonstrate that this species readily colonizes the root system of pines under aseptic conditions. Guerin-Laguette et al. (2000) obtained fruiting body primordia of *L. deliciosus* 1 year after inoculation of *Pinus sylvestris* seedlings in growth pouches and subsequently transferred them to containers. The *L. deliciosus* could be effectively used for controlled mycorrhizal plant production in nurseries as has been successfully done with other ectomycorrhizal fungi. Parladé et al. (2004) described different methods for inoculating seedlings of *Pinus pinaster* and *P. sylvestris* with edible *Lactarius* species under standard greenhouse conditions. All the inoculation methods tested, except the alginate-entrapped mycelium, were appropriate for the production of seedlings colonized with *L. deliciosus*. However, the percentage of colonized plants and the degree of colonization observed were highly variable depending on the inoculation method and the plant-fungal strain combination.

Because of their characteristic odor, flavor, and texture, "matsutake" mushrooms (fruiting bodies or sporocarps) of the ectomycorrhizal fungus *Tricholoma matsutake* are the most sought-after and expensive mushrooms in Japan. Recently, the annual harvest of matsutake mushrooms has declined dramatically as the result of *P. densiflora* trees dying from pine wilt disease caused by the nematode *Bursaphelenchus xylophilus*. Deforestation and modern forestry management practices have also been detrimental to matsutake growth (Wang et al. 1997; Gill et al. 2000). Despite nearly a century of research (Ogawa 1975a, b, 1977), attempts to cultivate matsutake have been unsuccessful. Yamada (1999) reported on the ability of *T. matsutake* isolates to form mycorrhizae using aseptic seedlings of *P. densiflora in vitro*. They germinated pine seeds aseptically on a nutrient agar medium, and pairs of 1-week-old seedlings were transplanted into polymethylpentene bottles containing autoclaved *Sphagnum* moss/vermiculite substrate. The substrate was saturated with nutrient medium containing glucose. At the same time, the bottles were inoculated with a *T. matsutake* isolate. The cultured *T. matsutake* mycelium formed true ectomycorrhizae with *P. densiflora* seedlings in vitro. Moreover, innovative inoculation techniques such as the recent "matsutake sheet" technique (Yoshimura 2004) could be helpful for the inoculation of mature trees in forest ecosystems and could be extended to other late-stage edible mycorrhizal fungi, such as *Boletus edulis*, *Cantharellus cibarius*, and *Amanita caesarea*, which have thus far not been domesticated. Guerin-Laguette et al. (2005) described successful inoculation of mature pine with *T. matsutake* using long root segments (ca. 5–10 mm diameter, 50 cm length) of 50-year-old *Pinus densiflora* trees; the long root segments were excavated, washed, auxin-treated (2–5 mg indole butyric acid, IBA, per root), and incubated in moist *Sphagnum* moss. After 12 months, short roots were regenerated of which approximately 90% were free of mycorrhizae. The mycorrhiza-free short roots were inoculated with mycelial pieces of *T. matsutake* and incubated further in a sterilized substrate. Four-and-a-half months later, roots putatively colonized by matsutake were

sampled near the inoculation points. The authors proposed that the localized inoculation technique was a key step in obtaining early-stage matsutake symbiotic structures *in situ* on a mature tree. Future work should focus on scaling up the inoculation trials *in situ* and on monitoring the persistence of matsutake mycorrhiza (Guerin-Laguette et al. (2005).

18.4.1 Formulation of ECM

In fact, the selection of an appropriate carrier is an important step in the development of a process for inoculant production. The mycelium in the inoculant must remain viable between the time of sowing and the time when receptive roots are formed. The nascent mycelium must resist adverse conditions such as drought, microbial antagonism, or predation by insects and other arthropods (Rossi et al. 2007). In studies to achieve a higher quality of inoculum and an improved production process. Krupa and Piotrowska-Seget (2003) used an alginate-immobilized inoculum of of mycorrhizal fungi to introduce the fubgi to the soil. They reported that the total concentration of cadmium in contaminated soil inoculated with ECM fungi was lower then in non-inoculated soil. As well, Kropáček et al. (1990) reported that they used mycelia of ECM fungi immobilized in alginate gel in a mixture with a silicate carrier-perlite. This inoculum was applied at sowing in forest nurseries to obtian resistant plants for afforestation of areas exposed to man-made stresses. Under both sterile and nonsterile conditions, the growth of seedings and mycorrhiza development were increased by inoculation with a strain Laccaria laccata. These formulation of ECM offers great flexibility as it allows addition of chemical additives to improve gel stability and conserve the inoculant (Mauperin et al. 1987). Inoculant beads can remain viable for several months under refrigeration, although the results vary between fungal species. *Hebeloma westraliense* and *Laccaria laccata* are relatively stable inoculants for more than 5 months; in contrast, the viability of *Elaphomyces* decreased to 40% after 1-month storage (Kuek et al. 1992).

An advantage of alginate gel is the possibility of preparing a multimicrobial inoculant. Douglas fir (*Pseudotsuga menziesii*) seedlings in two bare-root forest nurseries were inoculated with the ectomycorrhizal fungus *L. laccata*, together or not with one of five mycorrhiza helper bacteria isolated from *L. laccata* sporocarps or mycorrhizae and previously selected by in vitro and glasshouse screenings (Duponnois and Garbaye 1991). A dual inoculum composed of calcium alginate beads containing the two microorganisms was a valuable option for increasing the efficiency of ectomycorrhizal inoculation of planting stocks in forest nurseries.

Despite clear evidence from small-scale experiments that ectomycorrhizal fungi improves growth of the host plant, the use of inoculation in plantation forestry is not widespread. In contrast to arbuscular mycorrhizal inoculants, only relatively few ectomycorrhizal fungal inoculants have been commercialized (Table 18.2).

Table 18.2 Commercial ectomycorrhizal fungi inoculants produced through different processes by different companies (Rossi et al. 2007)

Commercial product	Type/process	Company, location
BioGrow Blend®	Spores	Terra Tech, LLC
MycoApply®-Ecto	Spores	Mycorrhizal Applications, Inc.
Mycorise Pro Reclaim®	Propagules ecto+endo	Symbio Technologies, Inc.
Myke® Pro LF3	Propagules	Premier Tech Biotechnologies
Mycor Tree®	Spores	Plant Health Care, Inc.
MycoRhiz®	Mycelium/Solid-state fermentation	Abbott Laboratories
Somycel PV	Mycelium/Solid-state fermentation	INRA-Somycel S.A.
Ectomycorrhiza Spawn	Mycelium/Solid-state fermentation	Sylvan Spawn Laboratory, Inc.
–	Mycelium/Submerged	Rhone Poilenc-INRA
Mycobead®	Mycelium/Submerged	Biosynthetica Pty. Ltd.

18.4.2 Storage of ECM

Ectomycorrhizal fungi are usually maintained by subculturing at approximately 25°C. Ito and Yokoyama (1983) and Jong and Davis (1987) demonstrated that some ectomycorrhizal fungi are preserved by freezing. Corbery and Le Tacon (1997) showed that the survival of ectomycorrhizal fungi after freezing at −196 or −80°C depends on cooling rate and species or strain. The optimum rate of cooling for ECM is −1°C per min. *Thelephora terrestris* and *Paxillus involutus* did not survive any freezing method. The resistance of *Cenococcum geophilum* to freezing may be related to its tolerance to water stress and high salinity. Hung and Molina (1986) reported that, in general, fresh inocula of *Laccaria laccata* and *Hebeloma crustuliniforme* were most effective; their effectiveness remained high for a month of storage and then declined rapidly for a short period, then slowly to the point of no mycorrhiza formation. The effectiveness declined more rapidly with lower inoculation rates. Storage at 2°C prolonged inoculum viability for at least 2 months over that of 21°C storage. Inoculum from different fungal species or isolates within a species responded to storage temperatures differently. *Pisolithus tinctorius* inoculum was the most sensitive: 1-month storage strongly reduced its effectiveness. The difference between 2 and 21°C storage was more obvious in *H. crustuliniforme* than in either isolate of *L. laccata*.

Tibbett et al. (1999) described a method for maintaining viable cultures of ectomycorrhizal *Hebeloma* strains in cold liquid culture medium. Isolates of *Hebeloma* spp., collected over a wide geographic range, were stored at 2°C for 3 years. All cultures survived this storage period and showed a greater time period and success rate than have previously been reported for the long-term storage of ectomycorrhizal basidiomycetes. Rodrigues et al. (1999) studied the viability of fragmented mycelia of *Pisolithus tinctorius* and *Paxillus involutus* entrapped in calcium alginate gel to determine the efficacy of producing ectomycorrhizal fungus inoculum. Fungi were grown in modified Melin-Norkrans (MMN) solution at 28°C before being fragmented in a blender and subsequently entrapped in

calcium alginate. *Paxillus involutus* mycelium was more than 90% viable when entrapped mycelia were 10–50 days old, and *Pisolithus tinctorius* attained its highest viability (55%) for 20- to 40-day-old mycelia. Gel-entrapped *Paxillus involutus* mycelium grew well at all temperatures after 30-day storage, but viability significantly decreased after 60-day storage at 6°C on dry filter paper. For gel-entrapped *Pisolithus tinctorius* mycelia, viability was greatest when stored at 25°C in 0.7 M $CaCl_2$. Entrapment of *Paxillus involutus* fragmented mycelia in calcium alginate beads under the conditions that they propose can be used successfully to produce inoculum. Lehto et al. (2008) grew isolates of *Suillus luteus*, *Suillus variegatus*, *Laccaria laccata*, and *Hebeloma* sp. in liquid culture at room temperature. Subsequently, they exposed samples to a series of temperatures between +5 and −48°C. Relative electrolyte leakage (REL) and regrowth measurements were used to assess damage. The REL test indicated that the lethal temperature for 50% of samples (LT_{50}) was between −8.3 and −13.5°C. However, in the regrowth experiment, all isolates resumed growth after exposure to −8°C and higher temperatures. As high as 64% of *L. laccata* samples, but only 11% in *S. variegatus*, survived at −48°C. There was no growth of *Hebeloma* and *S. luteus* after exposure to −48°C, but part of their samples survived −30°C (Lehto et al. 2008).

Here, we describe inoculant technologies; however, there is currently limited information regarding commercialized products. Therefore, the advent of inoculation technology on a broad scale is necessary, and the overall scientific evidence is important for justifying its use in increasing the economic productivity of forest plantations (Kuek 1994).

18.5 Discussion

Inoculation of plants with mycorrhizal fungi increases the survival and growth rates of seedlings and cuttings in greenhouse and natural conditions. The inoculation also improves the acclimatization of *in vitro* micropropagated plants and promotes earlier flowering and fruiting. These results have arisen because mycorrhizal plants are more efficient in the uptake of specific nutrients and more resistant to diseases caused by soil-borne pathogens. Inoculation of plants with mycorrhizae offers the possibility of reducing fertilizer and pesticide applications. Therefore, mycorrhizal inoculants are gaining popularity as “biofertilizers,” “bioprotectors,” and “biocontrol agents,” and the industry of mycorrhizal inoculum production is expanding worldwide.

To lower the risk of contamination by pathogenic organisms, crops are usually grown in soil-less potting mixes containing different ratios of perlite, vermiculite, peat moss, and composted forest products. Soil-less media also have a lower bulk density and provide better aeration and a higher water-holding capacity than do mineral soils. These artificial rhizosphere conditions may be advantageous to achieve rapid plant growth; however, their effects on mycorrhizal colonization are not well understood. The unpredictability of soil-less media to promote mycorrhizal

colonization can further be confounded by the multiple additives occurring in commercial mycorrhizal inoculants including carriers, fertilizers, humic acid, and soil conditioners. It is necessary to test the infectivity of commonly available commercial mycorrhizal inoculants in standard practices and to analyze plant growth response to inoculation with these products.

Entrepreneurs are currently developing inoculum production systems and marketing mycorrhiza. Still, however, technical difficulties exist for large-scale utilization of mycorrhizal inocula; additionally, numerous legal, ethical, and economical aspects of this technology must be addressed. It is important to fill gaps in fundamental knowledge and to optimize maintenance and application of mycorrhizal fungi in plant production systems. Producers and distributors of inocula should convince users that this technology is economically feasible. More applied studies are needed to aid food and plant production, particularly where sustainable methods of agriculture or horticulture are developing. Moreover, awareness on the part of the public must be encouraged regarding the potential of mycorrhizal technology for sustainable plant production and soil conservation.

References

Abdul-Khaliq, Gupta, M.L., Alam, A. 2001. Biotechnological approaches for mass production of arbuscular mycorrhizal fungi: current scenario and future strategies. In: Mukerji, K.G., Manoharachary, C., Chamola, B.P. (eds.), Techniques in Mycorrhizal Studies. Kluwer Academic Publishers, The Netherlands, pp. 299–312.

Adholeya, A. 2003. Commercial production of AMF through industrial mode and its large scale application. In: Proc. 4th Int. conf. Mycorrhizae (ICOM4), Montreal, Canada.

Akhtar, M.S. and Siddiqui, Z.A. 2008. Arbuscular mycorrhizal fungi as potential bioprotectants against plant pathogens. In: Siddiqui, Z.A., Akhtar, M.S., Futai, K. (eds.), Mycorrhizae: Sustainable Agriculture and Forestry. Springer, Dordrecht, The Netherlands, pp. 61–97, 362.

Akhtar, M.S., Siddiqui, Z.A. and Wiemken, A. 2011. Arbuscular mycorrhizal fungi and *Rhizobium* to control plant fungal diseases. Alternative farming systems, biotechnology, drought stress and ecological fertilization. Series Sustain. Agric. Rev. vol. 6 (Edit. Lichtfouse, E.) 390p. ISBN: 978-94-007-0185-49 (in press)

Ali, N.A. and Jackson, R.M. 1988. Effects of plant roots and their exudates on germination of spores of ectomycorrhizal fungi. Trans. Br. Mycol. Soc. 91: 253–260.

Allen, M.F. 1991. The Ecology of Mycorrhizae. Cambridge, UK: Cambridge University Press.

Allen, M.F., Swenson, W., Querejeta, J.I., Egerton-Warburton, L.M. and Treseder, K.K. 2003. Ecology of mycorrhizae: a conceptual frame work for complex interactions among plants and fungi. Annu. Rev. Phytopathol. 41: 271–303.

Bagyaraj, D.J. 1992. Mycorrhizal association in plants. In: Bagyaraj, D.J., Manjunath, A. (eds.), Manual of Mycorrhiza Technology. University of Agriculture Sciences, Bangalore, India.

Becard, G. and Piche, Y. 1992. Establishment of vesicular arbuscular mycorrhiza in root organ culture: review and proposed methodology. In: Norris, J., Read, D., Varma, A. (eds.), Techniques for the Study of Mycorrhiza. Academic Press, New York, NY, pp. 89–108.

Bécard, G. and Piché, Y. 1989. Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl. Enviro. Microbiol. 55:2320–2325.

Bhowmik S.N. and Singh C.S. 2004. Mass multiplication of AM inoculum: effect of plant growth-promoting rhizobacteria and yeast in rapid culturing of *Glomus mosseae*. Curr. Sci. 86: 705–709

Brundrett, M.C. Bougher, N., Dell, B., Grove, T, and Malajczuk, N. 1996. Working with mycorrhizas in forestry and agriculture. The Australian Center for International Agricultural Research. Monograph, Canberra, Australia. 375p.

Brundrett, M.C. 2002. Coevolution of roots and mycorrhizas of land plants. New Phytol. 154, 275–304.

Chabot, S., Becard, G. and Piche, Y. 1992. Life cycle of *Glomus intraradices* in root organ culture. Mycologia 84: 315–321

Chellappan, P., Christy, S.A.A. and Mahadevan, A. 2001. Multiplication of arbuscular mycorrhizal fungi on roots. In: Mukerji, K.G., Manoharachary, C., Chamola, B.P. (eds.), Techniques in Mycorrhizal Studies. Kluwer Academic Publishers, The Netherlands, pp. 285–297.

Chen, Y.L., Kang, L.H., Malajczuk, N. and Dell, B. 2006. Selecting ectomycorrhizal fungi for inoculating plantations in south China: effect of *Scleroderma* on colonization and growth of exotic *Eucalyptus globulus*, *E. urophylla*, *Pinus elliottii*, and *P. radiata*. Mycorrhiza 16: 251–259.

Clémençon, H., Emmett, V. and Emmett, E.E. 2004. Cytology and Plectology of the Hymenomycetes. Bibliotheca Mycologica. Vol 199. Berlin: J Cramer. 488 p.

Corbery Y. and Le Tacon F. 1997. Storage of ectomycorrhizal fungi by freezing. Ann. For. Sci. 54: 211–217

da Silva, F.S.B., Yano-Melo, A.M. and Maia, L.C. 2007. Production and infectivity of inoculum of arbuscular mycorrhizal fungi multiplied in a substrate supplemented with Tris-HCL buffer. Braz. J. Microbiol. 38: 752–755.

Dahlberg, A. and Stenlid, J. 1994. Size, distribution and biomass of genets in populations of *Suillus bovinus* (L.: Fr.) Roussel revealed by somatic incompatibility. New Phytol. 128: 225–234.

Dalpe, Y. 2004. Arbuscular mycorrhiza inoculum to support sustainable cropping systems, http://www.plantmanagementnetwork.org/pub/cm/review/2004/amfungi/.

Diop, T.A. 2003. *In vitro culture* of arbuscular mycorrhizal fungi: advances and future prospects. Afr. J. Biotechnol. 2: 692–697

Douds, D.D. and Schenck, N.C. 1990. Increased sporulation of vesicular-arbuscular mycorrhizal fungi by mainpulation of nutrient regimens. Appl. Environ. Microbiol. 56:413–418.

Douds, D.D. Jr. 2002. Increased spore production by *Glomus intraradices* in a split-plate monoxenic culture system by repeated harvest, gel replacement and re-supply of glucose to the mycorrhiza. Mycorrhiza 12: 163–167.

Douds, D.D. Jr., Nagahashi, G., Pfeffer, P.E., Reider, C. and Kayser, W.M. 2006. On-farm production of AM fungus inoculum in mixtures of compost and vermiculite. Bioresour. Technol. 97: 809–818.

Douds, D.D. Jr., Nagahashi, G. and Hepperly, P.R. 2010. On-farm production of inoculum of indigenous arbuscular mycorrhizal fungi and assessment of diluents of compost for inoculum production. Bioresour. Technol. 101: 2326–2330.

Duponnois, R. and Garbaye, J. 1991. Effect of dual inoculation of Douglas fir with the ectomycorrhizai fungus *Laccaria laccata* and mycorrhization helper bacteria (MHB) in two bare-root forest nurseries. Plant Soil 138: 169–176.

Epstein, E. 1972. Mineral Nutrition of Plants: Principles and Perspectives. Wiley, New York, NY. p 412.

Feldmann, F. and Idczak, E. 1992. Inoculum production of vesicular arbuscular mycorrhizal fungi for use in tropical nursery. In: Nooris, J.R., Read, D.J., Varma, A.K. (eds.), Methods in Microbiology, vol. 24. Academic Press, London, pp. 339–357.

Fortin, J.A., Becard, G., Declerck, S., Dalpe, Y., St-Arnaud, M., Coughlan, A.P. and Piche, Y. 2002. Arbuscular mycorrhiza on root-organ cultures: A review. Can. J. Bot. 80: 1–20.

Fries, N. 1976. Spore germination in Boletus induced by amino acids. Proc. K. Ned. Akad. Wet. Ser. C 79: 142–146.

Fries, N. 1978. Basidiospore germination in some mycorrhiza-forming hymenomycetes. Trends. Br. Mycol. Soc. 70: 319–324.

Fries, N. 1987. Somatic incompatibility and field distribution of the ectomycorrhizal fungus *Suillus luteus* (Boletaceae). New Phytol. 107: 735–739.

Futai, K., Taniguch, T. and Kataoka, R. 2008. Ectomycorrhizae and their importance in forest ecosystem. In: Siddiqui, Z.A., Akhtar, M.S., Futai K. (eds.), *Mycorrhizae:* Sustainable Agriculture and Forestry. Springer, Dordrecht, The Netherlands, pp. 241–285, 362.

Gentry, T.J., Rensing, C. and Pepper, I. 2004. New approaches for bioaugmentation as a remediation technology. Crit. Rev. Environ. Sci. Technol. 34: 447–494

Gianinazzi-Pearson, V., Branzanti, B. and Gianinazzi, S. 1989. *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7: 243–255.

Gianinazzi-Pearson, V., Dumas-Gaudot, E., Gollotte, A., Tahiri-Alaoui, A. and Gianinazzi, S. 1996. Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. New Phytol. 133: 45–57.

Gill, W.M., Guerin-Laguette, A., Lapeyrie, F. and Suzuki, K. 2000. Matsutake – morphological evidence of ectomycorrhizal formation between *Tricholoma matsutake* and host roots in a pure *Pinus densiflora* forest stand. New Phytol. 147: 381–388.

Guerin-Laguette, A., Plassard, C. and Mousain, D. 2000. Effects of experimental conditions on mycorrhizal relationships between *Pinus sylvestris* and *Lactarius deliciosus* and unprecedented fruit-body formation of the saffron milk cap under controlled soilless conditions. Can. J. Microbiol. 46: 790–799.

Guerin-Laguette, A., Matsushita, N., Lapeyrie, F., Shindo, K. and Suzuki, K. 2005. Successful inoculation of mature pine with *Tricholoma matsutake*. Mycorrhiza 15: 301–305.

Hung, L.-L. and Molina, R. 1986. Temperature and time in storage influence the efficacy of selected isolates of fungi in commercially produced ectomycorrhizal inoculum. For. Sci. 32: 534–545.

Hung, L.L. and Sylvia, D.M. 1988. Inoculum production by vesicular-arbuscular mycorrhizal fungi in aeroponic culture. Appl. Environ. Microbiol. 54: 353–357.

Hutchison, L.J. 1999. Lactarius. In: Cairney, J.W.G., Chambers, S.M. (eds.), Ectomycorrhizal Fungi: Key Genera in Profile. Springer, Berlin Heidelberg New York, pp. 269–285.

Ishida, T.A., Nara, K., Tanaka, M., Kinoshita, A. and Hogetsu, T. 2008. Germination and infectivity of ectomycorrhizal fungal spores in relation to their ecological traits during primary succession. New Phytol. 180: 491–500.

Ito, T. and Yokoyama, T. 1983. Preservation of basidiomycete cultures by freezing. IFO Res. Commun. 11: 60–70.

Iwase, K. 1992. Induction of basidiospore germination by gluconic acid in the ectomycorrhizal fungus *Tricholoma robustum*. Can. J. Bot. 70: 1234–1238.

Jarstfer, A.G. and Sylvia, D.M. 1999. Aeroponic culture of VAM fungi. In: Varma, A., Hock, B. (eds.), Mycorrhiza. Structure, Function, Molecular Biology and Biotechnology. 2nd edition. Springer, Berlin, pp. 427–441.

Johnson, C.R. and Hummel, R.L. 1985. Influence of mycorrhizae and drought stress on growth of Poncirus x Citrus seedlings. HortScience 20: 754–755.

Jong, S.C. and Davis, E.E. 1987. Germplasm preservation of edible fungi in culture through cryogenic storage. International symposium on scientific and technical aspects of cultivating edible fungi, University Park, Vol. 10, pp. 213–225.

Kikuchi, K., Matsushita, N. and Suzuki, K. 2006. Germination of *Suillus bovinus* spores in vitro. Nippon Kingakukai Kaiho 47: 37–40 (in Japanese with summary in English).

Kikuchi, K., Matsushita, N., Suzuki, K. and Hogetsu, T. 2007. Flavonoids induce germination of basidiospores of the ectomycorrhizal fungus *Suillus bovinus*. Mycorrhiza 17: 563–570.

Kim K.Y., Cho Y.S., Sohn B.K., Park R.D., Shim J.H., Jung S.J., Kim Y.W. and Seong, K.Y. 2002. Cold storage of mixed inoculum enhanced colonization and growth-promoting activity of *G. intraradices* compared to freshly prepared inoculum. Plant Soil 38: 267–272.

Kope, H.H. and Fortin, J.A. 1990. Germination and comparative morphology of basidiospores of *Pisolithus arhizus*. Mycologia 82: 350–357.

Krupa, P., Piotrowska-Seget, Z., (2003). Positive aspects of interaction between plants and mycorrhizal fungi originating from soils polluted with cadnium. Pol. j. environ. stud. 12: 723–726

Kropáček, K., Cudlin, P., and Mejstřík, V. (1990) The use of granulated ectomycorrhizal inoculum for reforestation of deteriorated regions. Agr. Ecosyst. Environ. 28: 263–269.

Kuek, C. 1994. Issues concerning the production and use of inoculants of ectomycorrhizal fungi in increasing the economic productivity of plantations. In: Robson, A.D., Abbott, L.K., Malajczuk, N. (eds.), Management of Mycorrhizas in Agriculture, Horticulture and Forestry. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 221–230.

Kuek, C., Tommerup, I.C. and Malajczuck, N. 1992. Hydrogel bead inoculants for the production of ectomycorrhizal eucalyptus for plantations. Mycol. Res. 96: 273–277.

Lamb, R.J. and Richards, B.N. 1974a. Inoculation of pines with mycorrhizal fungi in natural soils-I. Soil Biol. Biochem. 6: 167–171.

Lamb, R.J. and Richards, B.N. 1974b. Inoculation of pines with mycorrhizal fungi in natural soils-II. Soil Biol. Biochem. 6: 173–177.

Lehto, T., Brosinsky, A., Heinonen-Tanski, H. and Repo, T. 2008. Freezing tolerance of ectomycorrhizal fungi in pure culture. Mycorrhiza 18: 385–392.

Martín, M.P. and Gràcia, E. 2000. Spore germination and development of young mycelia in some rhizopogon species. Acta. Bot. Barc. 46: 31–46.

Mauperin, C.H., Mortier, F., Garbaye, J., Le Tacon, F. and Carr, G. 1987. Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. Can. J. Bot. 65: 2329–2336.

Melin, E. 1962. Physiological aspects of mycorrhizae of forest trees. In: Kozlowski, T.T. (ed.), Tree Growth. Ronald Press, New York, NY, pp. 247–263.

Menge, J.A. 1984. Inoculum production. In: Powell, C.L., Bagyaraj, D.J. (eds.), VA Mycorrhizae. CRC Press, Boca Raton, FL, pp. 187–203.

Mohammad, A., Khan, A.G. and Kuek, C. 2000. Improved aeroponic culture of inocula of arbuscular mycorrhizal fungi. Mycorrhiza 9: 337–339.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant 15: 473–497.

Nagarajan, S. and Singh, D.V. 1990. Long-distance dispersion of rust pathogens. Annu. Rev. Phytopathol. 28: 139–153.

Nemec, S. and Ferguson, J.J. 1985. A fluid-drilling applicator for applying VAM in the field. In: Molina, R. (ed.), Proceedings of the 6th North American Conference on Mycorrhizae. Oregon State University, Corvallis, p. 244.

Ogawa, M. 1975a. Microbial ecology of mycorrhizal fungus, *Tricholoma matsutake* Ito et Imai (Sing.) in pine forest I Fungal colony ('shiro') of *Tricholoma matsutake.* Bull. Gov. For. Exp. Stn. Tokyo 272: 79–121.

Ogawa, M. 1975b. Microbial ecology of mycorrhizal fungus, *Tricholoma matsutake* Ito et Imai (Sing.) in pine forest II Mycorrhiza formed by *Tricholoma matsutake.* Bull. Gov. For. Exp. Stn. Tokyo 278: 21–49.

Ogawa, M. 1977. Microbial ecology of mycorrhizal fungus, *Tricholoma matsutake* Ito et Imai (Sing.) in pine forest I The shiro of *T. matsutake* in the fungal community. Bull. Gov. For. Exp. Stn. Tokyo 297: 59–104.

Oort, A.J.P. 1974. Activation of spore germination in Lactarius species by volatile compounds of *Ceratocystis fagacearum*. Proc. K. Ned. Akad. Wet. Ser. C 77: 301–307.

Parladé, J., Pera, J. and Luque, J. 2004. Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions. Mycorrhiza 14: 171–176.

Rodrigues, L.S., Kasuya, M.C.M. and Borges, A.C. 1999. Viability of ectomycorrhizal fungus mycelium entrapped in calcium alginate gel. Mycorrhiza 8: 263–266.

Rossi, M.J., Furigo, A. Jr. and Oliveira V.L. 2007. Inoculant production of ectomycorrhizal fungi by solid and submerged fermentations. Food Technol. Biotechnol. 45: 277–286.

Sharma, A.K., Singh, C. and Akhauri, P. 2000.Mass culture of arbuscular mycorrhizal fungi and their role in biotechnology. Proc. Indian Natl. Sci. Acad. (PINSA) 66: 223–238.

Siddiqui, Z.A. and Mahmood, I. 1995. Role of plant symbionts in nematode management, A Review. Bioresour. Technol. 54*:* 217–226.

Siddiqui, Z.A. and Mahmood, I. 1998. Effect of a plant growth promoting bacterium, an AM fungus and soil types on the morphometrics and reproduction of *Meloidogyne javanica* on tomato. Appl. Soil Ecol. 8: 77–84.

Siddiqui, Z.A. and Pichtel, J. 2008. Mycorrhizae: an overview. In: Siddiqui, Z.A., Akhtar, M.S., Futai, K. (eds.), Mycorrhizae: Sustainable Agriculture and Forestry. Springer, Dordrecht, The Netherlands, pp. 1–35, 362.

Singh, G., Tilak, K.V.B.R. 2001. Techniques of AM fungus inoculum production. In: Mukerji, K.G., Manoharachary, C., Chamola, B.P. (eds.), Techniques in Mycorrhizal Studies. Kluwer Academic Publishers, The Netherlands, pp. 273–283.

Smith, S.E. and Read, D.J. 1997. Mycorrhizal Symbiosis. 2nd ed. Academic Press, London, 605 pp. ISBN 0-12-652840-3.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M. and Fortin, J.A. 1996. Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *G. intraradices* in an in vitro system in the absence of host roots. Mycol. Res. 100: 328–332.

Theodorou, C. 1971. Introduction of mycorrhizal fungi into soil by spore inoculation of seed. Aust. For. 35: 23–26.

Tibbett, M., Sanders, F.E. and Cairney, J.W.G. 1999. Long-term storage of ectomycorrhizal basidiomycetes (*Hebeloma* spp.) at low temperature. J. Basic Microbiol. 39: 381–384.

Trappe, J.M. 1962. Fungus associates of ectotrophic mycorrhizae. Bot. Rev. 28: 538–606.

Trappe, J.M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. Annu. Rev. Phytopathol. 15: 203–222.

Turk, M.A., Assaf, T.A., Hameed, K.M. and Al-Tawaha, A.M. 2006. Significance of mycorrhizae. World J. Agric. Sci. 2: 16–20.

Varma, A. and Schuepp, H. 1995. Mycorrhization of the commercially important micropropagated plants. In: Stewart, G.G., Russell, I. (eds.), Critical Reviews in Biotechnology. CRC Press, Canada, pp. 313–328.

Vassilev, N., Nikolaeva, I. and Vassileva, M. 2005. Polymer based preparation of soil inoculants: Applications to arbuscular mycorrhizal fungi. Rev. Environ. Sci. Biotechnol. 4: 235–243.

Von, A. 1998. State of commercial use of AMF-inoculum in Germany. In: Gianinazzi, S., Schuepp, H. (eds.), Arbuscular Mycorrhizas in Sustainable Soil-Plant Systems. Report of 1997 Activities, Cost Action 821, Iceland, p. 153.

Vosatka, M., Jansa, J., Regver, M., Sramek, F. and Malcova, R. 1999. Inoculation with mycorrhizal fungi – a feasible biotechnology for horticulture. Phyton Annu. Rev. Bot. 39: 219–224.

Wang, Y., Hall, I.R. and Evans, L.A. 1997. Ectomycorrhizal fungi with edible fruiting bodies. I. *Tricholoma matsutake* and related fungi. Econ. Bot. 51: 311–327.

Weathers, P.J. and Zobel, R.W. 1992. Aeroponics for the culture of organisms, tissues and cells. Biotechnol. Adv. 10: 93–115.

Webster, G., Poulton, P.R., Cocking, E.C. and Davey, M.R. 1995. The nodulation of micropropagated plants of *Parasponia andersonii* by tropical legume rhizobia. J. Exp. Biol. 46: 1131–1137.

Wu, C.-G., Lir, Y.S. and Huang, L.L. 1995. Spore development of *Entrophospora kentinensis* in an aeroponic system. Mycologia 87: 582–587.

Yamada, A., Maeda, K. and Ohmasa, M. 1999. Ectomycorrhiza formation of *Tricholoma matsutake* isolates on seedlings of *Pinus densiflora* in vitro. Mycoscience 40: 455–463.

Yoshimura, F. 2004.This is how far we've got! Matsutake cultivation. Toronto, 109 pp (in Japanese).

Zobel, R.W., Del Tredici, P. and Torrey J.G. 1976. Methods for growing plants aeroponically. Plant Physiol. 57: 344–346.