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review

Inoculant Production of Ectomycorrhizal Fungi by Solid and Submerged Fermentations

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Summary

Ectomycorrhizal fungi are very important for forestry practices. In order to develop controlled mycorrhization practices it is necessary to isolate and select ectomycorrhizal fungi that are able to colonize the intended plant species and that are efficient in promoting its growth under the environmental conditions prevailing in the plantation site. To be suitable for the inoculation of nursery seedlings, these fungi must be able to grow rapidly during large-scale cultivation and maintain high infectivity rates during storage. Even though several decades have passed since the first field-scale mycorrhizal experiments, the routine use of these fungi to inoculate plants is still not very common. The lack of suitable ectomycorrhizal inoculants in the market is one of the main factors contributing to this situation. This review presents and discusses techniques for the production and application of ectomycorrhizal inoculants, as well as the more recent studies aimed at developing reliable industrial production processes.

Key words: vegetative inoculant, controlled mycorrhization, bioreactors, carriers, immobilization, calcium alginate

Introduction

Mycorrhizas are mutualistic associations between roots and specific soil fungi that improve the absorption of water and nutrients by the plant (*1*). They occur in the majority of plant ecosystems and make important contributions to plant survival and growth (*2*). The importance of mycorrhizas in the growth of plants was demonstrated in the first, unsuccessful, attempts to establish forest plantations outside of their natural habitats, in regions where mycorrhizal fungi compatible with the plant species were not present (*3,4*). Success was only achieved when some type of mycorrhizal inoculant containing compatible fungal species was introduced.

Among several types of mycorrhizas, ectomycorrhizas are a specific group, most of which are formed by basidiomycetes. They are particularly important for the growth of plants of silvicultural interest, including species of *Eucalyptus*, *Fagus*, *Quercus* and *Pinus* (*5*). In the case of *Pinus*, the association with ectomycorrhizal fungi is absolutely necessary for the survival and growth of the plants (*2*).

Ectomycorrhizas contribute by increasing uptake of water and nutrients, particularly those nutrients presenting low mobility in the soil, such as phosphorus (*6–9*). This increased uptake is the result of a significant increase in the plant-soil interface, which can be attributed to two factors. Firstly, the hyphae of the ectomycorrhizal

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fungi grow out beyond the root zone and, secondly, colonized roots are, in general, more branched than the uncolonized roots (*10*). Ectomycorrhizas can also augment hydraulic conductivity inside the plant, the resistance of the plant to drought and soil-borne pathogens and can improve soil aggregation and structure (*11,12*). Through these different mechanisms, ectomycorrhizas promote plant growth and productivity even in low fertility or disturbed soils (*13*).

Besides being beneficial to plants, many ectomycorrhizal fungi are an important source of food for men and forest animals, both in temperate and in tropical regions, contributing to the economy of many human communities and to the maintenance and stability of forest ecosystems. As pointed out by Smith and Read (2), there is a great potential to exploit fruiting bodies of ectomycorrhizal fungi as commercial foods.

Although ectomycorrhizal fungi are naturally present in many soils, their ability to colonize and benefit plants is variable (*14*); therefore it is advantageous to inoculate the seedlings with specific strains that provide benefits to the plant in question, within the specific environment that it will experience (*15*). This so called 'mycorrhization control' is usually done by planting seedlings that have been previously inoculated in the nursery with the chosen fungal strain. This practice improves not only the survival of the seedlings upon transplanting but also their subsequent growth (*14,16*). The economic benefits of this practice, in terms of increased productivity, have been demonstrated in plantations in the United States of America and in France (*10,14,17*). However, in order for mycorrhizal inoculation to become a routine practice in nurseries, it is necessary to establish methods for inoculant production at industrial scale.

Mycorrhization control begins with the isolation and selection of efficient ectomycorrhizal fungi and culminates with the large-scale production and application of inoculants of these fungi (*13,18*). The present review focuses on the techniques for large-scale production of ectomycorrhizal inoculants. However, it also addresses related issues such as the initial selection of fungal isolates and the final formulation of a product that is suitable for inoculating seedlings. It will become clear that much more work is necessary in order to establish reliable large-scale processes for inoculant production.

Selection of Efficient Ectomycorrhizal Isolates

Isolates of ectomycorrhizal fungi are generally selected on the basis of their compatibility and efficiency, where compatibility means the ability to colonize roots while efficiency means the ability to promote growth of the plant host. The efficiency is usually tested by evaluating parameters such as the height of the inoculated plant, the diameter of the stem, the overall dry mass of the plant and the nutrient content of the plant, especially phosphorus and nitrogen (*19*). It may also be interesting to consider the ability of ectomycorrhizal isolates to metabolize organic nitrogen sources, given that in some soils low mineralization rates may lead to organic nitrogenous compounds being more abundant than mineral nitrogen (*2*). Moreover, some ectomycorrhizal fungi are able to utilize amino acids as a nitrogen source and could benefit host plants when growing in soils presenting high levels of these compounds (*20*).

Another important factor that should be considered, but which at the moment is seldom taken into account in the selection of mycorrhizal isolates, is the growth rate of the fungus when cultivated in large-scale bioreactors. Compared to free-living microorganisms, ectomycorrhizal fungi grow very slowly in laboratory conditions (*18, 21–24*) and this slowness of growth may cause operational problems in bioreactors. For example, during the long cultivation times that are necessary, mycelia may foul probes, air dispensers, nutrient inlets and drainage ports. Long-term cultivation also presents higher risks of contamination and of accumulation of toxic metabolites, which may reduce the viability of the inoculant (*25*).

Inoculant efficiency

The evaluation of the efficiency of an ectomycorrhizal isolate in promoting plant nutrition and growth under controlled conditions in a greenhouse is an important first step in selecting a strain for large-scale production of plant inoculants (*10,26,27*). However, to justify the investment in the establishment of a large-scale inoculant production plant, it is also necessary to demonstrate the efficiency of the inoculant in promoting plant growth under nursery and field conditions. In the nursery, these studies can be performed in periods up to 6 months for plants like *Eucalyptus* spp. and *Pinus* spp. However, under field conditions, it is necessary to follow plant development for several years and a far greater number of plants must be evaluated at this level (*17,28*).

Garbaye (*29*) reviewed 25 studies performed in experimental field plantations in different countries, in which plants inoculated in the nursery with selected ectomycorrhizal fungi were compared with uninoculated plants that were naturally colonized by native ectomycorrhizal fungi after transplanting. In the majority of these studies, the inoculated plants showed, after transplanting from the nursery to the field, an increase of 130 % in height, 40 % in terms of volume and 25 % in survival, when compared to controls. Therefore mycorrhization control programs may be considered as an alternative to conventional nursery practices to increase plant growth and productivity.

Ectomycorrhizal inoculants

The type of ectomycorrhizal material used for inoculation can affect the success of a mycorrhizal inoculation program. The inoculant must remain viable during storage and transport, maintaining its infectivity for several months after its production. Furthermore, the formulated inoculant must be easy to apply and must also be free of contamination by plant pathogens and any free-living microorganisms that could affect inoculant viability (*30*). Finally, the cost of the inoculant must be compatible with the financial resources available to the nursery; care must be taken to avoid raising seedling prices to uncompetitive levels (*31*).

Three main types of ectomycorrhizal inoculants have been used in nurseries during the last decades: soil, fungal spores and vegetative mycelia. In the earliest studies, a thin layer of soil obtained from natural forests, old

nurseries or established plantations was spread on the top of nursery bed and mixed with the soil or planting substrate (*4*). This method is still used in many parts of the world, particularly in developing countries. One problem with this type of inoculant is that large amounts of soil are required to inoculate nursery plants, but an even more important problem is the risk of introducing plant pathogens and weeds. Moreover, there is no precise information about the fungal species that are being introduced and their infection potential (*10*). Despite these disadvantages, soil inoculant is recommended if no other type of inoculant is available (*19*,*32*).

Fungal spores, obtained from fruiting bodies harvested in natural forests, old nurseries or established plantations, have also been used in many parts of the world (*33*). They are easy to obtain and easy to apply to plants. They can be added as pellets (*34*), mixed with the planting substrate, applied directly to seeds (*35*) or applied as a water suspension (*36*) through the nursery watering system (*10*). This type of inoculant is limited to those fungal species able to produce large numbers of spores and fruiting bodies and there is no certainty of their compatibility and efficiency towards the plant species to be cultivated. As spores are generally collected from multiple fruiting bodies, they tend to present a higher genetic variability than vegetative inoculant. The availability of spores is erratic during the year, hence the need to collect and store large numbers of fruiting bodies when they are abundant (*19*,*37*). According to Marx *et al.* (*19*), root colonization by this type of inoculant is slower than that presented by vegetative inoculant of the same fungal isolate.

Addition of mycelia obtained from pure cultures of ectomycorrhizal fungi, also called vegetative inoculant, has proven to be the most suitable method. Vegetative inoculants can be prepared from any fungus able to be cultivated in pure culture, allowing the use of selected isolates that have been previously tested in terms of their efficiency in promoting plant growth (*16*,*26*,*27*,*38*). Pure cultures are generally obtained from fruiting bodies or from mycorrhizas (*39–41*) and may be maintained indefinitely under laboratory conditions. For commercial production of vegetative inoculant, mycelium has to be grown in solid substrate or in liquid culture medium at several different scales. The next section evaluates the state of the art in the production of vegetative inoculants.

Production of Vegetative Ectomycorrhizal Inoculants

The required scale of production of vegetative inoculants of ectomycorrhizal fungi depends directly on the timber market. In 2005, world timber requirements were about 3.0 billion m^3 (*42*). Assuming a production of 400 m^3 per ha, it would be necessary to plant 12 billion seedlings per year in order to replant the exploited areas. If all seedlings were inoculated with selected ectomycorrhizal fungi then, considering that 14 000 containerized seedlings can be produced per m^3 of substrate and a typical application rate would be 5 g of dry mycelium per m^3 (*29*), it would be necessary to produce 4.3 t of mycelium per year. Although this amount of biomass may not appear large to those who work with organisms such as baker's yeast, it represents an enormous challenge to the inoculant production industry, especially if one considers the low growth rates of ectomycorrhizal fungi in culture.

In contrast to arbuscular mycorrhizal inoculants, which are presented as different types of commercial products and sold by several companies (*43*), only relatively few ectomycorrhizal fungal inoculants have been commercialized (Table 1, *44–52*). There is no information available in the literature to indicate which forest programs are making use of these inoculants, nor the magnitude of their application in terms of the number of seedlings inoculated. Moreover, commercial products containing ectomycorrhizal fungi that are advertised on the Internet contain fungal propagules whose nature and genetic quality are likely to be very variable, based on considerations already discussed in this review. In most of the cases, the fungal species in these products are not defined. Further, it would appear that these commercial products are based on methods other than the pure culture of vegetative mycelium.

There is a significant gap in the literature concerning the production and application of ectomycorrhizal fungal inoculants compared to the enormous volume of studies considering other aspects of the ectomycorrhizal symbiosis, such as the diversity of symbionts and the taxonomy, phylogeny and molecular biology of the fungi involved (*2*). This gap is still bigger if one considers vegetative inoculant and its application in forest practices.

Table 1. Commercial ectomycorrhizal fungi inoculants produced through different processes by different companies

Commercial product	Type/process	Company	Reference
BioGrow Blend®	Spores	Terra Tech, LLC	(*44*)
MycoApply®-Ecto	Spores	Mycorrhizal Applications Inc.	(*45*)
Mycorise Pro Reclaim®	Propagules ecto+endo	Symbio Technologies Inc.	(*46*)
Myke® Pro LF3	Propagules	Premier Tech Biotechnologies	(*47*)
Mycor Tree®	Spores	Plant Health Care, Inc.	(*48*)
MycoRhiz®	Mycelium/SSF	Abbott Laboratories	(*49*)
Somycel PV	Mycelium/SSF	INRA – Somycel S.A.	(*50*)
Ectomycorrhiza Spawn	Mycelium/SSF	Sylvan Spawn Laboratory, Inc.	(*51*)
–	Mycelium/Submerged	Rhône Poulenc – INRA	(*50*)
Mycobead®	Mycelium/Submerged	Biosynthetica Pty. Ltd.	(*52*)

SSF = solid-state fermentation

With respect to cultivation in bioreactors, we do not understand many important aspects of the biochemistry, physiology and kinetics of the growth of ectomycorrhizal fungi. In addition, little effort has been made into identifying the most appropriate bioreactor type for cultivation of these fungi, into characterizing mass transfer within bioreactors and into optimizing operating conditions. This lack of information is a major reason why large-scale production of ectomycorrhizal inoculants is so restricted.

Microbial inoculant production through solid-state fermentation and submerged liquid fermentation was reviewed by Walter and Paau (*53*). Many characteristics of the liquid system make this process attractive for culture and production of biological products. Liquid substrates are easily mixed, producing more uniform conditions for culture growth than solid substrates. They also allow easier and quicker changes of culture variables such as pH, dissolved oxygen, temperature, stirrer speed and nutrient concentration.

In comparison, solid-state fermentation has many disadvantages. Spatial homogeneity within the bed of solid particles is typically poor. Particles larger than about 3 mm in diameter will have anaerobic interiors, even when oxygen supply to the surface is unrestricted, limiting the growth of the mycelium within the particle (*54*). Solid--state fermentation has another disadvantage concerning the sterilization step. The poor heat transfer characteristics of the bed and the stabilizing effect of the particle surface on the microbial cells with which it is in contact contribute to a higher survival of cells (*55*), so that longer times are required to achieve sterilization. Besides the obvious risk of failure to sterilize the substrate, this problem increases the cost of the process. Moreover, solid-state fermentation systems are difficult to monitor, either by sampling or with probes. This difficulty, combined with rapid changes in local conditions due to microbial activity, means that important process variables such as pH, pO_2 and temperature are difficult to control (*56*). The resulting heterogeneities of the system, not only at the level of individual particles but also across the bed, mean that the system is so complex that it is difficult to establish accurate process models (*57*). As a result, scale--up of solid-state fermentation bioreactors is more complex than for submerged liquid fermentation bioreactors.

On the other hand, according to Cannel and Moo--Young (*58*), the main advantages of solid-state fermentation compared to submerged processes are the reduction of bacterial contamination due to the low water activity in solid substrates, the low costs of installation and the simplified design of bioreactors. Solid-state fermentation is particularly convenient for the production of inoculants of microorganisms that have simple nutritional requirements and which are able to decompose materials such as cellulose or lignin, since it allows the use of agricultural residues, moistened with either water or a solution of supplementary nutrients (*59*).

Cultivation of ectomycorrhizal fungi in solid substrates

Despite the problems listed above, solid-state fermentation has been the most common technique used to date for production of vegetative inoculants of ectomycorrhizal fungi. The preferred substrate for this process has been a mixture of peat and vermiculite, supplemented with a nutritive solution (*49*). This mixture may be prepared in different proportions, depending on the expected final pH, and is generally distributed in glass flasks or plastic bags, being inoculated with mycelium plugs or a mycelium suspension, obtained from a previous culture in solid or liquid medium, respectively. It then takes 2–4 months of incubation for the final product to be ready to inoculate seedlings (*16,26,60–62*). This method has been used for the propagation of ectomycorrhizal fungi in the laboratory, and also at industrial level for commercial production (*49*).

When applied to the planting substrate, the mycelium remains protected inside the vermiculite particles, where it can survive until receptive roots are produced by the host plant (*62*). Vermiculite is a low price material and absorbs nutrient solutions well. It also provides a bed structure that enables good aeration of the substrate. However, nutrients tend to diffuse into pores in the interior of the vermiculite particles, and this makes access of the fungus to the nutrients more difficult, delaying the exhaustion of sugar during fungal growth. This can be a problem because inoculants containing residual sugars are more susceptible to contamination. According to Garbaye (*29*), if all sugar in the substrate is utilized, there is no need to wash the inoculant before its application. It is interesting to avoid the washing step for both practical and economic reasons. Inoculation of plants with vegetative inoculants produced in solid substrates has been done in the USA (*10,49*), France (*17*), Mexico (*63*), Brazil (*64*), and Liberia (*65*), among other countries, with the inoculants showing high infectivity towards several plant species.

Among the ectomycorrhizal fungi applied as vegetative inoculants, *Pisolithus tinctorius* has been the most frequently applied, due to several factors. Firstly, the fungus has a wide geographical distribution and a wide host range; secondly, it tolerates environmental stress well and, thirdly, it is relatively easy to cultivate (*33,66*). For example, a vegetative inoculant of *Pisolithus tinctorius* improved mycorrhizal formation in *Pinus palustris* over that obtained with a spore-based inoculant (*67*). Vegetative inoculants of other species of ectomycorrhizal fungi, such as *Hebeloma crustuliniforme*, *Laccaria laccata*, *Suillus luteus*, *Cenococcum geophillum* and *Thelephora terrestris*, have also been tested with plant hosts of economical interest (*66*).

Submerged cultivation of ectomycorrhizal fungi

Techniques that are currently used for the submerged culture of microorganisms of industrial interest could be adapted for the production of ectomycorrhizal inoculants. Compared to solid-state fermentation, liquid fermentation requires less space and time since in liquid medium the contact between phases is maximized and nutrients are more efficiently utilized.

After cultivation in bioreactors, mycelium may be immobilized in calcium alginate gel (*60*) or other types of polymeric matrices. Nursery studies have shown that this type of inoculant is more efficient than those pro-

duced by solid-state fermentation, probably due to better survival of the fungus inside the gel when applied to the planting substrate (*68*). However, the performance of inoculant formulated in this manner does vary with the species involved (*69*). For some species, beads of alginate-immobilized mycelium retain 95 to 100 % viability after storage in water at 4 °C during 6 months (*70,71*). In the case of *Rhizopogon nigrescens*, such beads retained 100 % viability after 18 months under refrigeration and were able to colonize roots when applied to *P. taeda* seedlings under greenhouse conditions (*72*).

The high viability of the inoculant obtained by submerged fermentation is the result of the shorter periods of cultivation which, in turn, are related to the high mass transfer rates that are possible to obtain in this process. Lapeyrie and Bruchet (*25*) demonstrated that a cultivation period longer than 20 days lowers inoculum potential, which they defined as the energy of growth available for the infection of a host.

Despite being a very promising technique for the production of ectomycorrhizal fungal inoculants, submerged fermentation followed by gel immobilization is still of limited application, even two decades after its first application. The problem in the application of this technology is the relatively poor growth obtained in most cultivation systems. In addition, as these fungi do not sporulate in culture medium, it is necessary to disperse hyphal agglomerates mechanically when preparing a mycelial suspension for the inoculation of a bioreactor and this may result in damage to hyphae and a consequent reduction in viability (*25*). Contamination by saprophytic microorganisms is frequent during cultivation, and operational steps such as mycelium washing and immobilization increase both the risks of contamination and the production costs (*29*).

Another reason for the lack of application of submerged fermentation technology for the production of ectomycorrhizal inoculants is the lack of information on the biochemistry and physiology of ectomycorrhizal fungi while growing in liquid medium and on the engineering process in bioreactors. In the few studies published concerning the production of ectomycorrhizal inoculants in bioreactors (*60,68,71*), these aspects were not considered.

Pradella *et al.* (*73*) were the first to study the productivity of a submerged cultivation of an ectomycorrhizal fungus. Despite their best efforts to grow *Pisolithus tinctorius* in a stirred-tank bioreactor, they only attained a productivity of 0.15 g/(L·day). Nine years latter, Baroglio *et al.* (*21*) studied the submerged cultivation of another ectomycorrhizal fungus, *Suillus grevillei*, again in a stirred-tank bioreactor. Although *Suillus* spp. are more difficult to grow than *Pisolithus* spp., mycelium productivity in this latter study was higher, at about 0.25 g/(L·day), after 17 days of cultivation and with control of pH and nitrogen levels. More recently, Rossi *et al.* (*18*) characterized, for the first time, the growth kinetics of *Pisolithus microcarpus* during a submerged cultivation in a pneumatic airlift bioreactor. The productivity obtained, 0.48 g/(L·day), was significantly higher than that obtained in the earlier studies. Rossi *et al.* (*18*) pointed out the need for studies dealing with oxygen transfer during the cultivation of ectomycorrhizal fungi in liquid systems. In a later publication, they used an airlift bioreactor to study the growth kinetics of another ectomycorrhizal fungus, *Rhizopogon nigrescens* (*22*). The success of these studies in the airlift bioreactor indicated that this type of bioreactor is very adequate for the cultivation of these fungi. In fact, although airlift bioreactors have been used in bioprocesses for over 30 years (*74*), their use for mycorrhizal inoculant production is quite recent. Jolicoeur *et al.* (*75*) were the first to use an airlift bioreactor for the production of a mycorrhizal fungus, in their case an arbuscular mycorrhizal fungus.

In an attempt to provide engineering information necessary for development of a large-scale process for ectomycorrhizal fungus production, Rossi *et al.* (*23,76*) built an airlift bioreactor and studied its hydrodynamic parameters and oxygen transfer under different operational conditions. The specific oxygen uptake rate, the specific growth rate and the amount of oxygen required for cell maintenance were estimated during cultivation of *R. nigrescens*, at three levels of aeration, in the bioreactor (*24*). A productivity greater than 1 g mycelium/(L·day) was obtained. During these studies, Rossi (*24*) observed that the size of the initial mycelial propagules utilized to inoculate the bioreactor affected the specific growth rate and the critical oxygen concentration (the oxygen concentration above which cell growth is limited) during submerged cultivation in the bioreactor. He also observed that the increase in the size of the mycelial pellets increased the critical oxygen concentration and led to reduced oxygen transfer.

Rossi (*24*) also discovered that the addition of 0.2 % (m/V) of activated charcoal to mycelial suspensions, used for bioreactor inoculation and to alginate mycelial beads, helped to maintain the mycelial viability during more than 6 months. In this case the viability was independent of the fragmentation time used in preparing the inoculant formulation. These observations suggest that the loss of mycelium viability, which is a problem frequently encountered with ectomycorrhizal inoculants prepared after submerged fermentations, is not directly related to physical damage to the mycelium during the formulation step or in the bioreactor. It seems to be due to the action of metabolites produced by the fungus and liberated by broken hyphae (*24*). The chemical nature of such compounds remains to be ascertained.

Finally, Rossi (*24*) also built a 5-litre airlift bioreactor with external circulation. In one batch, this bioreactor has the capacity to produce the amount of mycorrhizal inoculant necessary to inoculate 300 000 seedlings. This quantity of seedlings would be enough for planting more than 200 ha. Based on his results, it is possible to estimate that 150 airlift bioreactors, with an internal volume between 50 and 100 L, operating throughout one year, could produce sufficient mycelium to inoculate more than 1 billion seedlings. This production would satisfy inoculant requirements of an important timber producer like Brazil. These results indicate the high potential that the submerged cultivation technology has for the production of ectomycorrhizal inoculants for the forestry sector.

Culture media and conditions for submerged fermentation

Ectomycorrhizal fungi require low molecular mass sugars and growth factors for optimal growth. As heterotrophic symbionts, they need carbonate compounds synthesized by their plant host. In culture, many isolates are able to use carbohydrates such as fructose, mannose and cellobiose (*77,78*). However, the best assimilated sugar is glucose (2).

When dealing with large-scale cultivation in batch systems, it is important to know the maximum cell concentration supported by the bioreactor in order to define the required nutrient concentration. The maximum cell concentration depends on the hydrodynamics and the oxygen transfer rate that can be achieved within the bioreactor. In most of the culture media used for the cultivation of ectomycorrhizal fungi, the glucose concentration ranges from 10 to 30 g/L (*18,52,73,79–81*). In an airlift bioreactor, operating at a specific air flow of 0.50 vvm, the carbohydrate concentration used was 16 g/L, and resulted in a maximum biomass concentration of 7 g/L (*24*).

The choice of an appropriate nitrogen source is important for optimizing the productivity of ectomycorrhizal fungal biomass. Since many ectomycorrhizal fungi can be cultivated in axenic culture, it is possible to investigate the effects of different nitrogen sources on their growth. Although several species are able to utilize NH_4^+ and NO_3^-, they seem to prefer the former (*20,82,83*). There are some isolates, however, that can utilize organic nitrogen sources, such as amino acids (*20,83,84*).

Modified Melin-Norkrans (MMN) medium (*40*) has been most commonly used for the cultivation of ectomycorrhizal fungi (*9,73,77,85–88*). In the modified medium, sucrose, present in the original medium (MN) at 2.5 g/L (*89*), is replaced by glucose at 10 g/L. This higher C concentration leads to a high C:N ratio and may result in the presence of residual sugar at the end of the cultivation.

Besides the nutritional aspects, one has to consider the action of physical factors on fungal growth in artificial media. Many isolates grow best between 18 and 25 °C (*90*), but are able to tolerate up to 42 °C (*91*). This wide range of temperatures for the cultivation of ectomycorrhizal fungi is probably related to the environmental conditions to which they are subjected in their original habitats. It is important to bear in mind that high temperatures reduce oxygen solubility in culture medium, and that oxygen availability is one of the most important factors controlling fungal growth under submerged conditions (*24,92*).

In general, ectomycorrhizal fungi grow better under slightly acid conditions, with optimal pH values between 4 and 6. It is necessary to control the pH of the culture medium in order to attain high productivities since, even if the pH of the culture medium is initially adjusted to the optimal value, fungal metabolism may produce organic acids from carbohydrates, and basic compounds from protein breakdown, promoting pH changes and limiting growth (*93*).

Carbonates added to culture media as nutrients may also act as pH buffers. Smith (*77*) and Rossi *et al.* (*18*) obtained high biomass productivities for *Pisolithus* spp. using culture media buffered at pH=5.4 with citric acid and calcium citrate. The Priddam-Gottlieb medium, utilized by Litchfield and Arthur (*79*), is buffered by the presence of KH_2PO_4/K_2HPO_4 and of proteins from the peptone and yeast extract. Despite their importance in culture media as nutrients and pH buffers, phosphates, if added in large quantities, may cause serious difficulties later when it is necessary for the fungus to adapt to low phosphorus levels in the soil (*52*).

Sterilization of culture media may affect the availability of carbohydrates for fungal growth. It is preferable to sterilize sugars separately to avoid formation of toxic compounds resulting from the reaction of sugars with the ammonium ion and amino acids (*93*). Autoclaving culture medium may also precipitate iron, manganese and zinc ions (*94*). This problem may, however, be avoided by the addition of small amounts of chelating agents such as EDTA, citric acid or polyphosphates. However, it is necessary to verify that the chelant does not inhibit microbial growth by immobilizing nutrients or by another toxic mechanism.

Choice of a carrier for ectomycorrhizal inoculants

Conversely to the mycelium produced by solid-state fermentation, where hyphae are protected inside vermiculite particles, mycelium produced by submerged fermentation is pure and concentrated and has to be protected from physical and biological factors before its application. 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 formulated mycelium must resist adverse conditions such as drought, microbial antagonism or predation by insects and other arthropods (*95,96*). Use of carrier materials is therefore crucial, with the added advantage that they can contribute to survival of the inoculant during storage and a better dispersion during the inoculation process itself.

Polymeric gels such as alginate, polyacrilamide and carrageenan have been utilized to immobilize enzymes, bacteria, fungi, plant and animal cells (*97*). They are particularly suitable for these purposes since they facilitate the control of bead size and cell concentration. These materials may possibly be utilized to carry mycelium of ectomycorrhizal fungi produced in liquid medium (*98,99*). In the case of alginate, a suspension of fragmented mycelium is mixed into a sodium alginate solution and slowly poured into a concentrated solution of calcium chloride. An ionic exchange between calcium and sodium takes place, producing gelled beads of calcium alginate that contain mycelium. The size of the beads can be easily controlled by the dispenser system. This procedure offers great flexibility since it allows addition of chemical additives to improve gel stability and conserve the inoculant (*70*). It is also possible to add plant nutrients and growth promoters.

The polymeric matrix of the alginate gel allows hyphae to grow inside the beads and expand towards its exterior (*71,99*). Another advantage is the possibility of preparing a multimicrobial inoculant containing more than one type of plant-growth-promoting microorgan-

ism, as proposed by Duponnois and Garbaye in relation to ectomycorrhizal fungi and mycorrhization helper bacteria (*100*).

Inoculant beads can remain viable for several months under refrigeration, although the results vary between fungal species. For example, relatively stable inoculants have been prepared from *Hebeloma crustuliniforme* (90 % viability after 5 months), *Hebeloma westraliensi* (80 % viability after 7 months), *Laccaria laccata* (80 % viability after 7 months), *Paxillus involutus* (90 % viability after 2 months) and *Rhizopogon nigrescens* (90 % viability after 18 months) (*70–72,88*). On the other hand, an inoculant of *Elaphomyces* sp. retained only 40 % viability after 1 month (*71*).

Nursery studies have shown that ectomycorrhizal inoculants immobilized in alginate gel improve plant growth when compared to traditional peat-vermiculite inoculants produced by solid-state fermentation (*60,68*). According to Kuek *et al.* (*71*) the better dispersal of the beads into the planting substrate and the protection of the mycelium offered by the alginate gel were the main factors contributing to the better quality and performance of the immobilized inoculant.

Conclusions

Mycorrhizas are a very important factor contributing to forest productivity. In the case of forest plantations, introduction of compatible and efficient ectomycorrhizal fungi is crucial to assure plant survival and growth. The establishment of a production technology for ectomycorrhizal fungal inoculants is an essential step in the direction towards the routine use of mycorrhizal fungi in forest nurseries. Submerged cultivation of ectomycorrhizal fungi is a convenient technique for this purpose and has many advantages in relation to solid-state fermentation, among them a higher viability and biomass productivity, smaller volumes of inoculant and lower costs. Inoculant production may be achieved using small bioreactors and the bioreactor costs may be minimized by the adoption of pneumatic bioreactors such as airlift bioreactors, whose construction and maintenance are less expensive than those of conventional stirred tank bioreactors. The mycelia that are produced in submerged culture should be immobilized in alginate gel or other polymeric carriers in order 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 will it be possible to obtain ectomycorrhizal fungal inoculants of high quality at an acceptably low cost and in quantities sufficient to meet the needs of the forestry industry.

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