Chapter 9
Techniques for Host Plant Inoculation with Truffles and Other Edible Ectomycorrhizal Mushrooms

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9.1 Introduction

Most of edible ectomycorrhizal mushrooms (EEMMs) are obligate symbionts. They cannot complete their life cycle without a host plant supplying the fungal mycelium with the photosynthates necessary to grow and develop (Karwa et al. 2011). For this reason, it is impossible to produce mature fruiting bodies of *Boletus* spp., *Tuber* spp., *Cantharellus* spp. or other EEMMs on organic substrates, as is done with saprobic edible mushrooms (Boa 2004). Currently, the production of EEMMs is almost totally via natural production. Only a few species, mainly truffles, are cultivated extensively around the world (Hall et al. 2003; Parladé 2007).

Production of well-mycorrhized plants, followed by their planting into suitable sites for soil and climate characteristics, is the crucial point in modern EEMM cultivation. The inability to cultivate most of the EEMMs is due to the difficulties in obtaining properly colonized plants and in maintaining their mycorrhizas in the field. During the past few years, the number of EEMM colonized plants available on the market has increased. This can mainly be attributed to the wide distribution and diffusion of truffle culture around the world. However, the traditional spore inoculation methodologies applied to *Tuber* spp. do not guarantee well-mycorrhized plants or consistent quality. Hence, some batches may result into being poorly colonized or not colonized at all, with the possibility of being heavily contaminated with other ectomycorrhizal (EM) fungi (Hall et al. 2003). In this context, new biotechnologies may certainly improve the efficiency of inoculation techniques for EEMMs. Moreover, recent advances in the genomics of EEMMs may reveal new insights into the biology and ecology of EEMMs, which are crucial for planning mycorrhization and cultivation programs tailored to each species

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(Martin et al. 2010). For example, the sexuality in truffles has been recently clarified (Paolocci et al. 2006; Rubini et al. 2011a) as the competitive interactions between strains of opposite mating types on root systems (Rubini et al. 2011b). Moreover, the identification of fungal genes involved in early symbiotic interactions and the characterization of enzymatic repertoires involved in fungal nutrition could improve the mycorrhization protocols for EEMMs. The aim of this chapter is to summarize the main methods currently used for inoculating EEMMs, including their preparation, application, limitations, and future perspectives.

9.2 Inoculum Types

Several types of natural and laboratory-produced EM fungal inocula have been used in the past to colonize plant roots in sterile, semi-sterile or non-sterile conditions (Repáč 2011). However, only three types of fungal material are extensively used as EEMM inoculum: spores (gamic inoculum), mycelial pure cultures (vegetative inoculum), and colonized roots (symbiotic inoculum).

9.2.1 Spore Inoculum

Spores are largely used for mycorrhizal synthesis in the greenhouse (semi-sterile conditions) but not to produce mycorrhizas in vitro. In fact, it is difficult to obtain sterile and vital spore inocula. The associated contaminants, such as bacteria or fungal parasites, may lead to unpredictable effects by favoring or selectively inhibiting root colonization (Bedini et al. 1999).

A fungal species is suitable for spore inoculation when (1) fruiting bodies are easily found and (2) its spores are produced in large amounts and can rapidly and extensively colonize the host plant root system (Lu et al. 1998). These characteristics made Gasteromycetes (*Pisolithus* spp., *Scleroderma* spp., *Rhizopogon* spp.) ideal candidates for infecting forest tree species with spores (Repáč 2011). Spore inoculum has also been successfully used for different EEMM species such as *Terfezia* spp. (see Chap. 14), *Lactarius* spp. and *Suillus* spp. (González-Ochoa et al. 2003). However, large-scale inoculation programs with spores have been applied in commercial nurseries only for *Tuber* species, and for more than 30 years, spores have been the preferred inoculum for producing infected plants with *Tuber melanosporum Vittad.*, *Tuber aestivum Vittad.*, and *Tuber borchii Vittad.* (Hall et al. 2007; Karwa et al. 2011).

There are many advantages to spore-based inoculations. Inoculum is relatively cheap, easy to prepare and less time consuming, and it does not require specialized equipment or training. Spores are obtained by crushing whole sporomata (in case of truffles) or only the fungal caps (in case of porcini or gilled mushrooms) using a

mortar and pestle or an electric mill or blender. To facilitate fungal tissue grinding, sterile water coupled with sand or other types of fine abrasive particles may be added into the crushing process. The spore suspension obtained can be injected into the planting hole or absorbed by sterile vermiculite that preserves the spores between lamellae, thus allowing inoculum placement around the root system. In the literature, there are many recommendations concerning the number of spores to provide to each plant. Although these values may vary according to species and experimental conditions, at least 10^6 spores per plant are generally recommended (Weden 2004; Hall et al. 2007).

Either fresh field-collected fruiting bodies or preserved fungal material can be used as source for spore inoculum. Preservation is often necessary because EEMM fruiting bodies are often not available at inoculation time (seasonal and/or irregular fruiting) so that short- or long-time preservation of spores is required. Moreover, preservation could be useful to reduce production costs, which are affected by the fluctuating economic value of EEMM fruiting bodies (e.g., truffles) used in spore inoculations.

Truffles are preferentially stored for short time in sterile moist sand at 4 °C. This method of preservation maintains unchanged their infective potential for up to 2 years (Zambonelli et al. 2010) and improves spore germination, perhaps due to the activity of microorganisms present in the fruiting bodies (Hall et al. 2007). Air-drying, freezing at −20 °C, and freeze-drying are simple and cheap practices commonly applied for long-time preservation of EEMMs.

A major concern with the spore inoculum technique has to do with the accidental presence of nontarget EM fungal propagules. If introduced, these undesired EM fungi may become established on the host plant root system where it may partially or completely replace the target species. This problem is relevant for truffles when large quantities of fruiting bodies are used to prepare the inoculum and, particularly, when low-grade fruiting bodies are used as inoculum (a tactic to reduce the cost of inoculum that we do not recommend). Low-grade truffles usually include immature or deteriorated fruiting bodies and nontarget taxa that may be difficult to identify. The incorporation of these less valuable and potentially more infective species of truffles can contaminate entire batches of plants (Ferrara and Palenzona 2001). In this context, *T. borchii* and *Tuber maculatum* Vittad. can contaminate *Tuber magnatum* Pico inocula, while *Tuber brumale* Vittad. or *Tuber indicum* Cooke & Massee can spoil *T. melanosporum* inocula (Tibiletti and Zambonelli 1999). The recent detection of *T. indicum* mycorrhizas in *T. melanosporum* truffle plantations in Europe and the USA (Murat et al. 2008; Bonito et al. 2011) demonstrates that lackadaisical applications of spore inoculum may lead to not only economic losses but also ecological damage through the introduction of invasive alien species. In order to avoid these problems, many countries require morphological and molecular taxonomic verification for all fruiting bodies used for inoculation. To this aim, rapid molecular identification based on direct PCR (Bonuso et al. 2006; Bonito 2009) might also be applied to reduce analytical costs and time.

9.2.2 *Inoculating with Mycelium*

Mycelium has been considered the most suitable inoculum source for infecting plants with EM fungal strains (Marx and Kenney 1982). However, the large-scale nursery application of this inoculum type strongly depends on the saprobic potential of the fungal species of interest and its ability to produce large amounts of mycelium in axenic conditions. Mycorrhizal synthesis using pure mycelial cultures has been obtained for many edible ectomycorrhizal (EEM) ascomycetes and basidiomycetes, either in greenhouse or in vitro conditions (Águeda et al. 2008; Danell and Camacho 1997; Giomaro et al. 2005; Yamada et al. 2001; Zambonelli et al. 2008). However, it is only extensively used for a few EEMM species such as *Lactarius deliciosus* (L.) Gray and *Tricholoma matsutake* (S. Ito & S. Imai) Singer (see Chap. 16). The main limitation in using EEMM mycelia as inoculum has to do with the difficulty in maintaining pure cultures and in producing adequate amounts of biomass for large-scale mycorrhization programs. This is particularly challenging for truffle species.

Mycelial inoculum has four main advantages over spore inoculum (1) lower contamination risks, (2) fewer problems with fluctuation in availability or cost, (3) higher rate of infection, and (4) better genetic tractability. The use of pure cultures can ensure that inocula are free of undesired EM fungi or contaminants that may inhibit root colonization of the target fungal species. Mycelia are also able to colonize the fine roots more rapidly than spores, thus reducing the risk of contamination during plant growth. For example, some mycelial strains of *T. borchii* are able to form mature mycorrhizas on *Quercus robur* L. in less than 1 month in semi-sterile conditions (Iotti, unpublished data). Moreover, vegetative inoculum enables the infection of plants with fungal genotypes specifically selected for infectivity, host plant affinity, ecological conditions or fruiting characteristics. This is important given that genetic differences between *Tuber* strains result in different levels of infection and in different affinities to the host plant both in vitro (Giomaro et al. 2000) and in greenhouse (Zambonelli et al. 2008) conditions. When suitable strains are available for mycelial expansion, it is possible to optimize the mycorrhization process, bypassing many constraints encountered when using spores as inoculum.

Mycelia of EEM fungi can be generated from either spores, ectomycorrhizas or fruiting bodies, but the success of this procedure depends on the quality of the source used (purity and age). In vitro germination of spores has been reported for a number of EEMM species including *T. melanosporum* (Fischer and Colinas 2005), *T. matsutake* (Murata et al. 2005), and *Cantharellus cibarius* Fr. (Danell 1994) yet is challenging for most EEMM species (Fries 1987; Nara 2009). Resulting mycelium from single spores is haploid (n), although basidiomycetes may quickly undergo plasmogamy to form dikaryons (n + n) if compatible monokaryons are available. Isolation of mycelia from ectomycorrhizas is more feasible, although the risk of contamination by other species of free-living, endophytic, or rhizospheric fungi or bacteria can be high. For this reason, ectomycorrhizas must first be surface sterilized in order to destroy propagules of contaminants but safeguard the vitality of the symbiotic fungus. To surface sterilize roots, excised root tips are washed two to

three times in sterile water by vortexing and spinning and then dipped in a calcium or sodium hypochlorite solution for 1 or a few minutes, according to the concentration of active chlorine. Alternatively, a 1–2-min bath in a 3–6 % hydrogen peroxide solution is used to sterilize root tips. After sterilization, mycorrhizal tips are rinsed three times with sterile distilled water and are placed on or submerged in Petri dishes filled with appropriate medium (see below) supplemented with antibiotics (e.g., 50–100 μg/ml of streptomycin) (Mukerji et al. 1998). Still, most pure cultures of EMM fungi have been obtained from fresh fruiting bodies, which offer lower contamination risks and easier isolation procedure. To isolate from fruiting bodies, small fragments of fungal tissues (<1 mm) are excised from the truffle gleba (preferentially from the white sterile veins) or from the inner tissues of the intersection zone between stipe and pileus (for epigeous fungi) and then transferred on agar plates. Isolations are more successful from fresh and immature fruiting bodies. With this approach, prompt growth of the hyphae from the excised fruiting body fragment and low levels of contamination are observed, even when no antibiotics are added to the culture medium (Iotti, unpublished results). Pure cultures isolated from basidiomata tissues are stable dikaryons and should be able to fruit after establishing symbiosis with the host plant. Some EEM basidiomycetes are also able to produce primordia or mature fruiting bodies in vitro, such as *Boletus reticulatus* Schaeff. (Yamanaka et al. 2000), *Lyophyllum shimeji* (Kawam.) Hongo (Ohta 1994) and *Phlebopus portentosus* (Berk. & Broome) Boedijn (Sanmee et al. 2010). In contrast, because pure cultures originated from ascomata of heterothallic ascomycetes such as *Tuber* spp. are composed only by homokaryotic maternal tissues, they are unable to fruit. For this reason, at least two compatible mycelial strains are necessary for producing fertile truffle orchards, as both mating types must be present to guarantee fruiting body production after planting (Rubini et al. 2007).

Once mycelium has been isolated on appropriated agar (more rarely Phytagel or Gelrite-based) media, isolates are incubated in the dark at 20–22 °C and maintained in active growth through repeated subculturing. The growth curve of a fungal colony developing on an agar plate proceeds in four phases (1) lag, (2) initial growth, (3) linear growth, and (4) staling (Hudson 1992). The shape of the curve is heavily dependent upon the fungal species and cultural conditions, but generally EEMM mycelia show longer phases and lower growth rates compared to saprobic fungi and other nonedible EM fungi (Iotti et al. 2002, 2005). For example, the lag phase of *Tuber* mycelia is commonly 7 days long and maximum growth rates are less than 1 mm per day. It is so for *T. magnatum* mycelia recently isolated in our laboratory, reaching the staling phase after one mm of radial growth in each subsequent culture. During the staling phase, many colonies lose their vitality and, consequently, the ability to generate new colonies. For this reason, it is necessary to cut an agar plug with hyphal tips from the edge of the fungal colony and to transfer it in a new plate when hyphae are still in active growth. Similarly, mycelial senescence is detrimental for root colonization.

Mycelial agar plugs and mycelial slurries from liquid cultures have been commonly used to synthesize ectomycorrhizas in a variety of in vitro systems (Petri plates, flasks, jars, tubes, etc.) (Repáč 2011). However, most common procedures

for preparing high amounts of mycelial inoculum come from the method proposed by Molina (1979). As a general rule, isolates are initially grown in flasks as submerged cultures (30–50 days). Actively growing mycelium is then mechanically fragmented, and the resulting mycelial slurry is used to inoculate tubes containing a mixture of sterile peat moss and vermiculite (1:29 v/v) moistened with the liquid medium (see Sect. 9.2.2.1). After a further 30–50 days of growth in the dark at 22 °C, the colonized substrate is removed from the tubes, washed with tap water and squeezed dry 5 times to remove fungal catabolites and residual nutrients. The vegetative inoculum is then mixed into an appropriate potting mixture or is localized around the plantlet root systems. This procedure enables one to obtain a rapid and uniform colonization of the substrate (Molina and Palmer 1982) but it is a time-consuming procedure. However, a number of biotechnology approaches can be applied to improve production processes of mycelial inoculum. Submerged fermentation in bioreactors is the most promising approach for efficient production of mycelia in liquid culture, and it also has recently been applied to EEMM species (Carrillo et al. 2004; Tang et al. 2008; Liu et al. 2009). Bioreactors allow production of higher amounts of fungal biomass in shorter time because fermentation parameters such as temperature, pH, oxygen, organic and inorganic nutrients, and fungal catabolites can be continuously controlled, maintaining the optimal conditions for mycelial growth.

Promising approaches for producing commercial mycorrhizal inocula also involve the use of various combinations of natural, semi-synthetic, and synthetic polymers (Siddiqui and Kataoka 2011). Entrapment of mycelial fragments in natural polysaccharide gels, such as calcium alginate, is a proven method to obtain efficient inocula for root colonization with different EM fungal species (Repáč 2011). However, this inoculant technology was unsuccessful for some EEMMs. In fact alginate-entrapped inocula of *L. deliciosus* (Parladé et al. 2004) and *Tuber* spp. (Kuek and Zambonelli, unpublished data) completely failed in forming mycorrhizas when mixed with the plant growth substrate, although viability of their mycelia was proven to be unaffected. More studies are needed to find and test alternative polymers that do not affect the colonization ability of the EEMM mycelia.

9.2.2.1 Culture Media

Many EEM fungi can be cultured in semi-synthetic and synthetic culture media, although the growth rate is extremely variable between species. Mycelia of *Boletus* spp., *Amanita* spp., *Tricholoma* spp., and *Lactarius* spp. are routinely maintained on the most common media (or their modified version) reported in the literature, such as potato dextrose agar (PDA), biotin–aneurin–folic acid (BAF) agar (Oort 1981), malt extract agar (MEA), and modified Melin-Norkrans (MMN) agar (Marx 1969). Specific media have also been developed to better support the growth of *C. cibarius* (modified Fries medium) (Danell 1994) and *Tuber* spp. (modified woody plant medium) (Iotti et al. 2002).

9.2.2.2 Preservation

Preservation of pure EEMM cultures is a priority. Beyond guaranteeing purity and viability of valuable EEMM strains, preservation has to guarantee the maintenance of their morphological, physiological and genetic integrity over time (Ryan and Smith 2004; Voyron et al. 2007). Many methods about fungal preservation are available (Nakasone et al. 2004), but only a few of them are suitable for EEMM species, in particular for *Tuber* mycelia (data not published). The choice of preservation method depends on the species of interest and project aims. Short-term preservation by repeated subculturing is simple, cheap and valid for most EEM fungi, but the risk of contamination as well as desiccation of the agar is high. Moreover, repeated transfers can lead to losses in vigor and infectivity due to mutations or modifications of gene expression (Coughlan and Piché 2005). To compensate this problem, in some cases, changes in media composition (i.e., alternation of nutrient-rich media with nutrient-poor media) can rejuvenate old cultures and minimize viability loss (Nakasone et al. 2004; Repáč 2011).

A low-cost method for long-term culture preservation is oil overlay. This consists of submerging a fungal colony grown on agar slants completely with oil. The tubes must be tightly sealed and the oil level must be checked periodically and refilled as necessary. Similarly, disks cut out of fungal growth on agar can be stored in tubes containing sterile distilled water. Cultures maintain highest vitality when stored at 4 °C (Richter 2008). Such conditions are valid only for some EEMM species. For example, long-term vitality of *Tuber* mycelia cannot be guaranteed in these conditions (personal observation).

Cryopreservation in electrical freezers at −80 °C is a technique with high survival rates (Kitamoto et al. 2002). In this method, small agar plugs sampled from actively growing fungal colonies are preserved into cryotubes containing the appropriate liquid media and dimethyl sulphoxide (DMSO) or 10 % glycerol as cryoprotectants. The cryotubes are then placed directly in a deep freezer. Some EM fungal species may remain viable under these conditions longer than others (Obase et al. 2011).

Another valuable ultra low temperature technique of preservation is cryopreservation in liquid nitrogen. It was assessed for the first time in 1953 for human sperm, and it is now becoming routine in mycology. For example, microbial collections at the American Type Culture Collection (ATCC) are cryopreserved in liquid nitrogen. In this process, hyphae are preserved by cooling to low sub-zero temperatures (−196 °C), at which all biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. Thus, cellular and genetic damage is prevented. The use of cryoprotectants is indispensable in preventing cellular damage resulting from decreases and increases in temperatures. Cryoprotectants generally have two modes of action: intracellular penetrating agents, such as DMSO and glycerol, and extracellular agents such as sugars (sorbitol, sucrose, lactose, trehalose, and others). By preserving cultures through deep freezing mutations are prevented, labor is reduced, infectivity is preserved, and cultures can readily be accessed. The main disadvantages of this technique are the high costs

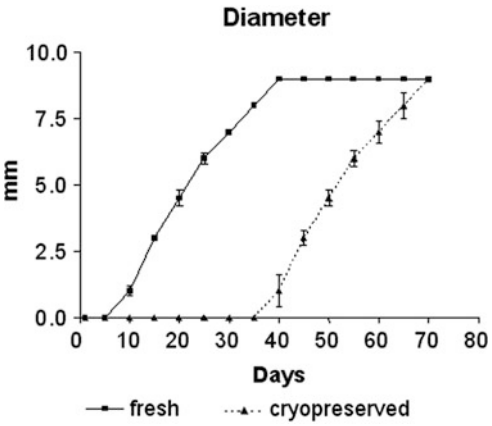

Fig. 9.1 Development of *T. borchii* mycelium before and after cryopreservation

required to purchase and maintain the deep freezing. Moreover, cryopreservation determines an increase in the lag phase of the first subculture after thawing. Cryopreservation success of pure cultures varies among species and strains, as concluded by Nagai et al. (2000).

The first successful attempts of cryopreservation with EM mushrooms were performed on *C. cibarius*, by Danell and Flygh (2002). We adopted such procedure on *Tuber* species after some small changes: replacement of DMSO by glycerol or addition of sucrose to DMSO. The first attempts of cryopreservation in liquid nitrogen of *T. borchii* were positive: after 2 months of cryopreservation, the two strains tested regenerated hyphae after thawing, although their lag phase was much longer than the one of the fresh transplanted mycelium (Fig. 9.1). However, once the lag phase was over, mycelium growth of the cryopreserved strains was similar to the one observed for the fresh strains. Similarly, initial attempts of deep freezing of *T. borchii* at −80 °C were successful too: after 2 months of cryopreservation followed by 20 days of direct storage −80 °C, the same strains developed hyphae, but in this case the lag phase was much shorter (8 days) (Piattoni et al. 2010). The same procedure of cryopreservation was applied to a single strain of *T. aestivum*. Again, tissue growth was re-established; however, the lag phase was much longer than observed for cryopreserved *T. borchii* strains (Piattoni et al. 2010).

9.2.3 Colonized Root Inoculum

Inoculum with colonized EM root tips allows one to obtain well-colonized plants regardless of the EEMM species. This approach exploits the high infectivity of hyphae emanated from active symbiotic tissues. This method was perfected for *Tuber* species to bypass the need of using spores, for instance, for species whose spores are difficult to germinate, such as *T. magnatum* (Gregori 2002) or for species which mycelial strains have low infectivity. The inoculum consists of whole

mycorrhized plants called "mother plants" (Zuccherelli 1990) or root fragments infected with the target EEMM (Chevalier and Grente 1973). In the first case, the mother plants, previously inoculated with spore or mycelium, are grown in the middle of large pots surrounded by sterile plantlets so that infected and uninfected root systems are in close contact. In the second case, mycorrhizal fragments are aseptically excised from the washed root system of the mother plant and then added to the potting mix next to the uninfected plantlet roots. Large-scale production of colonized root inoculum requires the use of numerous and well-infected mother plants initially obtained using sporal or mycelial inoculation techniques. However, the long-term maintenance of the mother plants in greenhouse conditions carries a high risk of spreading undesirable and competitive EM fungi, which can contaminate complete batches of plants. Although frequent and careful controls may be planned by growers, contaminated roots may escape detection. To reduce the risk of contaminations, colonized root inoculum may be obtained in vitro by mycelial inoculation. Over the last 50 years, a large number of in vitro techniques to grow whole plants or root organ cultures associated to EM fungi have been developed (Coughlan and Piché 2005; Giomaro et al. 2005), but they have been exploited only to improve the understanding of plant–fungus interactions. The better advances in this research sector involved the use of transformed root organs obtained by transferring of the Ri T-DNA plasmid of *Agrobacterium rhizogenes* into the host plant genome. Transformed organs show increased growth rate and branching without addition of exogenous plant growth regulators to the culture medium, and they can be used to produce mycorrhizas in axenic culture. However, transformed root clones of an EM plant able to establish EM association have only been obtained for *Cistus incanus* L. (Wenkart et al. 2001; Zaretsky et al. 2006). Mycorrhized transformed roots of *C. incanus* increased the hyphal growth of *T. melanosporum* mycelium and preserved its vitality and infectivity for months (Ventura et al. 2006). The characteristics of *C. incanus* root organs are appropriate for the long-term in vitro maintenance of EM isolates and of their infectiveness; moreover, they represent an alternative source of clonal inoculum for plant mycorrhization (Coughlan and Piché 2005). Recently, a method to obtain *T. magnatum* mycelium and mycorrhizas by using transformed roots has also been patented (Buee and Martin 2009).

9.3 Substrate

The type of soil used to grow inoculated plants affects the rate of root colonization by the target fungal species and potential contaminants. Donnini et al. (2009) and Zambonelli et al. (2009) demonstrated that the use of different soils affects the competitive abilities of truffle species. Indeed, natural soils from suitable areas have long been used as substrates to grow mycorrhized plants. However, large-scale operation for commercial purposes is not always feasible due to economic and ecological reasons. Given this, various potting mixes and soil-less plant growth

media have been developed by nurserymen. However, specific details about the composition of the potting mixes, inoculum amounts, amendments, and greenhouse conditions vary among nurserymen, and most remain trade secrets (Hall et al. 2003). Vermiculite, perlite, wood ash, bark, sand, dolomite, green compost, and peat moss are the most common ingredients used to prepare potting mixtures. In general, truffle species prefer low organic matter mixtures supplemented with calcium carbonate components (Pruett et al. 2009) to raise pH to ~8.0, whereas porcini mycorrhizas grow well in acid and peaty potting mix. Vaario et al. (2002) demonstrated that pine bark is a suitable substrate component for mycorrhization of *T. matsutake* on its host plant. Pot type, pot size, and shape used to grow inoculated plantlets are also crucial to develop appropriate highly branched root systems, optimize the effectiveness of the inoculum mass and facilitate good quality control (Palanzòn and Barriuso 2007).

9.4 Quality Control of the Mycorrhized Plants

Quality control measures are essential to successful EMM cultivation. A number of methods for the certification of truffle-in plants have been proposed by different scientists (Palanzòn and Barriuso 2007; Sisti et al. 2010). The percentage of fine roots colonized by the inoculated fungal species and the presence of undesirable EM associations are used as the main criteria for quality evaluation of mycorrhized plants. A high level of root colonization before transplanting is advisable for the development of secondary infections in the field. However, it is difficult to establish a suitable level of root colonization to guarantee the fungus persistence and spreading on roots after outplanting. The optimum level of mycorrhization mostly depends on the climatic and soil characteristics of the plantation site, which affect competitiveness, extent and rate of spreading of the EEMM inoculated species. Zambonelli et al. (2005) showed that a 30 % initial rate of root colonization with *T. aestivum* reached values of 50–70 % in mycorrhizal seedlings after 5 years when planted in a suitable soil. Hortal et al. (2009) found a higher persistence of *L. deliciosus–Pinus pinea* L. ectomycorrhizas with an initial colonization percentage over 50 %. A threshold colonization rate of about 30 % was considered acceptable by many authors (Wang and Hall 2004; Bencivenga et al. 1987; see also Chap. 14), although higher colonization levels of the target species favors the persistence of the introduced EEMM species after planting in field soils.

Although certification of commercially mycorrhizal plants is performed by some nurserymen, with the assistance of public research institutes, it is still not common for the industry and consistent standards are lacking. Also, performing large-scale quality control measures for entire plant batches is time-consuming and expensive. Its cost may represent up to 10–15 % of production costs (Bernardini, Raggi Vivai, personal communication). Moreover, previously such nursery sector has been

poorly defined and regulated by existing laws. At the assessment time, however, in few Italian regions mycorrhizal plants offered for sale are to be evaluated based on standard procedures (Bagnacavalli et al. 2012).

Root colonization by undesired fungal species may be due to negligent nursery management practices or inoculum contamination. In the first case common sources of contamination include airborne spores, insufficiently or non-sterilized potting mixtures and equipment (Brundrett et al. 2005). However, mycorrhizas of these opportunistic contaminants are relatively easy to detect with a microscope because they usually display distinctive anatomo-morphological characteristics. For example, mycorrhizas of *Pulvinula convexella* (P Karst.) Pfister (=*Pulvinula constellatio*) and *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička are common greenhouse contaminants but differ from truffle ectomycorrhizas on the basis of their mantle type and emanating hyphae (Amicucci et al. 2001). Inoculum contamination is much more difficult to detect because the contaminant species are congeneric with the target EEMM (see Sect. 15.2.1) and their mycorrhizas have often similar morphological features (Zambonelli et al. 1995; Kovács and Jakucs 2005; Águeda et al. 2008). Thus, quality control measures prior to outplanting are critical.

For these reasons, the use of molecular tools for quality control assessments is becoming more widely used across the EMM industry. This compensates for the limitations of morphological identification and helps to reduce the marketing of mycorrhized plants of poor quality. During the last 20 years, many PCR-based methods have been developed for species-specific identification of a large number of target fungi, including most of the valuable EEMM species (see Chap. 7). Most of these are based on the amplification of a small fragment of the fungal genome (usually the ITS regions of rDNA) with specific primers in single or multiplex PCR reactions (see Table 7.1). This approach is also amenable to direct PCR, thus saving labor and time costs (Iotti and Zambonelli 2006; Bonuso et al. 2006; Bonito 2009; Bonito et al. 2011).

It is undeniable that molecular analyses increase the cost of quality controls and, consequently, the price of mycorrhized plants. Yet, these costs are minimal compared to the cost of properly establishing an EMM orchard and are a wise safeguard against failure. Quality control measures will vary depending on inoculation procedure used. Generally, when using pure culture-based inocula, the molecular confirmation of the isolated strains coupled with morphological check for post-inoculation contaminants is sufficient. Similarly, when spore inoculation methods are applied, analyses should be carried out on inoculum prior to inoculating plants to guarantee that the correct species is being inoculated. Root systems and ectomycorrhizal colonization levels of inoculated plants ought to be checked prior to being sold or planted to confirm that roots are healthy and well colonized by the target species. If poorly mycorrhized plants are introduced in the field, nontarget fungal species may take over the root system resulting in orchard failure. A current challenge for the EEMM industry is the lack of quality control standards (that can be applied internationally) and the lack of independent labs available for testing inoculated plants using molecular and morphological approaches.

9.5 Conclusion

Unfortunately, recent advances in biotechnologies have been poorly utilized in large-scale production of mycorrhizal plants. Truffle-infected plants are still produced using the traditional spore inoculum techniques which, although efficient, do not offer the opportunity to use select fungal genotypes in producing mycorrhizal plants. In contrast, mycorrhizal plants from other EEMMs are routinely produced with mycelia, but we are not aware of any genetic screening programs in place for these isolates. Mycelium-based inoculation procedures offer the possibility for selecting fungal strains according to the different ecological conditions. This could be important for widely distributed species, such as *T. aestivum* and *T. borchii* in Europe, which exhibit great genetic diversity and perhaps contain cryptic species with different ecological requirements (Weden et al. 2005; Bonuso et al. 2010). Moreover, mycelium-based inoculations offer other advantages including lower risks of contamination and a more standardized level of colonization than is obtained with the more heterogeneous spore inoculum. Still, more research is needed on the physiology and nutritional requirements of each EEMM in order for large-scale culturing of their mycelia and reliable inoculum production. New insights into mycelial biomass production and symbiotic establishment are expected to result from recent mycorrhizal genome sequencing programs. Moreover, the development of mycelium-based technologies must be in step with the improvement and application of cryopreservation to avoid loss of valuable fungal genotypes and preserve EEMM biodiversity over time. To this end, a cryobank for EEMM species is advised.

In summary, modern biotechnologies developed at the end of the last century brought the first great opportunity for quality control of commercial EM plants inoculated with EEMMs. Molecular characterization of EEMMs minimizes the risk of misidentification and enables processing of a high number of samples resulting in a parallel saving of labor and expense. However, molecular identification of fungal symbionts is still not routine, even though costs of reagents and equipment continue to decrease and molecular tools continue to be developed for diagnostics. In order to further reduce costs and time involved in root-tip morphological typing and tip counting, quality control measures of commercially infected plants could be nearly entirely performed by molecular tools. In this way, the quantitative assays specifically developed to estimate the mean amount of biomass of an EEMM species on the roots of a plant batch as well as the level of contaminants can be optimized and may represent one of the main priorities of future research programs.

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