Chapter 3
Ectomycorrhizal Inoculum and Inoculation Techniques

Ivan Repáč

3.1 Introduction

ECM symbiosis plays an important role in physiology, ecology, resistance, production, and other aspects of life of a single trees, populations, and ecosystems of ectotrophic forest tree species. Among the various ways of improving the early growth and survival of forest plantations, controlled mycorrhization by inoculating seedlings with selected fungal strains is an energy-efficient and environment-friendly alternative to fertilization or soil tilling (Kropp and Langlois 1990; Marx 1991; Garbaye and Churin 1997). Trees planted particularly in soils deficient in ECM fungi (e.g., mine spoils, polluted areas, agricultural, and other treeless lands) and even in routine reforestation sites may benefit greatly from ECM inoculation (Marx 1991; Castellano 1996; Querejeta et al. 1998; Duñabeitia et al. 2004; Núñez et al. 2006), although the positive effect of inoculation is not consistent (Castellano 1996; Corrêa et al. 2006; Menkis et al. 2007; Baum et al. 2009). Several types of natural and laboratory-produced inocula and several application techniques have been used for seedling inoculation. Forest soil, litter, humus, or excised ectomycorrhizae were substituted by fungal spores and mycelial inoculum (Marx and Bryan 1975; Le Tacon et al. 1983; Duponnois and Garbaye 1991; Parladé et al. 1999). Vegetative inoculum appears to be the most appropriate and effective method of inoculation (Marx and Kenney 1982; Mortier et al. 1988; Garbaye and Churin 1997; Rincón et al. 2007). Artificial mycorhization with vegetative inoculum consists of several steps – selection of ECM fungi, isolation and maintenance of fungi in pure cultures, preparation of fungal inoculum, and inoculation of seedlings.

A large body of literature exists on ECM inoculum preparation and application in a research scale; a comprehensive review of the literature and of all aspects of this topic is impossible. Although there have been previous studies on inoculum preparation and inoculation techniques, this chapter attempts to bring together a

I. Repáč
Department of Silviculture, Technical University in Zvolen, Forestry Faculty, T.G. Masaryka 24, SK-96053, Zvolen, Slovak Republic
e-mail: repac@vsld.tuzvo.sk

M. Rai and A. Varma (eds.), *Diversity and Biotechnology of Ectomycorrhizae*, Soil Biology 25, DOI 10.1007/978-3-642-15196-5_3,

brief review of the major inoculum types and methods of their application developed and to offer a list of selected references as a source of other literature and detailed description of a particular material, procedure or product.

3.2 Isolation and Manipulation of Fungal Pure Cultures, Nutrient Media

Isolation, cultivation, and maintenance of ECM fungal cultures are crucial phases in artificial mycorrhization of forest tree seedlings (Molina and Palmer 1982). Many, but far from all, fungi can be cultivated in pure culture in the laboratory under controlled conditions. The fungal tissues are most commonly isolated from young and healthy fruit bodies as soon as possible after collection but can also be isolated from surface sterilized ectomycorrhizae, sclerotia, and rhizomorphs (Molina and Palmer 1982; Obase et al. 2009). The pieces of the tissues are usually transferred to sterile glass tubes on agar nutrient media. Tissue transfers and fungal cultures are incubated in the dark at 21–24°C for 2–6 weeks. Between the cultivation cycles the stock cultures are stored approximately for 2 months at 2–4°C. Higher rates of agar cultures are cultivated in Petri dishes and submerged cultures in Erlenmeyer flasks.

One of the most common nutrient media was the Modess modification of the Hagem semisynthetic formula (Molina and Palmer 1982). Modess medium was further modified (e.g., amended with thiamine by Timonen et al. 1997) and is still used (e.g., Niemi et al. 2006). Modified Melin–Norkrans medium (MMN) (Marx 1969) is probably the most used worldwide for experimental procedures (e.g. Fortin et al. 1980; Wong and Fortin 1989; Repáč 1996a; Gáper et al. 1999; Parladé et al. 1999). The original solution was a synthetic solution (no malt extract) with only 25 μg of thiamine and 2.5 g of glucose. MMN altered by Vrålstad et al. (2002) contained only 1.0 g of glucose to reduce possible adverse effects on ECM formation caused by high levels of exogenous carbon. MMN was also modified by Niemi et al. (2002) and Langer et al. (2008). Pachlewski medium (Pachlewski and Pachlewska 1974) was used, e.g., by Duponnois and Garbaye (1991) and Parladé et al. (1999). Moore et al. (1989) cultivated cultures of fungus *Cantharellus cibarius* on special species-specific C-medium. In the study of Guerin-Laguette et al. (2003), stock cultures were maintained on agar medium proposed by Ohta (1990), and mycelium was grown on Tween- or olive oil-supplemented A medium. Fungal biomass increased up to 15-fold as a result of olive oil incorporation.

Basic components of natural media are leachates of various crops such as potato, cabbage, wheat, and others. Although use of natural formulations is less frequent than semi-synthetic or synthetic ones, potato dextrose agar (PDA) was used in the past years, e.g., by Parladé et al. (1996a) and Giomaro et al. (2002). MMN (Marx 1969), BAF (Moser 1960), KHO (Šašek and Musílek 1967), and malt–peptone (50 ml of brewery wort + 5.0 g of peptone to 1,000 ml of H_2O) media are used for manipulation of fungal cultures in our laboratory of Department of Silviculture,

Forestry Faculty, Technical University in Zvolen, Slovakia (project VEGA 1/0516/09). The media are changed in recultivation cycles to support the vitality and resistance of cultures.

3.3 Synthesis of Ectomycorrhizae in Controlled Conditions

Syntheses of ECM in controlled conditions are important techniques for verification of symbiotic relationships (compatibility) between intended fungi and host trees, structural, physiological and biochemical studies, and ultimately the genetic control of ECM formation. Depending on the research goal and synthesizing apparatus either sterile, semi-sterile, or non-sterile techniques can be used. The purpose of this Section (and also Sect. 3.4) is not a comprehensive categorization, but just a review of some of the most often reported or meaningful inoculum types, methods of inoculum preparation, and inoculation procedures. For better understanding, see illustrations of the apparatus of synthesizing techniques, e.g., in the works of Peterson and Chakravarty (1991), Duponnois and Garbaye (1991), and other related literature cited.

3.3.1 Fungal Cultures Preparation

Fungi are applied into synthesis system most often as agar culture transfers. Usually, agar cultures are grown on nutrient agar in Petri plates for 3–4 weeks, then transferred onto fresh solid medium, and incubated for a few days. Several plugs (5–8 mm diameter) are then aseptically removed and placed around the roots in the synthesis apparatus. Mycelial agar plugs served as inoculum in Petri dish synthesis systems, e.g., in experiments of Wong and Fortin (1989), Brunner (1993), Gáper et al. (1999), Bending et al. (2002), Krüger et al. (2004), and Sarjala and Taulavuori (2004). Fortin et al. (1980) used plugs of mycelium in ECM synthesis in growth pouches, Högberg et al. (1999) and Giomaro et al. (2002) in vessels, and Vrålstad et al. (2002) in conical flasks. In Petri dishes, Herrmann et al. (2004) inoculated roots with a nylon sheet carrying 7–8 fungal plugs and Niemi et al. (2006) using two filter paper strips covered by mycelium.

Apart from agar cultures, fungi are usually introduced into synthesis system from liquid cultures (slurry of mycelium). Because the liquid cultures are shaken just before inoculation, the numerous mycelial fragments will yield rapid and uniform colonization of the synthesis substrate (Molina and Palmer 1982). Molina (1979) cultivated mycelium in glass tubes filled with MMN nutrient solution and small chips of broken glass; cultures were shaken periodically to fragment the mycelium. In experiments of Duponnois and Garbaye (1991), Brunner (1993), Parladé et al. (1996a), and Obase et al. (2009), fungal mycelium was grown for 3–4 weeks on a shaker in Erlenmeyer flasks containing liquid medium. The

mycelium was then washed in tap water to remove residual nutrients, homogenized in a blender for a few seconds, and resuspended in distilled water. Guerin-Laguette et al. (2003) collected mycelium of *Tricholoma matsutake* grown in liquid medium over nylon mesh filter, washed it thoroughly with and resuspended in 100 ml of a modified nutrient medium (NM). Flores et al. (2008) reported cultivation of *Laccaria bicolor* in flasks with semi-liquid MMN medium (1.5 g agar.l^{-1}) in the dark at 23°C. The mycelia obtained were filtered through a 65-μm net, washed in sterile water to eliminate sugars and to reduce the growth of contaminants in the seedling root systems, fragmented and homogenized by manual agitation, and then resuspended in distilled water (70–80 g mycelium.l^{-1}).

Vermiculite–peat (Marx and Bryan 1975) and alginate-bead (Le Tacon et al. 1983) inocula can be used in ECM synthesis at controlled conditions, even though they are primarily used in the operational inoculations. Preparation of these inoculum types is described in detail in Sect. 3.4.1.3. Mycelial plugs were laid on top of vermiculite–peat mixture (4:5–1:5, v:v) moistened to field capacity with modified liquid Pachlewski medium to prepare vermiculite–peat inoculum for synthesis in sterile conditions (Duponnois and Garbaye 1991). Corrêa et al. (2006) and Martín-Pinto et al. (2006) inoculated this substrate mixture with liquid fungal cultures. Alginate-bead inoculum of *Laccaria laccata* was used to synthesize ectomycorrhizae in aseptic conditions of glass test tubes (Duponnois and Garbaye 1991) and inoculum of *Amanita rubescens* and *Hebeloma sinapizans* in polyethylene pots in a plant growth cabinet (Kozdrój et al. 2007).

3.3.2 Inoculation Techniques

3.3.2.1 Sterile Techniques

Petri Dishes

Petri plates are probably the most extensively used method of ectomycorrhiza synthesis. Wong and Fortin (1989) described a Petri dish technique that avoids limitations of previous ones. In Petri dish filled with sugar-free agar medium, two sheets of nylon membrane sandwiched the root and were overlaid with a sheet of filter paper to keep the exposed surface of the roots moist. Cotton rolls were placed along the opposite edge of the Petri dish to absorb water which condensed during incubation. The filter paper was removed and the fungal plugs were placed on the membrane beside root laterals. Peterson and Chakravarty (1991) differentiated between simple system, divided Petri plates, sandwich technique, and nylon mesh method. Sandwich technique (mycelial plugs placed on cellophane sheets) was a method of synthesis experiments conducted by Langer et al. (2008) on *Populus tremula* plantlets. Mixture of vermiculite and peat seems to be a suitable substrate for Petri dish synthesizing system (Duponnois and Garbaye 1991; Bending et al. 2002; Sarjala and Taulavuori 2004). Duponnois and Garbaye (1991) observed pure

culture synthesis of *Pseudotsuga menziesii–Laccaria laccata* in autoclaved soil or silica sand. Gáper et al. (1999) studied synthesis of *Picea abies–Inocybe lacera* and *Suillus bovinus* in a mixture of perlite, peat, florex, and keramzit (7:2:2:1). Of course, substrate is always autoclaved or sterilized regardless of the purpose of ECM synthesis; in most instances substrate is disinfected or fumigated in other inoculation experiments, including operational conditions. To protect fungus and root system from direct illumination, Niemi et al. (2006) placed a semi-circle brown paper on the lower part of the lid of 14-cm Petri dish. To reduce the loss of plants during acclimatization, Krüger et al. (2004) placed a 90-mm Petri dish system in a 140-mm Petri dish in which humidity was regulated with moistened paper. Herrmann et al. (2004) transferred rooted micropropagated oak plantlets into 90-mm Petri dish rhizotrons. In this system, the roots grew two dimensionally inside the dish, whereas the shoot developed outside. Between inoculation and transfer to the growth chamber, the plants were kept in plastic bags moistened with wet cotton sheets to reduce adaptation shocks for the shoots.

Flasks and Jars

Some of the early attempts to synthesize ectomycorrhizae under sterile conditions were made by Melin using Erlenmeyer flasks containing sterile sand moistened with a nutrient solution into which an aseptically germinated seedling and single fungus culture were introduced (Peterson and Chakravarty 1991). Hacskaylo improved the method by using vermiculite instead of sand (Molina and Palmer 1982). Peterson and Chakravarty (1991) described Mason jars and Leonard jars, in which shoot of seedling remains in the environment and roots in aseptic conditions inside the jars. Fungal inoculum can be added into flasks as plugs of actively growing mycelium (Vrålstad et al. 2002) or as slurry of fungal hyphae (Brunner et al. 1992) either before, at time, or after seedling introduction. Vermiculite, peat, and particularly their mixture are mostly used as substrate since their advantages had been detected and confirmed for ECM synthesis. Martín-Pinto et al. (2006) transferred 7-day-old *Pinus nigra* seedlings to 45-ml sterile flasks containing 30 ml of substrate (MMN, peat or vermiculite) colonized by the ECM fungi.

Test Tubes

Pachlewski and Pachlewska (1974) achieved good results to synthesize ectomycorrhizae between *Pinus sylvestris* and more ECM fungi in test tubes containing water agar amended with thiamine. Molina (1979) used 300 × 38-mm glass test tubes filled with 110 ml of vermiculite and 10 ml of peat moss moistened with 70 ml of MMN nutrient solution to test a wide range of ECM fungi on *Alnus* species entirely enclosed in tubes. Technique described by Molina (1979), modified with double the amount of peat, was used by Parladé et al. (1996a). Within series of experiments

studying the biology of *Pseudotsuga menziesii–Laccaria laccata* symbiosis, Duponnois and Garbaye (1991) used glass test tubes (3 × 15 cm) filled with autoclaved peat–vermiculite (1:1, v:v) moistened to field capacity with modified Shemakanova mineral nutrient solution. Inoculation was carried out either with (1) peat–vermiculite inoculum mixed with substrate (1:10, v:v) or laid on top of the tube (1–2 cm), (2) alginate-bead inoculum (five beads laid on top of the tube), or (3) mycelial suspension (injected with a syringe or deposited with a pipette). The roots were maintained in axenic conditions, while the top of the plant developed outside the tube.

Polycarbonate Boxes

Guerin-Laguette et al. (2003) introduced mycorrhizal synthesis between *Tricholoma matsutake* and *Pinus densiflora* in the following substrate and apparatus: dry soil, vermiculite, and moist *Pinus densiflora* bark (pieces of about 1 cm^2) were mixed together (1:1:1/v:v:v) before addition of distilled water supplemented with olive oil. Wet substrate was autoclaved twice before distribution into sterile 920-ml, wide-mouth, polycarbonate boxes (9.5 × 9.5 × 11.5 cm) and inoculation with mycelial slurry (1 volume of slurry into 5 volumes of substrates). The substrate (approximately 300 ml per box) was applied as five layers (60 ml each), each layer being inoculated with the slurry before application of the following layer, i.e., 10 ml of slurry for the first two layers, 20 ml for the next two, 0 ml for the last one. Five sterile 12-day-old pine seedlings were then aseptically introduced into each box. The boxes were maintained 4 months at 23°C under a photosynthetic active radiation with a 16-h photoperiod.

3.3.2.2 Semi- and Non-sterile Techniques

Growth Pouches

Growth pouches were first introduced in detail by Fortin et al. (1980). Flat, transparent polyester pouches were used for synthesis of ectomycorrhizae between *Pinus strobus* and several ECM fungi. Fifteen milliliters of MMN solution was added, and a piece of glass tube was placed along the side of each pouch to add nutrient solution and water. The root system was laid directly onto the paper pad. As soon as formation of short roots initiated, plugs of actively growing mycelium were placed on the paper pad 3–5 mm from a short root primordium. Brunner (1993) used autoclaved polyethylene pouches 13 × 16 cm including a glass fiber paper with an activated charcoal paper disk attached. Pouches were filled with 10 ml of MMN liquid medium and one *Picea abies* seedling. After 2 months, mycelial disks were placed in the vicinity of the short roots. Two strips of foam were inserted to provide air space. Advantages of this technique are that numerous seedlings can be grown in a very small space, root system can be viewed without disturbing the roots or

the fungus, and roots are clean since substrate is not involved (Peterson and Chakravarty 1991).

Pots, Containers, Vessels

A brief review of several experiments can illustrate a variety of vessels, substrates, inoculum types, and methods of their applications used in non-sterile ECM synthesis techniques. Duponnois and Garbaye (1991) used transparent boxes, rootrainers, Hiko and "M" containers filled with soil or vermiculite–peat mixture (1:1, v:v) for growing Douglas fir seedlings in the glasshouse. Liquid, vermiculite–peat or alginate-bead inoculum were applied either by mixing with the substrate before filling the containers or by spreading the inoculum on the well-developed root system. Brunner (1993) synthesized ectomycorrhizae between *Picea abies* and *Hebeloma crustuliniforme* in autoclaved cuvettes of stainless steel (15 × 12 × 2 cm) filled with a vermiculite–peat moss mixture. Homogenized mycelium grown in liquid solution was introduced into cuvettes using an inverted pipette. Högberg et al. (1999) planted *Pinus sylvestris* seedlings in 0.5-l plastic pots filled with sand inoculated with ten mycelial agar plugs placed below the pine roots. Tissue blocks of *Tuber brumale* mycelium served for inoculation of micropropagated plantlets of *Tilia americana* and *Quercus pubescens* in the vessels (12 cm diameter × 18 cm height) filled with 800 ml of peat–vermiculite mixture (1:30, v:v) in experiment of Giomaro et al. (2002). Timonen et al. (1997) planted sterile *Pinus sylvestris* seedlings in plastic pots enclosed in sterile plastic bags to maintain moisture and prevent aerial contamination. After 25 days, the roots of one seedling were inoculated with three agar plugs (5 mm^3) of mycelium. Bogeat-Triboulot et al. (2004) and Flores et al. (2008) used mycelial suspension to inoculate *Pinus pinaster* with *Hebeloma cylindrosporum* in 0.4-1 pots filled with soil and *Abies guatemalensis* with *Laccaria bicolor* in 300-cm^3 containers filled with peat moss/vermiculite (1:1, v:v), respectively. Obase et al. (2009) soaked roots of *Populus maximowiczii* in MMN liquid medium containing cultured ECM fungal mycelia. Each seedling was planted in a plastic pot (60 × 60 × 100 mm) filled with heat-sterilized volcanic debris.

Aspray et al. (2006) and Corrêa et al. (2006) inoculated seedlings of *Pinus sylvestris* and *P. pinaster* with vermiculite–peat inoculum of *Laccaria bicolor*, *Lactarius rufus*, and *Pisolithus tinctorius* in 80-ml and 350-ml pots, respectively. Kozdrój et al. (2007) transferred *Pinus sylvestris* seedlings to polyethylene pots containing 150 g of autoclaved soil thoroughly mixed with alginate-bead inoculum of *Amanita rubescens* or *Hebeloma sinapizans* in ratio 15:1 (v:v). The inoculated seedlings were grown after inoculation in either growth (climatic) chamber (Brunner 1993; Högberg et al. 1999; Corrêa et al. 2006; Flores et al. 2008; Obase et al. 2009), culture room (Giomaro et al. 2002), growth cabinet (Bogeat-Triboulot et al. 2004; Kozdrój et al. 2007), or glasshouse (Timonen et al. 1997; Aspray et al. 2006).

3.4 Greenhouse and Nursery Inoculation

3.4.1 Inoculum Types

3.4.1.1 Natural Inoculum (Soil, Humus, Ectomycorrhizae)

Various organic matters, such as soil, litter, variable forms of humus, rotten wood, and ectomycorrhizae collected from forest plantations or mature stands were exploited especially in the beginning of the effort to inoculate forest tree seedlings. Collection of a large amount of viable inoculum of fungi adapted to the sites from which they were taken is relatively reliable and easy. A major drawback of natural inoculum is that the species of ECM fungi in the inoculum cannot be controlled. This inoculum may also contain harmful microorganisms and weeds in addition to the ECM fungi. Forest soil was used as a source of indigenous ECM fungi in experiments of Borchers and Perry (1990), Querejeta et al. (1998), and Wallander et al. (2005). Litter and diverse forms of humus from the forest floor including a range of materials from recently fallen needles on the surface to well-decomposed humus overlying the mineral soil were added to pot substrate (Parke et al. 1983; Repáč 1996a). Aucina et al. (2007) used pine and oak litter to affect development of bareroot seedlings. Rotten wood was collected from a Douglas-fir stand and fragmented by hand into small pieces about 1 cm in diameter for mixing in a growing medium (Kropp 1982). Natural inoculum is usually collected from stand or under individual tree of species which is inoculated. Ectomycorrhizae excised from root systems of trees were also used as inoculum on a limited basis in research trials (Marx and Kenney 1982). A great deal of time and care is required to obtain a sufficient quantity of viable ectomycorrhizae.

3.4.1.2 Basidiospores

Sporocarps and spores of various fungi were used as inoculum to form specific ectomycorrhizae on various forest tree species. Sporocarps are essentially spore inoculum, since their vegetative matrix is killed by dessication during drying or by decomposition when added to soil (Marx and Kenney 1982). Gastromycete, such as genera *Rhizopogon, Scleroderma,* and *Pisolithus*, produces numerous basidiospores that are easier to collect in large quantities than those of mushroom-produced ECM fungi. Advantages of using spores for inoculation are that spores require no extended growth phase under aseptic conditions like vegetative inoculum, spore inoculum is very light, and spores are able to survive storage from one season to the next. Major disadvantages are the lack of standard laboratory tests to determine spore viability, insufficiency of sporocarps of many fungi in any year, delay in ectomycorrhiza formation as compared with vegetative inoculum, and lack of genetic definition.

Basidiospores can be obtained and stored before application in principle either in dry state or blended with water. Basidiospores were obtained by crushing and sieving basidiocarps through a mesh screen (Rincón et al. 2001; Chen et al. 2006) in a closed plastic bag (Marx and Bryan 1975). Using these techniques, well over 1 kg (fresh weight) of basidiospores of *Pisolithus tinctorius* was collected in less than 3 h (Marx and Bryan 1975). The dry spores were stored in either small plastic bags or amber glass bottles in darkness at 5°C for 10 days before use (Marx and Bryan 1975), mixed with vermiculite for seedling inoculation (Rincón et al. 2001) or stored at 4°C for 1–18 months before blended with distilled deionized water (1:10, v:v) for 5 s on low speed (Chen et al. 2006). In experiments of Parladé et al. (1996b, 1999), Rincón et al. (2001), Hortal et al. (2008), and Becerra et al. (2009), spore suspensions were prepared by blending sporocarps with distilled or tap water using a blender at low or high speed until the spores were released. After collection, Parladé et al. (1996b) and Chen et al. (2006) dried sporocarps at 30–40°C for 48 h before blending and sieving, respectively. Núñez et al. (2006) cleaned and sterilized sporocarps of *Tuber melanosporum* by brief superficial flaming. To prepare the inoculum, Roldan et al. (1996), Querejeta et al. (1998), and Núñez et al. (2006) suspended spores in distilled water and stored the suspensions at 2–4°C until use. Required spore concentrations are prepared by serial dilution of spore suspension with water. Spore concentrations are counted using haemocytometer. The approximate number of spores contained per gram of dried sporocarp tissue was 10^7–10^{10} (Parladé et al. 1996b; Rincón et al. 2001) or 1.1 million basidiospores per milligram (Marx and Bryan 1975). Chen et al. (2006) obtained a concentration of 10^5 spores.ml^{-1} and Rincón et al. (2001) 10^3–10^8 spores.10 ml^{-1}.

3.4.1.3 Vegetative (Mycelial) Inoculum

Mycelial Suspension (Slurry)

This type of vegetative inoculum is more often used in a small-scale synthesis in controlled conditions than in nursery experiments (see Sect. 3.3.1). Mycelial slurry of *Pisolithus tinctorius, Suillus granulates,* and *Rhizopogon luteolus* was prepared by blending mycelial mats from liquid cultures with distilled water at high speed for less than 3 s (Cline and Reid 1982). One liter of sterile water contained 40 g of (fresh weight) mycelium. Gagnon et al. (1991, 1995) produced mycelial suspension of *Laccaria bicolor* in a fermentor. Starter content of living propagules was diluted five times with water and mixed with the substrate in a cement mixer to get a final content approximately 6.1×10^4 living propagules or 5.68 mg of dried mycelium per seedling. Gange et al. (2005) prepared mycelial slurry of *Laccaria laccata* maintained in sterile liquid culture on MMN medium by fragmenting the mycelium in a blender for 30 s.

Vermiculite–Peat (Substrate Carrier) Inoculum

Moser (1958) in Austria was one of the first to make a serious attempt to produce vegetative inoculum of ECM fungi. For production of inoculum, mycelium of *Suillus plorans* was first grown in liquid culture and then in sterile peat moss. Moser and other workers tested various organic materials as the final inoculum substrate, e.g., forest litter, sawdust, grain of cereals, corn, bark, and found that they were not as effective as peat moss (Marx and Kenney 1982). Although mycelium tends to grow around rather than into the particles of perlite substrate, Repáč (1996b) and Hönig et al. (2000) reported that a perlite–peat mixture appears to be a possible form of mycelial carrier. Vozzo and Hacskaylo (1971) grew mycelium of several fungi in polypropylene cups containing a 2:1 ratio of sterile peat moss and vermiculite moistened with nutrient solution. In the fundamental study of Marx and Bryan (1975), the inoculum containers were 2-L jars containing the mixture of 1,400 ml of vermiculite, 50 ml of finely divided peat moss, and 750 ml of liquid MMN medium with glucose. The containers were autoclaved for 30 min and each was inoculated with eight mycelium-agar disks of *Pisolithus tinctorius*. After 15 weeks at room temperature, the vermiculite particles were permeated with mycelium. To prepare mass inoculum for infestation of soil, mycelium was removed from the jars, passed through a 5-mm mesh screen, and held with two layers of cheesecloth while being leached with cool running tap water to remove nonassimilated nutrients.

Novel formulations of vermiculite–peat inoculum did not require leaching and drying before use (Garbaye et al. 1988; Marx and Cordell 1989). An important modification of original inoculum formulation was carried out on vermiculite:peat ratio of inoculum. Marx et al. (1982) reported that vermiculite–peat inoculum mixture produced by solid-substrate fermentation contained 5–10% peat moss by volume. Other vermiculite:peat ratios used were, e.g., 4:5–1:5 (Duponnois and Garbaye 1991), 9:1 (Garbaye et al. 1988; Baum et al. 2009), 10:1 (Machón et al. 2006), and 11:1 (Rincón et al. 2001). Blended mycelial starter cultures mixed with the substrate will reduce the time of incubation by half (Marx and Kenney 1982; Rincón et al. 2001). Depending on fungal properties, inoculum is incubated for 4–8 weeks at 24–25°C in the dark. The attempts to measure the quantity of mycelium in the vermiculite–peat inoculum did not give reliable results because of the peat that interferes with colorimetric measurements (Duponnois and Garbaye 1991).

Alginate-Bead Inoculum

Le Tacon et al. (1983) and Mauperin et al. (1987) have shown that mycelium grown in a liquid medium and entrapped in calcium alginate gel is a very efficient inoculum for ECM development and can be used as an alternative to the classical vermiculite–peat mixture. Mycelium in alginate-bead inoculum is better protected, survives longer, and has a longer lasting effect than when grown on a vermiculite–peat mixture (Mortier et al. 1988). For production of alginate-bead inoculum,

fungul cultures are grown in liquid medium. The mycelial pellets are washed in tap water, homogenized in a blender for 5–10 s, and resuspended in distilled water containing 10 $g.l^{-1}$ of sodium alginate and 30 $g.l^{-1}$ of powdered sphagnum peat. This suspension is pumped through a pipe with 5-mm holes above a 100 $g.l^{-1}$ $CaCl_2$ solution, each drop forming a bead of reticulated calcium alginate gel 3–4 mm in diameter. The beads are cured in $CaCl_2$ for 24 h at room temperature (for ensuring complete reticulation of the gel), washed in tap water to remove NaCl, stored in air-tight containers at room temperature to prevent drying, and used in the nursery (Mauperin et al. 1987).

Procedure of the French authors was modified by Kropáček and Cudlín (1989). In their work, sodium alginate and peat were substituted by agents Agricol and perlite, respectively, to obtain alginate paste containing the mycelium. Beads were cured in 5% calcium chloride for 30 min, rinsed with distilled water, dried to surface dryness (about 30 min in air filtered box), and stored in air-tight containers at 4°C until use. Alginate-bead inoculum was further used for inoculation of seedlings, e.g., in experiments of Duponnois and Garbaye (1991), Gagnon et al. (1991), Baum et al. (2000, 2002), and Repáč (2007). Parladé et al. (1999) prepared alginate-bead inoculum containing spores of *Rhizopogon* and mycelium of *Laccaria bicolor*. Mixed inoculum was prepared by adding spores plus fragmented mycelium (blended in autoclaved water) at different proportions to Pyrex flasks containing 20 $g.l^{-1}$ of an autoclaved water solution of sodium alginate. The content of flask was gently mixed, dropped into a 0.3-M water solution of $CaCl_2$ to polymerize, then washed with sterile distilled water, and kept at 4°C in plastic bags for 1 week.

3.4.2 Methods of Inoculum Application

Fumigation of nursery soil before inoculation improves ECM development because it lowers populations of soil microorganisms that can colonize introduced inocula, feeder root pathogens that damage roots and thus reduce ECM development, and/or indigenous competing ECM fungi. The seedlings can be inoculated with ECM inoculum during one of the following stages:

– Before seeds are sown or seedlings planted
– When seeds are sown or seedlings planted
– After seedlings emerge or seedlings planting

3.4.2.1 Basidiospores

Several inoculation techniques have been used:

– Mixing either crushed sporocarps or dry spores directly into soil or container medium

- Mixing spores with a moistened carrier, such as vermiculite, kaolin or sand, broadcasting onto soil and then mixing into the nursery soil or the growing medium of containers
- Dusting dry spores onto soil around young seedlings and leaching them into the root zone
- Suspending in water and drenching, irrigating, or injecting into growth substrate
- Dusting or spraying on roots of nonmycorrhizal seedlings
- Mixing with the pelletizing matrix and encapsulating or coating seeds before sowing

Marx et al. (1978) broadcast spores of *Pisolithus tinctorius* added to moist vermiculite onto soil and mixed inoculum with the soil using handtools. The spore quantities were 108, 324, or 648 mg of spores.m^{-2} of soil surface. Rincón et al. (2001) mixed dry spores of *P. tinctorius* and *Scleroderma verrucosum* included in vermiculite with potting substrate and filled containers with 10^3–10^8 spores per 175 ml of substrate.

Perhaps the most used method is application of spore water suspension to growth substrate. Marx and Bryan (1975) poured basidiospores of *P. tinctorius* (10 g suspended in 500 ml of distilled water with 1 drop of Tween 20) into microplots which were heavily watered to wash the basidiospores into the soil. Theodorou (1984) inoculated seeds of *Pinus radiata* with basidiospores (5.15×10^4 spores per seed) of *Rhizopogon luteolus* 1–2 days before sowing. Suspension of spores of *R. luteolus* (4.46×10^7 spores.ml^{-1}) was sprayed onto soil, and the spores were mixed with the soil by raking and rolling and by watering 1 day after sowing of inoculated seeds (i.e., 2 days after soil inoculation).

In experiments of Roldan et al. (1996) and Querejeta et al. (1998), water suspension of spores of *Pisolithus arhizus* was applied three times, 1 month apart, 12 weeks after sowing of *Pinus halepensis* to 300-ml plastic bags filled with 3:1 soil/peat mixture. Each plant received 5×10^5 spores per application. Molina et al. (1997) applied spore slurries of six *Rhizopogon* spp. to container-grown *Pseudotsuga menziesii* and *Pinus ponderosa* seedlings over two inoculations. For each inoculation, 10 ml of diluted spore suspension was pipetted onto the peat–vermiculite substrate. Chen et al. (2006) inoculated *Eucalyptus urophylla* seedlings 2 weeks after transplanting to plastic pots with 10 ml of spore suspension at a rate 10^6 spores per seedling. The spore slurry was added to a 2–3 cm deep hole near the plant using a 5-ml pipette. Parladé et al. (1996b), Rincón et al. (2001), and Hortal et al. (2008) inoculated 1-month-old container-grown seedlings with spore suspension of several ECM fungi at the rate of 10^2–10^8 spores per seedling. Núñez et al. (2006) applied spore suspension of *Tuber melanosporum* by injecting manually into each seedling's pot substrate (400 ml of light and dark peat and vermiculite, 2:1:1) approximately 7.5×10^5 spores at 3–8 cm depth. In the work of Becerra et al. (2009), 1 ml of spore suspension of two *Alpova* species containing 10^6 spores was inoculated at the base of *Alnus acuminata* seedlings.

3.4.2.2 Natural and Vegetative Inoculum

In inoculation programs, since hyphae cannot grow from the inoculum to roots, inoculum must be placed in the rooting zone of seedlings where roots can grow into the inoculum (Marx and Kenney 1982). In principle, natural and vegetative inoculum can be applied by three methods:

- Mixed with the rooting medium
- Banded or layered below seeds or seedlings
- Suspended in water and poured onto seedlings or dipping seedlings into the slurry before planting (except of beads)

Bareroot and container-grown seedlings and cuttings of numerous tree species were inoculated with vegetative inocula of many ECM fungi in greenhouse and nursery experiments. *Pisolithus tinctorius* (Marx and Bryan 1975; Marx et al. 1982; Marx and Cordell 1989; Vijaya and Srivasuki 1999; Rincón et al. 2001) and genus *Laccaria* (Mortier et al. 1988; Kropáček and Cudlín 1989; Gagnon et al. 1995; Garbaye and Churin 1997; Parladé et al. 1999; Baum et al. 2002; Gange et al. 2005; Machón et al. 2006) have been the most often tested fungi in inoculation experiments. ECM seedlings or cuttings are produced through inoculation of different growing substrates. Soil and peat mixed with vermiculite or other substrate components – the standard substrates for production of planting stock of forest tree species, are convenient and most frequently used for ECM inoculation.

Unequivocally, the most preferred technique of application of organic matter and vegetative inoculum is mixing it with a growth substrate. Amount of inoculum mixed with substrate in pot experiments is most commonly expressed as a ratio of inoculum and substrate by volume. Organic matter (humus, litter, rotten wood) is added to substrate in a larger ratio (1:4 to 1:1) (Kropp 1982; Parke et al. 1983; Repáč 1996a) than vegetative inoculum. Vermiculite–peat inoculum was mixed with potting substrate at the proportion 1:4 (Rincón et al. 2001), 1:10 (Hortal et al. 2009), as well as 1:64 (Rincón et al. 2001). Baum et al. (2009) expressed a ratio of soil:vermiculite-peat inoculum mixture (10:1) by weight. Parladé et al. (1999) and Baum et al. (2000, 2002) carried out inoculation by mixing alginate beads with potting substrate in the proportion 1:20 (v:v). Before filling containers, Gagnon et al. (1991) mixed alginate beads with the substrate so that each seedling received a volume of 17 ml of beads. Gagnon et al. (1995) mixed liquid inoculum with substrate in a cement mixer to get a final volume of 30 ml of mycelial slurry per seedling.

In nursery bed experiments, vegetative inoculum was broadcast evenly over nursery bed (0.5–2.8 $l.m^{-2}$) and incorporated into the upper 10–12 cm of substrate with handtools (Marx et al. 1978; Mortier et al. 1988; Duponnois and Garbaye 1991; Repáč 1996b; Garbaye and Churin 1997). Marx and Bryan (1975) reported application of *P. tinctorius* inoculum to fumigated soil at a ratio 1:8 (v:v) in upper 10 cm layer of plot.

Cline and Reid (1982) injected 20 ml of mycelial slurry into a root zone of container-grown seedlings at a depth of 6–8 cm using a glass syringe. Garbaye et al.

(1988), Machón et al. (2006), and Gange et al. (2005) added 50 ml of vermiculite–peat inoculum and 20 ml of mycelial slurry, respectively, into containers in contact with the roots of transplanted seedlings. Aucina et al. (2007) placed a layer of pine or oak litter on the surface of the nursery bed soil with seedlings to influence the growth and ECM communities of seedlings. Kropáček and Cudlín (1989) and Repáč (2007) evenly spread alginate-bead inoculum below surface of substrate immediately before seed sowing.

Perhaps the most capable way of expression of amount of vegetative inoculum is in a dry weight of mycelium, enabling the comparison of inoculum rate between experiments of different patterns. Unfortunately, to ascertain quantity of mycelium in vermiculite–peat carrier is almost impossible. Nevertheless, Mortier et al. (1988) reported 1–2 g of mycelium (dry weight) per m^2 in this type of inoculum. Mortier et al. (1988), Duponnois and Garbaye (1991), Vijaya and Srivasuki (1999), and Repáč (2007) referred 2 g, 2–10 g, 4 g, and 5 g of mycelium in dry weight per m^2, respectively, in alginate-bead inoculum. In the pot experiments, each seedling received either 6.82 mg (Gagnon et al. 1991) or 12 mg (Gagnon et al. 1995) of dried mycelium or 20–80 mg of mycelium in fresh weight (Parladé et al. 1999).

3.5 Field Inoculation

Inoculation of seedlings at the time of field planting is time consuming, requires more inoculum, and the introduced fungus must be compatible with native microorganisms and climatic conditions of the planting site (Riffle and Maronek 1982). Thus, nursery inoculated seedlings are more frequently outplanted (Roldan et al. 1996; Garbaye and Churin 1997; Núñez et al. 2006; Rincón et al. 2007) than inoculated at the time or after field planting. Castellano (1996) reviewed most of available literature (including unpublished data) on outplanting performance of ECM-inoculated seedlings and provided insight to the fungus-inoculum type-host-location combinations in field experiments. Inoculum types and application methods in the field are very similar to nursery inoculation of container-grown seedlings. Various natural organic materials and organic wastes are often used and may be considered as a simple source of ECM fungi propagules. Hallsby (1995) planted Norway spruce seedlings to mounds containing forest floor material from the F- and H-layers. Querejeta et al. (1998) added 150 ml of pine forest soil (top 20 cm of mineral soil) to the planting holes of *Pinus halepensis* seedlings at the time of planting. Organic materials, particularly decayed wood or mixtures containing decayed wood were equal or superior to mineral substrates for supporting ECM activity on planted seedlings (Harvey et al. 1997). Larcheveque et al. (2006) incorporated fresh co-composted sewage sludge and greenwastes (20 or 40 $kg.m^{-2}$ of compost) into the soil at each stem of 1-year-old seedlings of *Quercus ilex*, *Pinus halepensis*, and *P. pinea*.

In field experiments with laboratory produced inoculum, e.g., Baum et al. (2002) applied 50 ml of alginate beads containing mycelium of *Laccaria laccata* around

each *Populus trichocarpa* cutting (1.5-m long annual shoots, diameter 13–17 mm) in 50-mm soil depth. Duñabeitia et al. (2004) inoculated seedlings few days after outplanting (and reinoculated 5 months later) by watering at 5 cm around the stem with 200 ml of spore slurry of *Scleroderma citrinum*, *Pisolithus arhizus*, and *Leccinum scabrum*. Menkis et al. (2007) wrapped root systems of *Pinus sylvestris* and *Picea abies* seedlings in a filter paper containing mycelium of *Cenococcum geophilum*, *Piceirhiza bicolorata* or *Hebeloma crustuliniforme*, overlaid with damp peat–sand mixture, wrapped in a paper towel, and planted seedlings on poor sandy soil.

3.6 Commercial Inoculum

It is relatively simple to produce sufficient volumes of inoculum for small research studies, but it is extremely difficult to produce sufficient quantities of inoculum to support commercial nursery inoculation. Since 1976, several formulations of vegetative and spore inocula of "super-strain" of *Pisolithus tinctorius* were produced commercially (e.g., inoculum trademarked MycoRhiz®) and used in large scale for inoculation of millions of bareroot and containerized pine and oak seedlings in USA (Marx 1991). In France, a company called Somycel produced vegetative beads inoculum, which substantially increased reforestation effort of coniferous trees followed by nursery inoculation (Kropp and Langlois 1990).

Numerous entities of international, national, or local importance produce and offer ECM fungal inocula for commercial purposes. For example, several European producers of mycorrhizal fungi inocula constituted organization called "Federation of European Mycorrhizal Fungi Producers" (FEMFiP) in 2003, aimed to achieve and maintain high standards for these products in Europe (Federation of European Mycorrhizal Fungi Producers 2010). The most products of FEMFiP contain arbuscular fungi and are intended to horticulture, agriculture, and landscape sectors. The companies PlantWorks Ltd. (Great Britain, product TerraVital), Biorize (France, product Ectorize), and Symbio-m (Czech Republic, product Ectovit) offer ECM inocula for inoculation of forest tree species.

Spore and mycelial inocula are used in operational scale to match the ECM fungi to the host and environment in which they are required to thrive. Natural biostimulants and ingredients supporting the development of ECM symbiosis (natural humates, sea-grass extracts, ground minerals) are usually added to commercial inoculum. Inoculation techniques are in principle the same as described in Sects. 3.4.2 and 3.5. Compared to research-scale inoculum formulations, gel formulation (slurry), prepared by diluting ECM inoculum containing naturally degradable granules of water-retaining gel in water, is more frequently used in a commercial scale. The slurry can be sprayed onto the substrate before seed sowing, into the rooting zone of transplanted seedlings, or root systems of seedlings can be dipped into the slurry. The production and marketing of inocula on a commercial scale increased recently and further potential appears to be quite large.

3.7 Conclusion

There are a variety of ECM inoculum types, inoculum preparation methods, and inoculation techniques to initiate the development of ectomycorrhizae on forest tree seedlings. The selection of fungi, inoculum type, and inoculation method all depends on the intended purpose of inoculation. Better understanding of the structure and functioning of ECM symbiosis and better performance of inoculated seedlings under nursery and field conditions are the ultimate goals of research on artificial mycorrhization, and therefore further development of inoculation methods in operational conditions is desirable. Although sterile conditions are a faraway from natural ones, techniques of pure culture synthesis are necessary for compatibility, structural, physiological, molecular, and other studies.

Application of ECM inoculum to substrate does not guarantee that ectomycorrhizae will develop on a host plant. Success of inoculation depends on the type and age of inoculum used, inoculum dose, timing of inoculation, inoculum placement in the growing medium, etc. (Riffle and Maronek 1982; Mortier et al. 1988; Rodrigues et al. 1999). Besides inoculum and inoculation pattern, interspecific and intraspecific host–fungus variation, environmental conditions, seedling production practices, and other factors are responsible for seedling response to inoculation (Kropp and Langlois 1990; Castellano 1996; Menkis et al. 2007; Duponnois et al. 2008). Despite of (or just because of) inconsistency of effects of introduced fungi on performance of inoculated seedlings, more research is needed on the screening of potential host–fungus species and genotype combinations and the host–fungus–environment interactions to optimize the effect of fungi on plants. Also, researchers might be able to make much progress in simplifying the application of ECM fungi in seedling inoculation. Because of natural complexity and diversity of ECM relationships, we have to realize that their understanding will neither be completed nor their use completely reliable. Nevertheless, when we consider extensive area of treeless lands and adverse forest sites required artificial regeneration, the importance of artificial mycorrhization of seedlings as reforestation and afforestation management tool is obvious.

References

Aspray TJ, Frey-Klett P, Jones JE, Whipps JM, Garbaye J, Bending GD (2006) Mycorrhization helper bacteria: a case of specifity for altering ectomycorrhiza architecture but not ectomycorrhiza formation. Mycorrhiza 16:533–541

Aucina A, Rudawska M, Leski T, Skridaila A, Riepsas E, Iwanski M (2007) Growth and mycorrhizal community structure of *Pinus sylvestris* seedlings following the addition of forest litter. Appl Environ Microb 73:4867–4873

Baum C, Schmid K, Makeschin F (2000) Interactive effects of substrates and ectomycorrhizal colonization on growth of poplar clone. J Plant Nutr Soil Sci 163:221–226

Baum C, Stetter U, Makeschin F (2002) Growth response of *Populus trichocarpa* to inoculation by the ectomycorrrhizal fungus *Laccaria laccata* in a pot and a field experiment. For Ecol Manage 163:1–8

Baum C, Toljander YK, Eckhardt KU, Weih M (2009) The significance of host-fungus combinations in ectomycorrhizal symbioses for the chemical quality of willow foliage. Plant Soil 323:213–224. doi:10.1007/s111040099928x

Becerra AG, Menoyo E, Lett I, Li CY (2009) *Alnus accuminata* in dual symbiosis with *Frankia* and two different ectomycorrhizal fungi *(Alpova austroalnicola* and *Alpova diplophloeus)* growing in soilless growth medium. Symbiosis 47:85–92

Bending GD, Poole EJ, Whipps JM, Read DJ (2002) Characterisation of bacteria from *Pinus sylvestris – Suillus luteus* mycorrhizas and their effects on root-fungus interactions and plant growth. FEMS Microbiol Ecol 39:219–227

Bogeat-Triboulot MB, Bartoli F, Garbaye J, Marmeisse R, Tagu D (2004) Fungal ectomycorrhizal community and drought affect root hydraulic properties and soil adherence to roots of *Pinus pinaster* seedlings. Plant Soil 267:213–223

Borchers SL, Perry DA (1990) Growth and ectomycorrhiza formation of Douglas-fir seedlings grown in soils collected at different distances from pioneering hard-woods in southwest Oregon clear-cuts. Can J For Res 20:712–721

Brunner I (1993) Production of ectomycorrhizal *Picea abies – Hebeloma crustuliniforme* seedlings for ecological studies: effects of synthesis techniques on the morphology of the symbiosis. Water Air Soil Poll 68:231–240

Brunner I, Amiet R, Zollinger M, Egli S (1992) Ectomycorrhizal syntheses with *Picea abies* and three fungal species: a case study on the use of an in vitro technique to identity naturally occurring ectomycorrhizae. Mycorrhiza 2:89–96

Castellano MA (1996) Outplanting performance of mycorrhizal inoculated seedlings. In: Mukerji KG (ed) Concepts in mycorrhizal research. Kluwer Academic Publishers, Netherlands, pp 223–301

Chen YL, Kang LH, Dell B (2006) Inoculation of *Eucalyptus urophylla* with spores of *Scleroderma* in a nursery in south China: comparison of field soil and potting mix. For Ecol Manage 222:439–449

Cline ML, Reid CPP (1982) Seed source and mycorrhizal fungus effects on growth of containerized *Pinus contorta* and *Pinus ponderosa* seedlings. For Sci 28:237–250

Corrêa A, Strasser RJ, Martins-Loução MA (2006) Are mycorrhiza always beneficial? Plant Soil 279:65–73

Duñabeitia M, Rodríguez N, Salcedo I, Sarrionandia E (2004) Field mycorrhization and its influence on the establishment and development of the seedlings in a broadleaf plantation in the Basque country. For Ecol Manage 195:129–139

Duponnois R, Garbaye J (1991) Techniques for controlled synthesis of Douglas-fir – *Laccaria laccata* ectomycorrhizal symbiosis. Ann Sci For 48:641–650

Duponnois R, Kisa M, Prin Y, Ducousso M, Plenchette Ch, Lepage M, Galiana A (2008) Soil factors influencing the growth response of *Acacia holosericea* A. Cunn. ex G. Don to ectomycorrhizal inoculation. New For 35:105–117

Federation of European Mycorrhizal Fungi Producers (2010) http://www.fenfip.com. Cited 15 Feb 2010

Flores R, Díaz G, Honrubia M (2008) Mycorrhizal synthesis and basidioma primordium formation between *Abies guatemalensis* and *Laccaria bicolor*. Nova Hedwigia 87:153–160

Fortin JA, Piché Y, Lalonde M (1980) Technique for the observation of early morphological changes during ectomycorrhiza formation. Can J Bot 58:361–365

Gagnon J, Langlois CG, Garbaye J (1991) Growth and ectomycorrhiza formation of container-grown red oak seedlings as a function of nitrogen and inoculum type of *Laccaria bicolor*. Can J For Res 21:966–973

Gagnon J, Langlois CG, Bouchard D, Le Tacon F (1995) Growth and ectomycorrhizal formation of container-grown Douglas-fir seedlings inoculated with *Laccaria bicolor* under four levels of nitrogen fertilization. Can J For Res 25:1953–1961

Gange AC, Gane DRJ, Chen Y, Gong M (2005) Dual colonization of *Eucalyptus urophylla* S.T. Blake by arbucular and ectomycorrhizal fungi affects levels of insect herbivore attack. Agric For Entomol 7:253–263

Gáper J, Repáč I, Cudlín P (1999) In vitro mycorrhizae syntheses of *Inocybe lacera* and *Suillus bovinus* with *Picea abies*. In: Moreno G et al (eds) Book of abstracts of the XIII Congress of European Mycologists, Alcalá de Henares (Madrid), p 41

Garbaye J, Churin JL (1997) Growth stimulation of young oak plantations inoculated with the ectomycorrhizal fungus *Paxillus involutus* with special reference to summer drought. For Ecol Manage 98:221–228

Garbaye J, Delwaulle JC, Diangana D (1988) Growth response of Eucalypts in the Congo to ectomycorrhizal inoculation. For Ecol Manage 24:151–157

Giomaro G, Sisti D, Zambonelli A, Amicucci A, Cecchini M, Comandini O, Stocchi V (2002) Comparative study and molecular characterization of ectomycorrhizas in *Tilia americana* and *Quercus pubescens* with *Tuber brumale*. FEMS Microbiol Lett 216:9–14

Guerin-Laguette A, Vaario LM, Matsushita N, Shindo K, Suzuki K, Lapeyrie F (2003) Growth stimulation of Shiro-like mycorrhiza forming mycelium of *Tricholoma matsutake* on solid substrates by non-ionic surfactants or vegetable oil. Mycol Prog 2:37–44

Hallsby G (1995) Field performance of outplanted Norway spruce – effects of organic-matter amendments and site preparation. Can J For Res 25:1356–1367

Harvey AE, Page-Dumroese DS, Jurgensen MF, Graham RT, Tonn JR (1997) Site preparation alters soil distribution of roots and ectomycorrhizae on outplanted western white pine and Douglas-fir. Plant Soil 188:107–117

Herrmann S, Oelmüller R, Buscot F (2004) Manipulation of the onset of ectomycorrhiza formation by indole-3-acetic acid, activated charcoal or relative humidity in the association between oak microcuttings and *Piloderma croceum*: influence on plant development and photosynthesis. J Plant Physiol 161:509–517

Högberg P, Högberg MN, Quist ME, Ekblad A, Näsholm T (1999) Nitrogen isotope fractionation during nitrogen uptake by ectomycorrhizal and non-mycorrhizal *Pinus sylvestris*. New Phytol 142:569–576

Hönig K, Riefler M, Kottke I (2000) Survey of *Paxillus involutus* (Batsch) Fr. inoculum and fruitbodies in a nursery by IGS-RFLPs and IGS sequences. Mycorrhiza 9:315–322

Hortal S, Pera J, Parladé J (2008) Tracking mycorrhizas and extraradical mycelium of the edible fungus *Lactarius deliciosus* under field competition with *Rhizopogon* spp. Mycorrhiza 18:69–77

Hortal S, Pera J, Parladé J (2009) Field persistence of the edible ectomycorrhizal fungus *Lactarius deliciosus*: effects of inoculation strain, initial colonization level, and site characteristics. Mycorrhiza 19:167–177

Kozdrój J, Piotrowska-Seget Z, Krupa P (2007) Mycorrhizal fungi and ectomycorrhiza associated bacteria isolated from an industrial desert soil protect pine seedlings against Cd(II) impact. Ecotoxicology 16:449–456

Kropáček K, Cudlín P (1989) Preparation of granulated mycorrhizal inoculum and its use in forest nurseries. In: Vančura V, Kunc F (eds) Interrelationships between microorganisms and plants in soil. Academia, Praha, pp 177–182

Kropp BR (1982) Rotten wood as mycorrhizal inoculum for containerized western hemlock. Can J For Res 12:428–431

Kropp BR, Langlois CG (1990) Ectomycorrhizae in reforestation. Can J For Res 20:438–451

Krüger A, Peškan-Berghöfer T, Frettinger P, Herrmann S, Buscot F, Oelmüller R (2004) Identification of premycorrhiza-related plant genes in the association between *Quercus ro*bur and *Piloderma croceum*. New Phytol 163:149–157

Langer I, Krpata D, Peintner U, Wenzel WW, Schweiger P (2008) Media formulation influences in vitro ectomycorrhizal synthesis on the European aspen *Populus tremula* L. Mycorrhiza 18:297–307. doi:10.1007/s0057200801825

Larcheveque M, Ballini C, Korboulewsky N, Montes N (2006) The use of compost in afforestation of Mediterranean areas: effects on soil properties and young tree seedlings. Sci Total Environ 369:220–230

Le Tacon F, Jung G, Michelot P, Mugnier M (1983) Efficacité en pépinič̈re forestiére d'un inoculum de champignon ectomycorhizien produit en fermenteur et inclus dans une matrice de polyméres. Ann Sci For 40:165–176

Machón P, Santamaría O, Pajares JA, Alves-Santos FM, Diez JJ (2006) Influence of the ectomycorrhizal fungus *Laccaria laccata* on pre-emergence, post-emergence and late damping-off by *Fusarium moniliforme* and *F. oxysporum* on *Scots pine* seedlings. Symbiosis 42:153–160

Martín-Pinto P, Pajares J, Díez J (2006) In vitro effects of four ectomycorrhizal fungi, *Boletus edulis*, *Rhizopogon roseolus, Laccaria laccata* and *Lactarius deliciosus* on *Fusarium* damping off in *Pinus nigra* seedlings. New For 32:323–334. doi:10.1007/s1105600690067

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH (1991) The practical significance of ectomycorrhizae in forest establishment. In: Hägglund B (ed) Ecophysiology of ectomycorrhizae of forest trees. The Marcus Wallenberg foundation symposia proceedings: 7, Stralins Tryckeri AB, Grycksbo, Sweden, pp 54–90

Marx DH, Bryan WC (1975) Growth and ectomycorrhizal development of Loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. For Sci 21:245–254

Marx DH, Cordell CE (1989) The use of specific ectomycorrhizas to improve artificial forestation practices. In: Whipps JM, Lumsden RD (eds) Biotechnology of fungi for improving plant growth. Press Syndicate of the University of Cambridge, British Mycological Society, New York, pp 1–26

Marx DH, Kenney DS (1982) Production of ectomycorrhizal fungus inoculum. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathology Society, St. Paul, MN, pp 131–146

Marx DH, Morris WG, Mexal JG (1978) Growth and ectomycorrhizal development of Loblolly pine seedlings in fumigated and nonfumigated nursery soil infested with different fungal symbionts. For Sci 24:193–203

Marx DH, Ruehle JL, Kenney DS, Cordell CE, Riffle JW, Molina RJ, Pawuk WH, Navratil S, Tinus RW, Goodwin OC (1982) Commercial vegetative inoculum of *Pisolithus tinctorius* and inoculation techniques for development of ectomycorrhizae on container-grown tree seedlings. For Sci 28:373–400

Mauperin C, Mortier F, Garbaye J, Le Tacon F, Carr G (1987) Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. Can J Bot 65:2326–2329

Menkis A, Vasiliauskas R, Taylor AFS, Stenlid J, Finlay R (2007) Afforestation of abandoned farmland with conifer seedlings inoculated with three ectomyorrhizal fungi – impact on plant performance and ectomycorrhizal community. Mycorrhiza 17:337–348

Molina R (1979) Pure culture synthesis and host specificity of red alder mycorrhizae. Can J Bot 57:1223–1228

Molina R, Palmer JG (1982) Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathology Society, St. Paul, MN, pp 115–129

Molina R, Smith JE, McKay D, Melville LH (1997) Biology of the ectomycorrizal genus, *Rhizopogon*. New Phytol 137:519–528

Moore LM, Jansen AE, Van Griensen LJLD (1989) Pure culture synthesis of ectomycorrhizas with *Cantharellus cibarius*. Acta Bot Neerl 38:273–278

Mortier F, Le Tacon F, Garbaye J (1988) Effects of inoculum type and inoculation dose on ectomycorrhizal development, root necrosis and growth of Douglas fir seedlings inoculated with *Laccaria laccata* in a nursery. Ann Sci For 45:301–310

Moser M (1958) Die künstliche Mykkorhizaimpfung an Forstpflanzen. I. Erfahrungen bei der Reinkulture von Mykorrhizapilzen. Forstwiss Centralbl 77:32–40

Moser M (1960) Die Gattung Phlegmacium (Schleimköpfe). Verlag Julius Klinkhardt, Bad Heilbrunn, Germany, 440 pp

Niemi K, Vuorinen T, Ernstsen A, Häggman H (2002) Ectomycorrhizal fungi and exogenous auxins influence root and mycorrhiza formation of Scots pine hypocotyl cuttings in vitro. Tree Physiol 22:1231–1239

Niemi K, Julkunen-Titto R, Häggman H, Sarjala T (2006) *Suillus variegatus* causes significant changes in the content of individual polyamines and flavonoids in Scots pine seedlings during mycorrhiza formation in vitro. J Exp Bot 58:391–401. doi:10.1093/jxb/erl209

Núñez JAD, Serrano JS, Barreal JAR, González JAS (2006) The influence of mycorrhization with *Tuber melanosporum* in the afforestation of a Mediterranean site with *Quercus ilex* and *Quercus faginea*. For Ecol Manage 231:226–233

Obase K, Tamai Y, Yajima T, Miyamoto T (2009) Mycorrhizal synthesis of four ectomycorrhizal fungi in potted *Populus maximowiczii* seedlings. Mycoscience 50:143–145

Ohta A (1990) A new medium for mycelial growth of mycorrhizal fungi. T Mycol Soc JPN 31:323–334

Pachlewski R, Pachlewska J (1974) Studies on symbiotic properties of mycorrhizal fungi of pine (*Pinus sylvestris* L.) with the aid of the methods of mycorrhizal synthesis in pure cultures on agar. For Res Inst Press, Warsaw, Poland, 228 pp

Parke JL, Linderman RG, Trappe JM (1983) Effects of forest litter on mycorrhiza development and growth of Douglas-fir and western red cedar seedlings. Can J For Res 13:666–671

Parladé J, Álvarez IF, Pera J (1996a) Ability of native ectomycorrhizal fungi from northern Spain to colonize Douglas-fir and other introduced conifers. Mycorrhiza 6:51–55

Parladé J, Pera J, Alvarez IF (1996b) Inoculation of containerized *Pseudotsuga menziesii* and *Pinus pinaster* seedlings with spores of five species of ectomycorrhizal fungi. Mycorrhiza 6:237–245

Parladé J, Alvarez IF, Pera J (1999) Coinoculation of containerized Douglas-fir (*Pseudotsuga menziesii*) and maritime pine (*Pinus pinaster*) seedlings with the ectomycorrhizal fungi *Laccaria bicolor* and *Rhizopogon* spp. Mycorrhiza 8:189–195

Peterson RL, Chakravarty P (1991) Techniques in synthesizing ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology, vol 23. Academic Press Limited, London, pp 75–106

Querejeta JI, Roldán A, Albaladejo J, Castillo V (1998) The role of mycorrhizae, site preparation, and organic amendment in the afforestation of a semi-arid mediterranean site with *Pinus halepensis*. For Sci 44:203–211

Repáč I (1996a) Inoculation of *Picea abies* (L.) Karst. seedlings with vegetative inocula of ectomycorrhizal fungi *Suillus bovinus* (L.:Fr.) O. Kuntze and *Inocybe lacera* (Fr.) Kumm. New For 12:41–54

Repáč I (1996b) Effects of forest litter on mycorrhiza formation and growth of container-grown Norway spruce (*Picea abies* [L.] Karst.) seedlings. Lesnictví 42:317–324

Repáč I (2007) Ectomycorrhiza formation and growth of *Picea abies* seedlings inoculated with alginate-bead fungal inoculum in peat and bark compost substrates. Forestry 80:517–530

Riffle JW, Maronek DM (1982) Ectomycorrhizal inoculation procedures for greenhouse and nursery studies. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathology Society, St. Paul, MN, pp 147–155

Rincón A, Alvarez IF, Pera J (2001) Inoculation of containerized *Pinus pinea* L. seedlings with seven ectomycorrhizal fungi. Mycorrhiza 11:265–271

Rincón A, de Felipe MR, Fernández-Pascual M (2007) Inoculation of *Pinus halepensis* Mill. with selected ectomycorrhizal fungi improves seedling establishment 2 years after planting in a degraded gypsum soil. Mycorrhiza 18:23–32

Rodrigues LS, Kasuya MCM, Borges AC (1999) Viability of ectomycorrhizal fungus mycelium entrapped in calcium alginate gel. Mycorrhiza 8:263–266

Roldan A, Querejeta I, Albaladejo J, Castillo V (1996) Growth response of *Pinus halepensis* to inoculation with *Pisolithus arhizus* in a terraced rangeland amended with urban refuse. Plant Soil 179:35–43

Sarjala T, Taulavuori K (2004) Fluctuation in free and conjugated polyamines in Scots pine seedlings after changes in temperature and daylength. Acta Physiol Plant 26:271–279

Šašek V, Musílek V (1967) Cultivation and antibiotic activity of mycorrhizal basidiomycetes. Folia Microbiol 12:515–523

Theodorou C (1984) Mycorrhizal inoculation of pine nurseries by spraying basidiospores onto soil prior to sowing. Aust For 47:76–78

Timonen S, Tammi H, Sen R (1997) Outcome of interactions between genets of two *Suillus* spp. and different *Pinus sylvestris* genotype combinations: identity and distribution of ectomycorrhizas and effects on early seedlings growth in N-limited nursery soil. New Phytol 137:691–702

Vijaya T, Srivasuki KP (1999) Effect of inoculum type and inoculation dose on ectomycorrhizal development and growth of Acacia nilotica seedlings inoculated with *Pisolithus tinctorius* in a nursery. Biologia 54:439–442

Vozzo JA, Hacskaylo E (1971) Inoculation of *Pinus caribaea* with ectomycorrhizal fungi in Puerto Rico. For Sci 17:239–245

Vrålstad T, Schumacher T, Taylor AFS (2002) Mycorrhizal synthesis between fungal strains of the *Hymenoscyphus ericae* aggregate and potential ectomycorrhizal and ericoid hosts. New Phytol 153:143–152

Wallander H, Fossum A, Rosengren U, Jones H (2005) Ectomycorrhizal fungal biomass in roots and uptake of P from apatite by *Pinus sylvestris* seedlings growing in forest soil with and without wood ash amendment. Mycorrhiza 15:143–148

Wong KKY, Fortin JA (1989) A Petri dish technique for the aseptic synthesis of ectomycorrhizae. Can J Bot 67:1713–1716