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Inoculation of containerized *Pseudotsuga menziesii* and *Pinus pinaster* seedlings with spores of five species of ectomycorrhizal fungi

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Abstract Container-grown *Pseudotsuga menziesii* and *Pinus pinaster* seedlings were inoculated with water suspensions of spores of five ectomycorrhizal fungi commonly found in northeastern Spain. *Pseudotsuga menziesii* seedlings were inoculated with basidiospores of *Melanogaster ambiguus*, or *Rhizopogon subareolatus*, or with ascospores of *Tuber maculatum*. *Pinus pinaster* seedlings were inoculated with basidiospores of *Melanogaster ambiguus*, *Rhizopogon roseolus* or *Scleroderma citrinum*. The spore concentrations were 10^2–10^7 spores per seedling for *Melanogaster ambiguus* (in *Pseudotsuga menziesii*) and *Rhizopogon subareolatus*, 10^3–10^7 for *Melanogaster ambiguus* (in *Pinus pinaster*), *Rhizopogon roseolus*, and *Scleroderma citrinum*, and 10^2–10^4 for *Tuber maculatum*. *Melanogaster ambiguus* colonized more short roots in a larger proportion of plants at 10^7 spores per seedling than at any other rate. The highest colonization by *Rhizopogon subareolatus* was obtained at 10^4 spores per seedling and higher, and all inoculated plants became infected at 10^6 spores per seedling and higher. *Tuber maculatum* colonized a high percentage of short roots at all rates tested; the proportion of infected plants was over 80% at 10^3–10^4 spores per plant, decreasing to 50% at 10^2 spores per plant. *Rhizopogon roseolus* colonized the highest number of short roots on nearly all the inoculated plants when applied at 10^5 spores per seedling and higher. *Scleroderma citrinum* colonized a high percentage of short roots on all inoculated plants when applied at 10^5 spores per seedling and higher. The abundance of sporocarps of *Melanogaster ambiguus*, *Rhizopogon subareolatus*, *Rhizopogon roseolus* and *Scleroderma citrinum* and their colonization ability at relatively low rates allows these spores to be used as ectomycorrhizal inocula on a large scale.

Key words Ectomycorrhizae · *Pseudotsuga menziesii* · *Pinus pinaster* · *Melanogaster ambiguus* · *Rhizopogon roseolus* · *Rhizopogon subareolatus* · *Scleroderma citrinum* · *Tuber maculatum* · Spore inoculation

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Introduction

A delay or lack of mycorrhizae formation in forest nurseries has long been known to lead to stunted, nutrient-deficient seedlings (Mitchell et al. 1937; Pryor 1956; Trappe and Strand 1969) or to an excessive loss of plants during forestation of treeless areas and reclamation of adverse sites (Marx 1980). Frequently, substrate constituents used in container production lack viable propagules of ectomycorrhizal fungi, and seedlings grown at high nutrient levels in containers usually show erratic and deficient ectomycorrhizae formation, except for seedlings ectomycorrhizal with *Thelephora terrestris* Fr. (Castellano and Molina 1989). Nonmycorrhizal seedlings grow well in artificial substrates if enough water and soluble nutrients are supplied but, after outplanting, their capacity to absorb water and nutrients from the soil to meet growth and transpiration demands may be impaired (Kropp and Langlois 1990). Seedlings with mycorrhizae are better prepared than nonmycorrhizal seedlings to initiate soil exploration and, therefore, have a better chance of survival and early growth once in a reforestation site (Kropp and Langlois 1990).

To ensure good ectomycorrhizae development, containerized seedlings must be inoculated with appropriate fungi in the nursery (Ruehle and Marx 1977; Trappe 1977). Research on mycorrhizae has emphasized the use of vegetative mycelium as the most suitable material for inoculation of nursery seedlings with selected fungal strains (Trappe 1977; Marx 1980; Marx et al. 1991). However, high quantities of axenically pro-

duced, viable inoculum are needed for large-scale nursery application.

Spores of some ectomycorrhizal fungi are a relatively abundant and inexpensive source of inoculum. They were already used in the 18th century in attempts to enhance truffle production before the true nature of mycorrhizae was known (Trappe 1977). Spores have since been used to inoculate bareroot and container-grown conifer seedlings (Marx 1980; Azevedo 1982; Castellano and Trappe 1985; Marx et al. 1991; Massicotte et al. 1994). Some of the advantages of spore inocula over vegetative inocula are the lack of a growth phase in pure culture, the lower bulk, and the possibility of storing the spores of certain fungi. Among the disadvantages are problems in determining spore viability, the irregular fruiting of the sporocarps, the delay in forming mycorrhizae compared with vegetative inocula, and the lack of genetic definition of the spores (Marx and Kenney 1982).

The local, regular abundance of sporocarps of the ectomycorrhizal fungi *Melanogaster ambiguus* (Vitt.) Tul. & Tul., *Rhizopogon roseolus* (Corda ex Sturm) Fr., *Rhizopogon subareolatus* Smith, *Scleroderma citrinum* Pers., and *Tuber maculatum* Vitt. in northern Spain represents a large supply of spores. The genus *Rhizopogon* has been tested successfully as ectomycorrhizal inoculum (Castellano and Molina 1989; Massicotte et al. 1994) and some species are among the most widely studied ectomycorrhizal fungi. The genus comprises diverse ectomycorrhizal fungi mostly associated to *Pinus* species, although some of them have been reported to be host specific with *Pseudotsuga menziesii* (Mirb.) Franco (Molina and Trappe 1982). Molina (1980) reported the failure of mycelial inocula of *Rhizopogon vinicolor* Smith and *Rhizopogon parksii* Smith to colonize containerized *Pseudotsuga menziesii* seedlings and only limited success in bareroot nurseries. Limited results were also obtained when a vegetative inoculum of *Rhizopogon roseolus* (Corda ex Sturm) Fr was used to inoculate *Pinus taeda* L. (Ford et al. 1985) and *Pinus pinea* L. (Mousain et al. 1987). In contrast, spores of *Rhizopogon colossus* Smith, *Rhizopogon parksii*, *Rhizopogon subcaerulescens* Smith, and *Rhizopogon vinicolor* have been successfully used to inoculate container-grown *Pseudotsuga menziesii* seedlings (Castellano et al. 1985; Berch and Roth 1993; Massicotte et al. 1994). It has been reported that *Rhizopogon vinicolor* favors the recovery of plants under water stress (Parke et al. 1983), increases field performance of *Pseudotsuga menziesii* seedlings after the first growing season (Castellano and Trappe 1985), and may inhibit the growth of some root pathogens (Zak 1971). Spores of various *Rhizopogon* species have also been used to inoculate *Pinus radiata* D. Don (Theodorou 1971; Theodorou and Bowen 1970, 1973; Lamb and Richards 1974; Donald 1975; Chu-Chou and Grace 1985), *Pinus ponderosa* Dougl. ex Laws (Massicotte et al. 1994), *Pinus elliottii* Little & Dorman (Lamb and Richards 1974) and *Pinus halepensis* (Miller) (Torres and Honrubia 1994). Among the *Rhizopogon* species collected regularly in Spain, *Rhizopogon subareolatus* is the only fungus reported to be host specific with *Pseudotsuga menziesii* (Molina and Trappe 1982, 1994; Ho and Trappe 1987). The origin of this exotic fungal species and its mode of entry into Spain remain unknown (Alvarez et al. 1993).

The genus *Melanogaster*, an ectomycorrhizal fungus associated with conifer and hardwood species (Alvarez et al. 1993), has been rarely used as ectomycorrhizal inoculum. Recently, Massicotte et al. (1994) found that spore slurries of *Melanogaster euryspermus* (Zeller) Zeller & Dodge were ineffective in forming ectomycorrhizae with *Pseudotsuga menziesii*.

The inoculation of nursery seedlings with commercially valuable *Tuber* species using either spores or mycelium is well documented (Chevalier and Grente 1978). Nevertheless, the only previous inoculation experiment with *Tuber maculatum* was carried out by Fontana (1967) who successfully used crushed fruitbodies of this fungal species to inoculate *Pinus strobus* L.

Successful inoculation of container-grown *Pinus pinaster* Ait. with spores of the ectomycorrhizal fungi *Amanita muscaria* (L ex Fr) Pers ex Hook, *Pisolithus tinctorius* (Pers) Coker & Couch, *Rhizopogon luteolus*, *Russula cyanoxantha* Schff ex Fr , *Scleroderma citrinum*, *Suillus granulatus* (L ex Fr) Kuntze and *Tricholoma terreum* (Schff ex Fr) Kummer was reported by Azevedo (cited in Marx 1980; Azevedo 1982). *Scleroderma citrinum* has been found to be commonly associated with various tree species in nurseries as well as in plantations and forests (Schramm 1966; Garrido 1984; Ingleby et al. 1985). This fungus has been used as ectomycorrhizal inoculum in the greenhouse and nursery to increase *Pinus resinosa* Ait. outplanting success (Richter and Bruhn 1987). Colonization of *Pinus pinaster* seedlings with mycorrhizas of *Scleroderma citrinum* has been obtained using a mycelial inoculum (Takacs 1961) or seeds pelleted with spores (Azevedo 1982).

For the practical use of spores as inoculum it is necessary first to establish the optimal application rates. In this study, we have used spore suspensions of five ectomycorrhizal fungi to inoculate containerized *Pseudotsuga menziesii* and *Pinus pinaster* seedlings. Both tree species are commonly used for reforestation in northern Spain. The objectives of our study were to determine 1) the colonization ability of the spores, and 2) the effect of a range of spore concentrations of each fungus on ectomycorrhizae formation and seedling growth.

Materials and methods

Sporocarps of *Melanogaster ambiguus*, *Rhizopogon subareolatus* and *Tuber maculatum* were collected in spring-autumn 1990 in the Montseny Range, Girona, northeastern Spain, at 1100 m elevation in a pH 5.5 sandy soil beneath 40- to 50-year-old *Pseudotsuga menziesii*. Sporocarps of *Rhizopogon roseolus* were collected in spring 1991 in Sta. Pellaia, Les Gavarres Range, Girona,

at 100 m elevation, in a pH 5.6 sandy soil, beneath *Pinus pinaster* in a mixed stand of *Pinus pinaster* and *Quercus suber* L. Sporocarps of *Scleroderma citrinum* were collected in spring 1991 in Oihanberri nursery, Bizkaia, Basque Country, at 50 m elevation, in a pH 5.4 clayey soil, beneath old-growth *Quercus robur* L. After collection and identification, the fungi were cleaned with a brush, identified, dried at 40 °C for 48 h, and kept at room temperature until use. Voucher specimens of each fungal species were deposited in the Real Jardín Botánico de Madrid Herbarium, Madrid, Spain (references MA-Fungi 28325 and MA-Fungi 31749 for *Melanogaster ambiguus*, MA-Fungi 31748 for *Rhizopogon roseolus*, MA-Fungi 28351 for *Rhizopogon subareolatus*, MA-Fungi 31747 for *Scleroderma citrinum*, and MA-Fungi 28382 for *Tuber maculatum*).

Seeds of *Pseudotsuga menziesii* (seed zone 261, lot No 313–1980) and *Pinus pinaster* (origin Aquitaine, CEMAGREF lot No 81245) were surface sterilized with H_2O_2 for 30 min and stratified at 5 °C for 30 days. Root-trainer Spencer-Lemaire (Spencer 1974) containers (175-ml capacity) were filled with a potting substrate of Floratorf peat (Floragard, Oldenburg, Germany) and vermiculite (Termita grade 2, Asfaltex, Barcelona, Spain) in the proportion 1:1 (v:v). The substrate was previously autoclaved (120 °C, 1 kg/cm^2) for 1 h. The final pH of the pot mixture measured in water was 5.5. Two seeds were sown in each container and later thinned to 1 seedling per container.

Spore suspensions of each fungus were prepared in spring 1991 by blending chopped sporocarps in tap water with a Waring blender at low speed until the spores were released. The approximate number of spores contained per gram of dried sporocarp tissue was 10^9 for *Melanogaster ambiguus* and *Scleroderma citrinum*, 10^{10} for *Rhizopogon* spp., and 10^7 for *Tuber maculatum*. Ranges of spore concentration were prepared by serial dilution in water. Initial spore concentrations and subsequent dilutions were counted using a Newbauer haemacytometer. For *Pseudotsuga menziesii* inoculations, spore suspensions of *Melanogaster ambiguus* and *Rhizopogon subareolatus* were applied at: 0, 10^2, 10^3, 10^4, 10^5, 10^6 or 10^7 spores per seedling. Spores of *Tuber maculatum* were applied at only 0, 10^2, 10^3 or 10^4 spores per seedling because higher concentrations could not be obtained. For *Pinus pinaster* inoculations, spore suspensions of *Melanogaster ambiguus*, *Rhizopogon roseolus* and *Scleroderma citrinum* were applied at 10^3, 10^4, 10^5, 10^6 or 10^7 spores per seedling.

Sixty-four 1-month-old seedlings per treatment were inoculated with 10 ml of spore suspension containing the appropriate number of spores (control seedlings received 10 ml of water per plant). Treatments were established in a completely randomized design. Plants were grown in a shaded greenhouse with a controlled environment (20–25 °C and >40% relative humidity). Supplementary light was provided with high-pressure sodium vapor lamps to ensure a 16-h photoperiod with a minimum light intensity of 200 μmol s^{-1} m^{-2}. Plants were watered when needed (usually 3 times per week) and fertilized every 3 weeks with 10 ml per seedling of a solution containing 17-6–18 NPK fertilizer (Kristalon, DSM Agro Specialities, Utrecht, Holland) and micronutrients (Fetrilon13 and Hortrilon, BASF). Each plant received at each fertilization 3.65 mg N, 1.29 mg P, 3.87 mg K, 0.35 mg Fe, 0.07 mg Mg, 0.06 mg Mn, 0.06 mg Cu, 0.01 mg Zn, 0.01 mg B, and 0.01 mg Mo.

After 5 months, plants were examined for the presence of ectomycorrhizae in all fungal treatments. Height, root collar diameter, shoot and root biomass and number of mycorrhizal seedlings were measured for all plants in each treatment. Twenty *Pseudotsuga menziesii* and 10 *Pinus pinaster* seedlings were randomly taken from each fungal treatment to assess the percentage of colonized short roots. Because of the high number of seedlings involved, pre-trials were performed to determine the best method of counting ectomycorrhizae. To standardize the sample, lateral roots were taken from three different levels of the entire root system from each plant (top, middle and bottom), cut into small pieces (ca. 1 cm), and mixed in a Petri dish containing water. The percentage of mycorrhizal short roots for each plant was assessed by counting at least 200 short roots from the mixture of segmented roots under the stereomicroscope. Data were analyzed by analysis of variance (ANOVA). Means were compared by Tukey's multiple range test ($P \leq 0.05$). Percentages of mycorrhizae were transformed with the arcsin equation to reduce the error variance (Snedecor and Cochran 1980) before performing the ANOVA.

Results

Pseudotsuga menziesii

Plants inoculated with *Melanogaster ambiguus* and *Tuber maculatum* were harvested after 5 months, when the ectomycorrhizae were clearly developed. As ectomycorrhizae formed by *Rhizopogon subareolatus* were not observed until 2 months later, the assessment was carried out after 7 months. There was no difference in height after 5 months between control plants and plants inoculated with either *Melanogaster ambiguus* or *Tuber maculatum* (Table 1). However, the seedlings inoculated with 10^7 spores per seedling of *Rhizopogon subareolatus* appeared to grow better than noninoculated plants; after 7 months, the seedlings had the commercial standard size recommended for outplanting.

Melanogaster ambiguus did not form ectomycorrhizae if the spore concentration was below 10^5 spores per seedling. The proportion of infected plants ranged from 27% at the rate of 10^5 spores per seedling to 53% at 10^7 spores per seedling (Table 1). The percentage of colonized short roots increased with increasing concentrations of spores from 13% at 10^5 spores per seedling to a maximum of 65% at 10^7 spores per seedling. However, the differences between 10^6 and 10^7 spores per plant were not significant (Fig. 1a). Ectomycorrhizae formed by *Melanogaster ambiguus* were golden brown and developed simple branching with long, twisted elements and a velvety surface. Rhizomorphs were long and abundant, with the same color as the mantle (Fig. 2a).

Rhizopogon subareolatus formed ectomycorrhizae with treatments of 10^4 spores per plant and higher. All the plants inoculated at the rate of 10^6 and 10^7 spores per plant became ectomycorrhizal (Table 1). The application of increasing concentrations of spores per plant from 10^4 to 10^7 produced similar percentages of short root colonization (33–49%) (Fig. 1a). Ectomycorrhizae formed by *Rhizopogon subareolatus* were white with dirty brown patches, simple or pinnate. The elements were covered by abundant white mycelium which spread over the surface of lateral roots. Rhizomorphs were abundant and brown colored (Fig. 2b). The outer zone of the mantle of the mycorrhizae of *Rhizopogon subareolatus* had characteristic thick-walled brown hyphae, also clearly visible in the peridium of the sporocarp, as it is known for *Rhizopogon* species in the section *Villosuli* (Smith and Zeller 1966).

Tuber maculatum formed ectomycorrhizae at all spore rates tested. The proportion of ectomycorrhizal plants was over 80% at 10^3–10^4 spores per seedling and decreased to 50% at 10^2 spores per seedling (Table 1).

Table 1 Growth and percentage of ectomycorrhizal container-grown *Pseudotsuga menziesii* seedlings inoculated with three ectomycorrhizal fungi at different spore rates. Means in the same column within a fungal treatment sharing a common letter are not significantly different by Tukey's test ($P \leq 0.05$)

Fungus	Spore rate per seedling	Stem caliper (mm)	Shoot height (cm)	Total dry weight (g)	Mycorrhizal seedlings (% total)
Melanogaster ambiguus	10^7	2.3 a b	21.2 a	1.6 a b	53
	10^6	2.8 b	19.9 a	2.1 b	47
	10^5	2.4 a b	17.8 a	1.6 a b	27
	10^4	2.4 a b	17.9 a	1.5 a	0
	10^3	2.2 a	16.5 a	1.2 a	0
	10^2	2.3 a	17.3 a	1.3 a	0
	0	2.2 a	17.1 a	1.2 a	0
Rhizopogon subareolatus	10^7	3.8 a	26.6 c	3.5 b	100
	10^6	3.6 a	20.4 a	2.8 a b	100
	10^5	3.6 a	27.2 b c	3.4 a b	50
	10^4	3.5 a	24.1 a b c	2.9 a b	43
	10^3	3.6 a	22.6 a b c	2.9 a b	0
	10^2	3.5 a	22.7 a b c	2.7 a	0
	0	3.3 a	21.5 a b	2.4 a	0
Tuber maculatum	10^4	2.3 a	17.3 a	1.5 a b	88
	10^3	2.4 a	18.1 a	1.4 a b	82
	10^2	2.7 a	21.4 a	2.1 b	50
	0	2.3 a	16.7 a	1.3 a	0

The percentage of colonized short roots ranged from 42% at 10^2 spores per seedling to 73% at 10^4 spores per seedling. No significant differences were detected (Fig. 1a). Ectomycorrhizae formed by *Tuber maculatum* were orange-brown, with simple to pyramidal pinnate, apparently smooth elements densely covered by short spicules characteristic of *Tuber* species (Fig. 2c).

Pinus pinaster

All the plants were assessed after 5 months. No statistical differences were detected in total dry weight among plants inoculated with *Melanogaster ambiguus* or in height of plants inoculated with *Rhizopogon roseolus* and *Scleroderma citrinum* (Table 2). None of the growth parameters could be related to the abundance of ectomycorrhizae. After 5 months the seedlings reached sizes adequate for their use in reforestation.

Melanogaster ambiguus formed ectomycorrhizae with containerized *Pinus pinaster*, but the effectivity of inoculation varied with the spore concentration applied. More than 70% of the inoculated plants remained nonmycorrhizal at the rate of 10^3 spores per seedling (Table 2). Increasing the spore concentration above 10^4 spores per seedling led to ectomycorrhizae formation on nearly 90% of the inoculated plants. The degree of ectomycorrhizae formation increased with increasing spore concentrations reaching 82% of the short roots colonized at 10^6 spores per seedling or higher (Fig. 1b). Ectomycorrhizae formed by *Melanogaster ambiguus* were golden brown with simple to dichotomous elements covered with abundant rhizomorphs and extramatrical mycelium with the same color as the mantle (Fig. 2d).

Rhizopogon roseolus formed ectomycorrhizae on more than 90% of the inoculated plants at all spore application rates (Table 2). The percentage of colonized short roots also increased with increasing spore concentration. The 10^3 spore application rate resulted in significantly fewer ectomycorrhizae than higher application rates, but no significant differences were detected among the three highest rates (Fig. 1b). Ectomycorrhizae formed by *Rhizopogon roseolus* were white to pinkish, cottony with generally dichotomous elements covered with abundant extramatrical mycelium and white rhizomorphs (Fig. 2e).

Scleroderma citrinum formed ectomycorrhizae at all the spore rates tested. The application of 10^3 spores per seedling resulted in less than 40% of the inoculated plants infected (Table 2) and less than 20% of colonized short roots (Fig. 1b). When the spore application rate was increased to 10^5 or above, all the seedlings developed ectomycorrhizae and the degree of colonized short roots was significantly increased to 84% or higher (Fig. 1b). Ectomycorrhizae formed by *Scleroderma citrinum* were bright white with interwoven mycelial strands on the surface. Elements were dichotomous to coralloid and densely covered with abundant extramatrical mycelium and long, white rhizomorphs (Fig. 2f).

Discussion

The relative colonization ability of spores applied in water suspensions was estimated for five fungal species. To our knowledge, this is the first report of containerized *Pseudotsuga menziesii* seedlings colonized with either *Melanogaster ambiguus*, *Rhizopogon subareolatus* or *Tuber maculatum*, and of *Pinus pinaster* seedlings

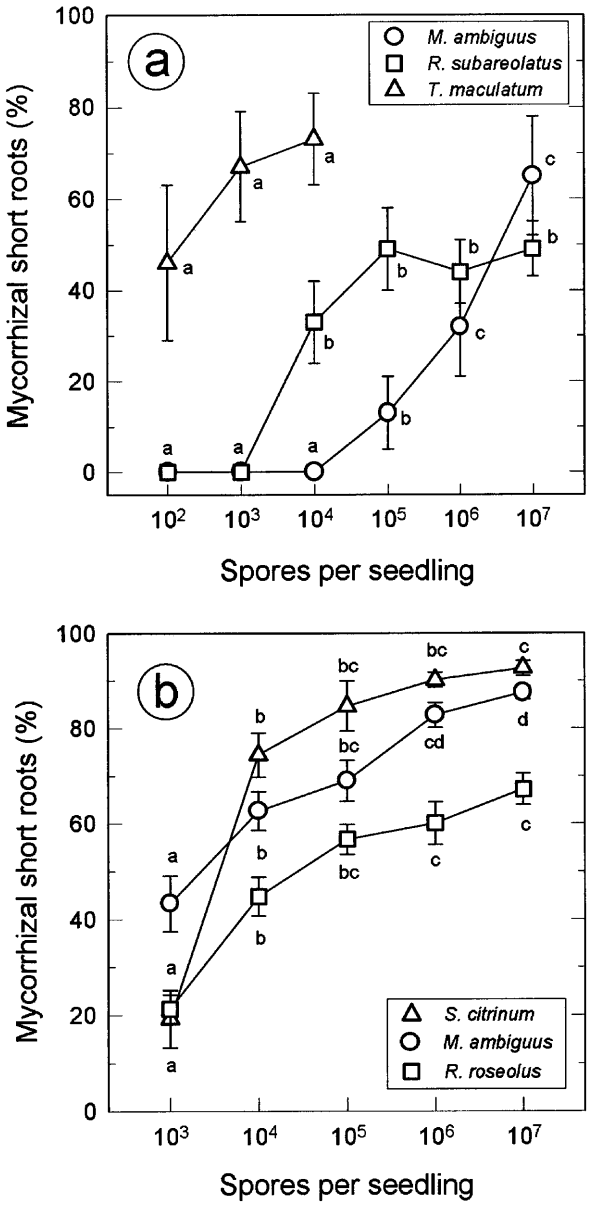

Fig. 1 Percentages of ectomycorrhizal short roots formed on container-grown *Pseudotsuga menziesii* (**a**) and *Pinus pinaster* (**b**) seedlings inoculated with different spore concentrations of five ectomycorrhizal fungi (*Melanogaster ambiguus*, *Rhizopogon roseolus*, *Rhizopogon subareolatus*, *Scleroderma citrinum*, *Tuber maculatum*). Values in the same fungal treatment sharing the same letter are not different by Tukey's test ($P \leq 0.05$). *Bars* represent the internal standard errors for each mean

colonized with either *Melanogaster ambiguus* or *Rhizopogon roseolus*. According to our results, to obtain high percentages of mycorrhizal seedlings and colonized short roots utilizing only moderate amounts of inocula, the application rates needed are 10^6 spores per seedling for *Melanogaster ambiguus* (for both tree species), 10^6 spores per seedling for *Rhizopogon subareolatus*, 10^3 spores per seedling for *Tuber maculatum*, 10^5 spores per seedling for *Rhizopogon roseolus*, and 10^5 spores per seedling for *Scleroderma citrinum*.

Melanogaster ambiguus was a less effective colonizer of *Pseudotsuga menziesii* than of *Pinus pinaster* in spite of its occurrence in *Pseudotsuga menziesii* plantations. So far, the ectomycorrhizal relationships of the genus *Melanogaster* have been rarely studied. Sporocarps of *Melanogaster* spp. have been found associated with *Pseudotsuga menziesii* (Molina and Trappe 1982; Alvarez et al. 1993), *Pinus* spp., *Quercus* spp. (Molina and Trappe 1982), *Abies* spp., and *Tsuga* spp. (Massicotte et al. 1994). Experimental evidence for an association by pure culture synthesis has only been demonstrated for *Pseudotsuga menziesii* (Molina and Trappe 1982; Parladé et al. 1996) and *Pinus pinaster* (Pera and Alvarez 1995). Extensive searching for sporocarps under other host trees and pure culture synthesis tests are needed to elucidate the host range of *Melanogaster* spp.

Rhizopogon subareolatus showed higher effectivity than *Melanogaster ambiguus* in forming ectomycorrhizae with *Pseudotsuga menziesii* at relatively low spore rates. Castellano et al. (1985) reported effective application rates for spores of *Rhizopogon vinicolor* and *Rhizopogon colossus* similar to those given here for *Rhizopogon subareolatus*. The ease of isolation from sporocarps and the fast growth in culture led us to use this and other species of *Rhizopogon* as a mycelial inoculum in parallel trials under similar conditions (Parladé 1992; Pera 1992), but spores were the only effective propagule for colonizing inoculated seedlings. Similar results were reported by Molina (1980), who suggested that the failure of mycelial inoculum was due to the disturbance experienced in inoculum preparation.

Tuber maculatum was the most effective fungus in forming ectomycorrhizae with *Pseudotsuga menziesii* at low spore rates. However, the sporocarps of this species are small and the number of spores produced low, and thus inoculation of a high number of seedlings may not be feasible. The morphology of the ectomycorrhizae formed by *Tuber maculatum* closely matches the description by Chu-Chou and Grace (1983) of naturally occurring ectomycorrhizae of *Tuber* spp. with *Pseudotsuga menziesii* in New Zealand.

The effectivity of a *Scleroderma citrinum* spore inoculum cannot be compared to previous results of Takacs (1961), Azevedo (1982) and Richter and Bruhn (1987) because their application rates were not specified.

Inoculation with spore suspensions proved to be a suitable method for obtaining seedlings ectomycorrhizal with fungi that do not grow or grow slowly in culture. It may also prove to be an alternative method for inoculating nursery seedlings with fungi that cannot withstand the disturbance involved in vegetative inoculum preparation or do not survive until young seedlings produce short roots susceptible to colonization.

Genetic diversity in spore inoculum can be enormous, particularly if spores are collected from many areas and combined into a single inoculum. In such a situation it can be expected that different results will be obtained using inocula from different collections. Some

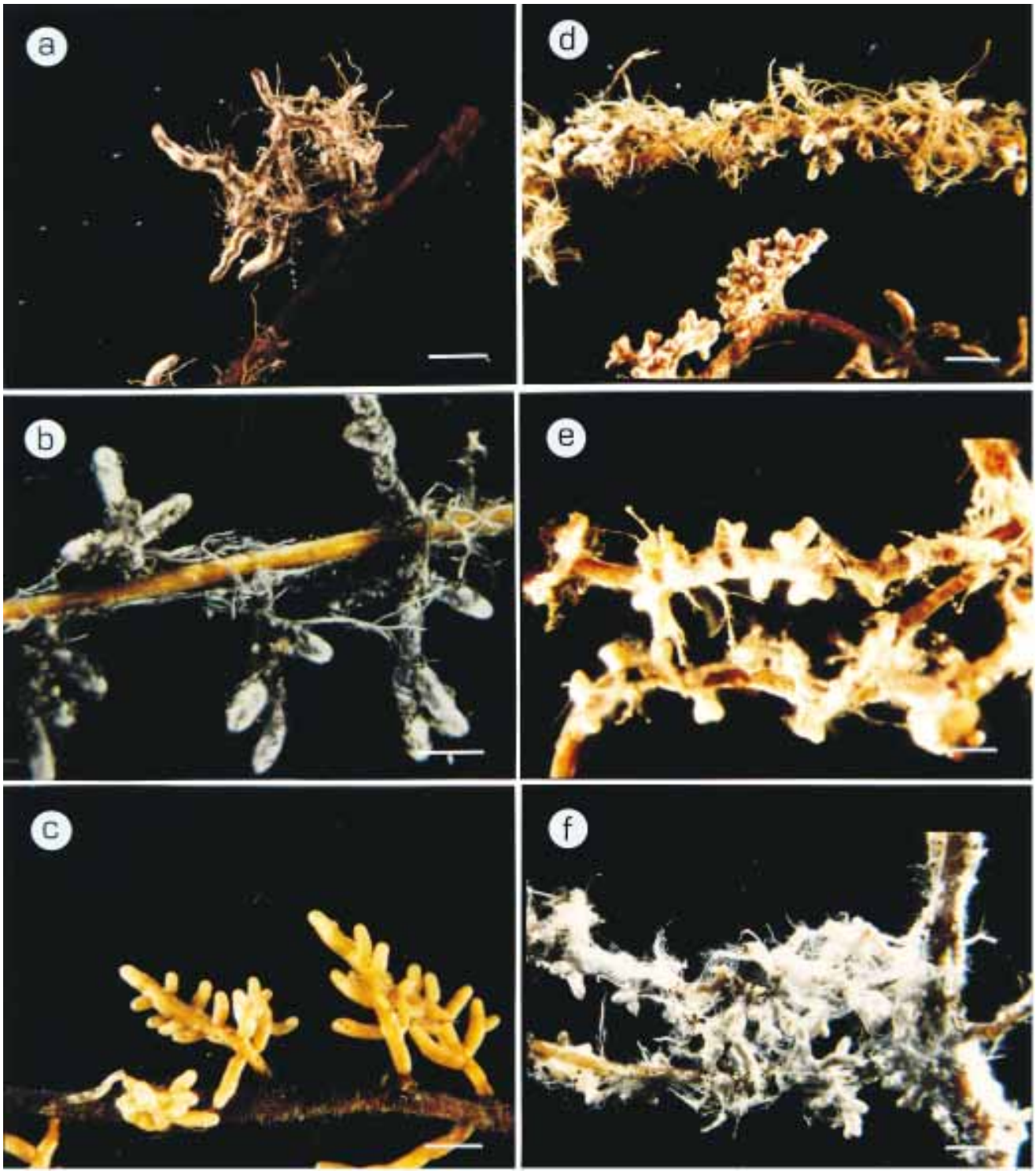

Fig. 2 Stereomicroscope photographs of ectomycorrhizae formed by **a** *Melanogaster ambiguus* + *Pseudotsuga menziesii* (×4), **b** *Rhizopogon subareolatus* + *Pseudotsuga menziesii* (×4.5), **c** *Tuber maculatum* + *Pseudotsuga menziesii* (×4), **d** *Melanogaster ambiguus* + *Pinus pinaster* (×3.5), **e** *Rhizopogon roseolus* + *Pinus pinaster* (×4.5), **f** *Scleroderma citrinum* + *Pinus pinaster* (×3.5); *bars* 2.5 mm

of the fungi used in our inoculation experiments (*Melanogaster ambiguus*, *Rhizopogon roseolus*, and *Rhizopogon subareolatus*) produce sporocarps every year in the same location, and even beneath the same trees, providing a stable source of inoculum. Comparisons between inocula obtained from different areas would help to define possible differences and to select suitable spore origins, in a process similar to the selection of seed provenance for forest trees (Marx 1991).

Table 2 Growth and percentage of ectomycorrhizal container-grown *Pinus pinaster* seedlings inoculated with three ectomycorrhizal fungi at different spore rates. Means in the same column within a fungal treatment sharing a common letter are not significantly different by Tukey's test ($P \leq 0.05$)

Fungus	Spore rate per seedling	Stem caliper (mm)	Shoot height (cm)	Total dry weight (g)	Mycorrhizal seedlings (% total)
Melanogaster ambiguus	10^7	2.1 a	22.9 a	1.2 a	95
	10^6	2.1 a	24.1 a	1.6 a	89
	10^5	2.0 a	24.2 a	1.3 a	87
	10^4	2.0 a	24.3 a	1.4 a	91
	10^3	2.0 a	22.9 a	1.5 a	27
Rhizopogon roseolus	10^7	2.1 a	23.2 a b	1.3 a	100
	10^6	2.1 a	24.4 b	1.2 a	100
	10^5	2.2 a	23.8 a b	1.3 a	96
	10^4	2.2 a	25.1 b	1.3 a	100
	10^3	2.0 a	21.2 a	1.2 a	90
Scleroderma citrinum	10^7	2.8 a	24.7 a b	2.9 a	100
	10^6	2.7 a	31.2 a b	3.4 a	100
	10^5	2.6 a	31.7 b	3.6 a	100
	10^4	2.8 a	25.4 b	3.7 a	87
	10^3	2.9 a	27.7 a	3.9 a	37

Melanogaster ambiguus, *Rhizopogon subareolatus*, *Rhizopogon roseolus* and *Scleroderma citrinum* all produced abundant rhizomorphs, which are considered to play an important role in water transport to host plants (Duddridge et al. 1980). Plants ectomycorrhizal with fungi that produce rhizomorphs are considered more drought tolerant than nonmycorrhizal plants or plants mycorrhizal with fungi that do not produce rhizomorphs (Parke et al. 1983; Boyd and Hellebrand 1991). This may be a very important consideration when selecting fungi for inoculation of seedlings for reforestation in Mediterranean areas, where plants are to subject severe dry periods due to the irregular distribution of rainfall during the year.

The formation of ectomycorrhizae was not consistently related with an increase in plant growth for any of the fungi studied. Statistically significant differences in biomass production of *Pseudotsuga menziesii* seedlings after 7 months were only found between control plants and plants inoculated with *Rhizopogon subareolatus* at 10^7 spores per seedling. This difference, however, was not detected between control plants and plants inoculated with 10^6 spores per seedling, which formed percentage of mycorrhizas similar to that obtained with 10^7 spores per seedling. Although some authors have reported growth stimulation of pine seedlings ectomycorrhizal with *Rhizopogon* species (Theodorou and Bowen 1970; Torres and Honrubia 1994), *Pinus pinaster* growth was not significantly affected by any inoculation treatment in our work. Riffle and Tinus (1982) reported that height, stem caliper and weight of containerized Ponderosa pine inoculated with *Rhizopogon roseolus* were not significantly different to those of controls. Castellano and Molina (1989) reviewed many different fungal inoculations successful with container seedlings. Many of these symbionts, including species of *Rhizopogon* and *Scleroderma*, had little or no effect on container seedling growth in the nursery. The application of nutrients by fertirrigation, as in the present study, and the use of artificial potting substrate in containers generally obscures the growth-promoting effect of ectomycorrhizae in the nursery (Marx and Barnett 1974; Molina 1979, 1980). For the most part, the growth of pines, spruces and firs was frequently unaffected or depressed, instead of stimulated (Castellano and Molina 1989). Castellano et al. (1985) discussed the possibility of underestimating the root dry weight due to the fungal biomass destroyed while processing seedlings inoculated with fungi that produce large amounts of rhizomorphs. Nevertheless, some ectomycorrhizal fungi that do not increase seedling growth in the nursery increase seedling field performance (Thomas and Jackson 1983; Stenström et al. 1985).

From the results obtained here, it would be feasible to use spore inocula of the most efficient fungi to produce ectomycorrhizal plants on a large scale. Further studies are needed to elucidate the effectiveness of *Melanogaster ambiguus*, *Rhizopogon* spp., and *Scleroderma citrinum* in promoting the performance of *Pseudotsuga menziesii* and *Pinus pinaster* containerized seedlings in commercial nurseries and field plantations. Long-term field experiments are currently being carried out with *Pseudotsuga menziesii* seedlings ectomycorrhizal with *Melanogaster ambiguus* and *Rhizopogon subareolatus*, to compare the effect of inoculation with different fungi on survival and growth of outplanted seedlings. These studies are aimed at selecting ectomycorrhizal fungi which are suitable for improving standard reforestation practices with this tree species in northern Spain.

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References

Alvarez IF, Parladé J, Trappe JM, Castellano MA (1993) Hypogeous mycorrhizal fungi of Spain. Mycotaxon 47:201–217

Azevedo N (1982) Ectomicorrizas del *Pinus pinaster* Sol. ex Ait. Boletín de la Estación Central de Ecología (ICONA) 11:37–42

Berch SM, Roth AL (1993) Ectomycorrhizae and growth of Douglas-fir seedlings preinoculated with *Rhizopogon vinicolor* and outplanted on eastern Vancouver Island. Can J For Res 23:1711–1715

Boyd CD, Hellebrand KE (1991) Assessment of the effect of mycorrhizal fungi on drought tolerance of conifer seedlings. Can J Bot 69:1764–1771

Castellano MA, Molina R (1989) Mycorrhizas. In: Landis TD, Tinus RW, McDonald SE, Barnett JP (eds) The container tree manual, vol 5: The biological component: nursery pests and mycorrhizas. U S D A Forest Service Agriculture Handbook 674, Washington DC, pp 101–167

Castellano MA, Trappe JM (1985) Ectomycorrhizal formation and plantation performance of Douglas-fir nursery stock inoculated with *Rhizopogon* spores. Can J For Res 15:613–617

Castellano MA, Trappe JM, Molina R (1985) Inoculation of container-grown Douglas-fir seedlings with basidiospores of *Rhizopogon vinicolor* and *R. colossus*: effects of fertility and spore application rate. Can J For Res 15:10–13

Chevalier G, Grente J (1978) Application practique de la symbiose ectomycorhizienne: production a grande echelle de plants mycorhizes par la truffe (*Tuber melanosporum* Vitt.). Mushroom Sci X:483–505

Chu-Chou M, Grace LJ (1983) Characterization and identification of mycorrhizas of Douglas-fir in New Zealand. Eur J For Pathol 13:251–260

Chu-Chou M, Grace LJ (1985) Comparative efficiency of the mycorrhizal fungi *Laccaria laccata*, *Hebeloma crustuliniforme* and *Rhizopogon* species on growth of radiata pine seedlings. N Z J Bot 23:417–424

Donald DGM (1975) Mycorrhizal inoculation for pines. S Afr For J 92:27–29

Duddridge JA, Malbari A, Read DJ (1980) Structure and function of ectomycorrhizal rhizomorphs with special reference to their role in water transport. Nature 287:834–836

Fontana A (1967) Sintesi micorrizica tra *Pinus strobus* e *Tuber maculatum*. G Bot 101:298–299

Ford VL, Torbert JL, Burger JA, Miller OK (1985) Comparative effects of four mycorrhizal fungi on loblolly pine seedlings growing in a greenhouse in a Piedmont soil. Plant Soil 83:215–221

Garrido N (1984) Notes on *Scleroderma citrinum* Pers. (Mycota-Gasteromycetes) in *Pinus radiata* forests in Chile. Nova Hedwigia Kryptogamenkd 40:511–516

Ho, I, Trappe JM (1987) Enzymes and growth substances of *Rhizopogon* species in relation with mycorrhizal hosts and infrageneric taxonomy. Mycologia 79:553–558

Ingleby K, Last FT, Mason PA (1985) Vertical distribution and temperature relations of sheathing mycorrhizas of *Betula* spp. growing on coal spoil. For Ecol Manag 12:279–285

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

Lamb RJ, Richards BN (1974) Inoculation of pines with mycorrhizal fungi in natural soil. I. Effects of density and time of application of inoculum and phosphorus amendment on mycorrhizal infection. Soil Biol Biochem 6:167–171

Marx DH (1980) Ectomycorrhiza fungus inoculations: a tool for improving forestation practices. In: Mikola P (ed) Tropical mycorrhiza research. Oxford University Press, Oxford, pp 13–71

Marx DH (1991) The practical significance of ectomycorrhizas in forest establishment. In: Ecophysiology of ectomycorrhizas of forest trees. Symposium proceedings. The Marcus Wallenberg Foundation 7:54–90

Marx DH, Barnett JP (1974) Mycorrhizas and containerized forest tree seedlings. In: Tinus RW, Stein WI, Balmer WE (eds) Proceedings of the North American Containerized Forest Tree Seedling Symposium, Great Plains Agriculture Council Publication 68, Denver, Colo, pp 85–91

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, Minn, pp 131–146

Marx DH, Ruehle JL, Cordell CE (1991) Methods for studying nursery and field response of trees to specific ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology, vol 23, Academic Press, London, pp 384–411

Massicote HB, Molina R, Luoma DL, Smith JE (1994) Biology of the ectomycorrhizal genus *Rhizopogon*. II. Patterns of host-fungus specificity following spore inoculation of diverse hosts grown in monoculture and dual culture. New Phytol 126:677–690

Mitchell HL, Finn RF, Rosendahl RO (1937) The relation between mycorrhizas and the growth and nutrient absorption of coniferous seedlings in nursery beds. Black Rock For Pap 1:57–73

Molina R (1979) Ectomycorrhizal inoculation of containerized Douglas-fir and lodgepole seedlings with six isolates of *Pisolithus tinctorius*. For Sci 25:585–590

Molina R (1980) Ectomycorrhizal inoculation of containerized western conifer seedlings. USDA Forest Service Research Note PNW-357

Molina R, Trappe JM (1982) Patterns of ectomycorrhizal host specificity and potential among Pacific Northwest conifers and fungi. For Sci 28:423–458

Molina R, Trappe JM (1994) Biology of the ectomycorrhizal genus *Rhizopogon*. I. Host associations, host-specificity and pure culture syntheses. New Phytol 126:653–675

Mousain D, Falconnet G, Gruez J, Chevalier G, Tillard P, Bousquet N, Plassard C, Cleyet-Marel JC (1987) Controlled ectomycorrhizal development of mediterranean forest seedlings in the nursery. First results and prospects. Proceedings of the 7th North American Conference on Mycorrhizas, Gainesville, Fla, p 29

Parke JL, Linderman RG, Black CH (1983) The role of ectomycorrhizas in drought tolerance of Douglas-fir seedlings. New Phytol 95:83–95

Parladé J (1992) Técnicas de inoculación de abeto de Douglas [*Pseudotsuga menziesii* (Mirb.) Franco] con hongos ectomicorrícicos y su aplicación en reforestación. PhD thesis, Universitat Autònoma de Barcelona

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

Pera J (1992) Selección de hongos ectomicorrícicos de *Pinus pinaster* Ait. para su aplicación en reforestación. PhD thesis, Universitat Autònoma de Barcelona

Pera J, Alvarez IF (1995) Ectomycorrhizal fungi of *Pinus pinaster*. Mycorrhiza 5:193–200

Pryor LD (1956) Chlorosis and lack of vigour in seedlings of Ranantherous species of *Eucalyptus* caused by lack of mycorrhiza. Proc Linn Soc N S W 81:91–96

Richter DL, Bruhn JN (1987) *Scleroderma* spp. ectomycorrhizae for use in greenhouse and nursery to increase *Pinus resinosa* outplanting success. Mycol Soc Am Newsletter 38:45

Riffle JW, Tinus RW (1982) Ectomycorrhizal characteristics, growth, and survival of artificially inoculated Ponderosa and Scots pine in a greenhouse and plantation. For Sci 28:646–660

Ruehle JL, Marx DH (1977) Developing ectomycorrhizas on containerized pine seedlings. U S D A Forest Service Research Note SE-242

Schramm JM (1966) Plant colonization studies on black wastes from anthracite mining in Pennsylvania. Trans Am Philos Soc 56:1–194

Smith AH, Zeller SM (1966) A preliminary account of the North American species of *Rhizopogon*. Mem N Y Bot Gard 14:1–178

Snedecor GW, Cochran WG (1980) Statistical methods. Iowa State University Press, Ames, Iowa

Spencer HA (1974) To "engineer" the container. In: Tinus RW, Stein WI, Balmer WE (eds) Proceedings of the North American Containerized Forest Tree Seedling Symposium, Great Plains Agriculture Council Publication 68, Denver, Colo, pp 229–232

Stenström E, Ek M, Unestam T (1985) Prolonged effects of initially introduced mycorrhizas of pine plants after outplanting. In: Physiological and genetical aspects of mycorrhizas. Proceedings of the First European Symposium on Mycorrhizas, Dijon. INRA, Paris, pp 503–506

Takacs EA (1961) Algunas especies de hongos formadores de micorrizas en árboles forestales en la Argentina. Rev For Argentina 5:80–82

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

Theodorou C, Bowen GD (1970) Mycorrhizal response of radiata pine in experiments with different fungi. Aust For Sci 34:183–191

Theodorou C, Bowen GD (1973) Inoculation of seeds and soil with basidiospores of mycorrhizal fungi. Soil Biol Biochem 5:765–771

Thomas GW, Jackson RM (1983) Growth responses of Sitka spruce seedlings to mycorrhizal inoculation. New Phytol 95:223–229

Torres P, Honrubia M (1994) Inoculation of containerized *Pinus halepensis* (Miller) seedlings with basidiospores of *Pisolithus arhizus* (Pers) Rauschert, *Rhizopogon roseolus* (Corda) Th M Fr and *Suillus collinitus* (Fr) O Kuntze. Ann Sci For 51:521–528

Trappe JM (1977) Selection of fungi for ectomycorrhizal inoculation in nurseries. Annu Rev Phytopathol 15:203–222

Trappe JM, Strand RF (1969) Mycorrhizal deficiency in a Douglas-fir region nursery. For Sci 15:381–389

Zak B (1971) Characterization and classification of mycorrhizas of Douglas-fir. II. *Pseudotsuga menziesii* + *Rhizopogon vinicolor*. Can J Bot 49:1079–1084