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Inoculation of containerized *Pinus pinea* L. seedlings with seven ectomycorrhizal fungi

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Abstract Containerized *Pinus pinea* L. seedlings are commonly used for reforestation in the Mediterranean area. While there is an increasing knowledge of the potential ectomycorrhizal fungi associated with *Pinus pinea*, few studies exist of inoculation techniques with selected ectomycorrhizal fungi. We tested seven ectomycorrhizal fungi for their effectiveness with containerized *Pinus pinea* seedlings. *Hebeloma crustuliniforme*, *Laccaria laccata* and *Pisolithus tinctorius* were applied as vegetative inocula while *Melanogaster ambiguus*, *Pisolithus tinctorius*, *Rhizopogon luteolus*, *Rhizopogon roseolus* and *Scleroderma verrucosum* were tested as spore inocula. The inoculum of each fungus was tested at several application rates. Among the fungi tested as vegetative inocula, the highest percentages of ectomycorrhizas were obtained with *H. crustuliniforme* at all rates tested. The ectomycorrhizas formed by *L. laccata* varied from 11% to 40% depending on the inoculum rate applied. Vegetative inoculum of *Pisolithus tinctorius* was only effective at the highest inoculum rates and gave mycorrhization percentages around 60%. *Pisolithus tinctorius* applied as a spore inoculum formed ectomycorrhizas at a frequency of about 50% at the effective inoculum rates. The rest of the fungi applied as spore inocula produced more than 50% of ectomycorrhizas at the effective spore concentrations. These included the highest percentages of ectomycorrhizas (>80%) obtained with both *Rhizopogon* species. Differences in growth due to inoculation with the different fungi were not detected and in some cases inoculation even reduced the total biomass accumulated by seedlings. All seedlings reached a size suitable for transplantation.

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Introduction

Pinus pinea has been traditionally used in reforestation and afforestation programs in the Mediterranean region, mainly to produce edible nuts and for soil conservation purposes (Montero et al. 1997). Mediterranean climatic features, such as summer drought, reduce *P. pinea* natural regeneration and increase the risk of erosion in deforested zones (Masetti and Mencuccini 1991). Containerized seedlings are commonly used for establishing *P. pinea* plantations (Montero et al. 1997). Mycorrhization can be particularly important for seedling performance under adverse conditions such as those imposed by a Mediterranean climate (Querejeta et al. 1998). Ectomycorrhizas ameliorate the physiological status of seedlings mainly by improving water and nutrient uptake from the soil (Smith and Read 1997). Ectomycorrhizas can play an important role in the protection of plants against environmental stress factors such as drought, pathogenic agents or heavy metal pollution (Marx 1973; Boyd and Hellebrand 1991; Leyval et al. 1997). The fertilization and the sanitary treatments within the nursery usually adversely impact on mycorrhization of seedlings. Controlled inoculation techniques are useful as an additional nursery culture method to increase field performance of out-planted seedlings (Cordell et al. 1987; Mousain et al. 1987). The pre-selection of ectomycorrhizal fungi is a critical step for establishing nursery inoculation programs (Trappe 1977). The selection criteria are based on the physiological and ecological differences between different fungi and even between fungal strains (Wong et al. 1990; Marx et al. 1992). These criteria include the symbiotic compatibility of fungus and host, the ecological adaptability of the mycorrhizal fungus to the site of transplantation, the ability of the fungus to compete against native fungi and the ease of inoculum produc-

tion. Once compatibility of plant and fungus is established, the development of suitable methods for inoculum production and application is necessary. Vegetative inocula of selected fungal strains have been frequently recommended as the method of choice (Marx 1980; Brundrett et al. 1996). However, large quantities of viable inoculum are needed for application on an operational scale and the storage of vegetative inoculum usually adversely influences its effectiveness. Spore inoculum is commonly used because of its easy application and the availability of large quantities of spores from few sporocarps. Moreover, growth in pure culture is not required and spores can tolerate long storage periods. The main disadvantage of spore inoculum is the large genetic variability, the lack of reliable laboratory methods to determine spore viability and the delay in mycorrhization compared with vegetative inoculum (Brundett et al. 1996). Selection of the most suitable inoculation method for a particular fungus and the optimization of its application in the nursery requires tests of application of effective inoculum rates.

We previously began the selection process of ectomycorrhizal fungi for *P. pinea* (Rincón et al. 1999). We reported on the ability of several fungi to form mycorrhizas with *P. pinea* and the morphological characterization of the mycorrhizas formed. In this present work, we tested the effectiveness of seven ectomycorrhizal fungi to produce containerized ectomycorrhizal *P. pinea* seedlings. We present the effectiveness of two types of inoculum (vegetative and spore) for obtaining ectomycorrhizal *P. pinea* seedlings and the effect of a range of inoculum concentrations on ectomycorrhiza formation and seedling growth.

Materials and methods

Fungal material

Sporocarps of *Hebeloma crustuliniforme* (Bull.ex St. Amans), *Laccaria laccata* (Scop.ex Fr.) Quél., *Melanogaster ambiguus* (Vitt.) Tul. & Tul., *Pisolithus tinctorius* (Pers.) Coker & Couch, *Rhizopogon luteolus* (Fr. & Nordh.), *Rhizopogon roseolus* (Corda ex. Sturm) Fr. and *Scleroderma verrucosum* (Bull ex. Pers. ss. Grév.) were collected in nurseries and mixed forests of *Pinus pinea* in different locations of Catalonia (northeastern Spain). After collection, taxonomic identification and isolation in pure culture, the sporocarps of the different fungi were dried at 40°C for 48 h and kept at room temperature for further use (herbarium of the DPV-IRTA). Pure culture isolates were obtained as described by Rincón et al. (1999).

Plant material

Pinus pinea seeds were collected in 1994 and 1995 from natural forests in the Montnegre and Montseny sierras in Catalonia. Before germination, seeds were rinsed in running tap water overnight, surface disinfected by shaking for 30 min in 30% (v:v) H_2O_2 and washed in four changes of sterile distilled water.

Production of fungal inocula

Two types of inoculum were tested: vegetative inoculum produced in a peat-vermiculite substrate and spore inoculum. *H. crustuliniforme* (126), *L. laccata* (127) and *Pisolithus tinctorius* (93) were tested as vegetative inocula. Mycelial suspensions of each fungus were obtained by growing in liquid MMN medium (Marx 1969) (5 plugs per 10 ml) at 25°C for 4 weeks. Once grown, 40–60 ml of each fungal suspension was transferred to 1-l bottles containing an autoclaved mixture of peat-vermiculite (50–550 ml, 20 min, 120°C) moistened with 350 ml of liquid MMN medium (glucose reduced to 2.5 g/l). The inocula were incubated at 25°C in the dark for 1 month before use. *M. ambiguus*, *R. luteolus* and *R. roseolus* were applied as spore suspensions. The sporocarps of the different fungi were blended in distilled water at low speed until the spores were released. For each fungus, initial spore concentration was measured with an hematocytometer and the bulk spore suspension was serially diluted to obtain spore concentrations of 10^3–10^8 spores/10 ml for *Rhizopogon* spp. and 10^3–10^7 spores/10 ml for *M. ambiguus*. *Pisolithus tinctorius* and *S. verrucosum* basidiospores were removed from the sporocarps by sieving through a 0.5-mm mesh and counted with an hematocytometer. One gram of *Pisolithus tinctorius* and *S. verrucosum* sporocarps contained 16×10^8 and 5×10^8 spores, respectively. Dry basidiospores of each fungus were mixed with vermiculite for seedling inoculation.

Seedling inoculation and culture conditions

A potting substrate containing equal volumes of peat and vermiculite, autoclaved (60 min, 120°C) and with a final pH (in water) of 5.5, was used to fill Rootrainer Spencer-Lemaire containers (175 ml capacity). The vegetative inocula of each fungus tested were mixed with the potting substrate before filling containers at rates of 1:4, 1:8, 1:16, 1:32 and 1:64 inoculum:substrate (v:v). Dry spores of *Pisolithus tinctorius* and *S. verrucosum* included in vermiculite were mixed with the potting substrate and the containers filled with 10^3–10^8 spores per 175 ml of substrate.

Two disinfected *Pinus pinea* seeds were sown in each container and seedlings were thinned to one per container after emergence. One-month-old seedlings were used for inoculation with spore suspensions. Seedlings were inoculated with 10 ml of the spore suspension at the desired concentration from 10^3 to 10^8 spores per seedling (for *M. ambiguus* the highest spore concentration tested was 10^7 spores per seedling). In each experiment, a control treatment of non-inoculated seedlings was established. In all experiments, a total of 16 replicates were set up in each treatment. Seedlings were grown in a greenhouse with a photoperiod of 16 h (minimum 200 µmol s^{-1} m^{-2}) provided by high pressure sodium vapor lamps. Greenhouse temperature oscillated between 18 and 25°C and relative humidity was higher than 40%. Seedlings were fertilized every 3 weeks with 10 ml per seedling of a solution of 20–7–19 Peter's (Grace-Sierra) fertilizer (1.8 g/l) and the micronutrients preparations Fetrilon (0.12 g/l) and Hortrilon (0.28 g/l).

Measured parameters and statistical analysis

Five months after inoculation, when ectomycorrhizas were clearly developed, seedlings in all treatments were harvested and their roots washed free of substrate. The ectomycorrhizas formed by each inoculated fungus were identified according to morphological criteria derived from results previously obtained in synthesis trials (Rincón et al. 1999). Each seedling root was cut into 2- to 3-cm segments and the percentage of ectomycorrhizal short roots was assessed by counting at least 200 randomly selected short roots under the stereomicroscope. All plants were measured for stem height and root collar diameter. The seedling shoots and roots were oven dried (60°C, 48 h) to obtain the total dry weight. Data were analyzed by one-way analysis of variance (ANOVA) and significant differences among treatments were separated by Tukey's test ($P<0.05$). Percentages of ectomycorrhizas were arc-sin transformed before performing ANOVA (Snedecor and Cochran 1980).

Table 1 Growth and percentage of ectomycorrhizal *Pinus pinea* seedlings inoculated with different rates of vegetative inoculum of *Hebeloma crustuliniforme*, *Laccaria laccata* or *Pisolithus tinctorius*. For each fungal treatment, means in the same column followed by a different letter are significantly different by Tukey's test ($P \leq 0.05$). Inoculum rate is inoculum:substrate (v:v)

Fungus	Inoculum rate	Stem diameter		Shoot height		Total dry weight		Mycorrhizal seedlings %
		mm		cm		g		
Hebeloma crustuliniforme	1:4	3.4	a	24.7	ab	3.0	a	92
	1:8	3.5	a	23.5	ab	3.4	a	100
	1:16	3.6	a	23.2	a	3.1	a	100
	1:32	3.4	a	25.9	b	3.3	a	100
	1:64	3.4	a	23.7	ab	3.2	a	100
	Control	3.7	a	24.9	ab	3.4	a	0
Laccaria laccata	1:4	3.9	a	26.1	c	3.6	a	92
	1:8	4.0	a	20.6	a	3.6	a	100
	1:16	4.1	a	24.9	bc	3.7	a	100
	1:32	4.1	a	22.2	ab	4.2	a	100
	1:64	4.0	a	22.7	abc	3.7	a	100
	Control	4.2	a	22.1	ab	3.6	a	0
Pisolithus tinctorius	1:4	3.5	a	24.2	a	3.1	a	93
	1:8	3.5	ab	25.8	a	3.0	a	83
	Control	3.7	b	24.7	a	3.5	a	0

Results

Vegetative inoculum

Almost all seedlings were colonized when inoculated with vegetative inoculum of *H. crustuliniforme* (Table 1) and the percentage of ectomycorrhizal short roots was higher than 80% at all inoculum rates tested (Fig. 1). All seedlings inoculated with vegetative inoculum of *L. laccata* became colonized except at the 1:4 inoculum rate (92%) (Table 1). The percentages of ectomycorrhizal short roots formed by *L. laccata* at the different inoculum rates varied from 11% to 40% (Fig. 1). The percentage of ectomycorrhizas obtained at the 1:16 rate was significantly higher than the other inoculation rates, except for 1:4. Vegetative inoculum of *Pisolithus tinctorius* only formed ectomycorrhizas when applied at the 1:4 and 1:8 inoculum rates (Table 1). Seedlings showed 58% and 67% of ectomycorrhizal short roots at the 1:4 and 1:8 rates, respectively, with no significant differences between the two treatments (Fig. 1). Inoculation with *Pisolithus tinctorius* significantly decreased the stem diameter of seedlings when applied at the highest inoculum rate, while inoculation with *H. crustuliniforme* and *L. laccata* did not affect this parameter (Table 1). For all three experiments, no significant differences were detected in total dry weight between inoculated and non-inoculated seedlings (Table 1). Seedlings in all treatments reached a standard suitable for transplantation.

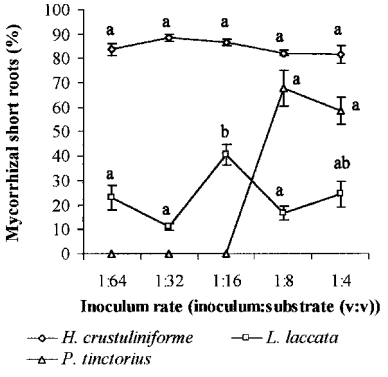

Fig. 1 Percentages of ectomycorrhizal short roots of containerized *Pinus pinea* seedlings inoculated with vegetative inoculum of *Hebeloma crustuliniforme*, *Laccaria laccata* or *Pisolithus tinctorius*. Means in each fungal treatment with the same letter are not significantly different by Tukey's test ($P \leq 0.05$). Non-effective inoculum rates are not included in the statistical analysis

Spore inoculum

Melanogaster ambiguus did not form ectomycorrhizas when spores were applied below 10^6 spores per seedling. All seedlings became mycorrhizal when 10^7 spores per seedling were applied and 72% of them were colonized at the spore rate of 10^6 (Table 2). The percentage of ectomycorrhizal short roots obtained at the 10^7 rate was significantly higher than at the 10^6 rate (Fig. 2). Ectomycorrhizas formed by *M. ambiguus* were golden-brown with a thick mantle and abundant brown rhizomorphs. Spore inoculum of *Pisolithus tinctorius* was effective when applied at10^6–10^8 spores per seedling (64–100% of colonized seedlings). Ectomycorrhizas did not form at spore rates of 10^3–10^5 (Fig. 2). The percentages of ectomycorrhizal short roots obtained at the 10^6, 10^7 and 10^8 spore rates were close to 50% (52%, 43% and 52%, respectively) and were not significantly different. All seedlings were colonized when inoculated with *R. luteolus* independent of spore concentration, except at the lowest (57% of mycorrhizal seedlings) (Table 2). The percentage of ectomycorrhizal short roots obtained at rates 10^4 and 10^5 (64% and 67%) were significantly lower than those obtained at rates 10^6, 10^7 and 10^8 (>80%) (Fig. 2). The percentage of ectomycorrhizas obtained at the rate of 10^7 was significantly higher than at 10^3. *R. luteolus*

Table 2 Growth and percentage of ectomycorrhizal *Pinus pinea* seedlings inoculated with different spore rates of *Melanogaster ambiguus*, *Pisolithus tinctorius*, *Rhizopogon luteolus*, *R. roseolus* or *Scleroderma verrucosum*. For each fungal treatment, means in the same column followed by a different letter are significantly different by Tukey's test ($P \leq 0.05$)

Fungus	Spores per seedling	Stem diameter		Shoot height		Total dry weight		Mycorrhizal seedlings %
		mm		cm		g		
Melanogaster ambiguus	10^7	4.2	a	19.2	a	3.9	a	100
	10^6	4.4	a	23.7	b	4.6	ab	72
	Control	4.5	a	26.2	b	4.8	b	0
Pisolithus tinctorius	10^8	3.7	a	33.8	a	3.4	a	100
	10^7	4.0	ab	32.5	a	3.5	a	100
	10^6	4.1	b	30.9	a	4.0	b	64
	Control	4.0	ab	38.1	b	4.2	b	0
Rhizopogon luteolus	10^8	3.5	a	19.1	b	3.0	a	100
	10^7	3.7	ab	17.8	ab	3.1	ab	100
	10^6	3.7	ab	16.5	a	3.5	bc	100
	10^5	3.9	b	16.5	a	3.6	bc	100
	10^4	3.8	b	19.6	bc	3.6	bc	100
	10^3	3.9	b	21.4	c	3.8	c	57
	Control	3.8	b	16.7	a	3.9	c	0
Rhizopogon roseolus	10^8	3.6	a	27.9	ab	3.3	ab	100
	10^7	3.7	a	29.1	b	3.3	ab	100
	10^6	3.6	a	27.2	ab	3.3	ab	100
	10^5	3.5	a	27.5	ab	3.1	a	100
	10^4	3.7	a	27.3	ab	3.5	ab	100
	10^3	3.6	a	26.3	ab	3.1	a	80
	Control	4.0	a	23.3	a	4.2	b	0
Scleroderma verrucosum	10^8	3.7	ab	17.1	a	3.6	ab	100
	10^7	3.7	a	19.4	bc	3.2	a	92
	10^6	3.8	ab	20.3	c	3.9	b	100
	10^5	3.9	ab	18.4	ab	3.7	b	86
	Control	4.0	b	17.9	ab	4.7	c	0

ectomycorrhizas were white-beige forming a cotton mantle, coralloid branching and abundant white rhizomorphs. All seedlings became colonized by *R. roseolus* at all the spore rates tested, except the lowest (10^3), in which 80% of the seedlings showed ectomycorrhizas (Table 2). The percentage of ectomycorrhizal short roots obtained at the different spore rates varied between 68% and 81% (Fig. 2). No significant differences were detected between the different spore rates except at 10^3, where the percentage of ectomycorrhizal short roots was significantly lower than at 10^5 (Fig. 2). *S. verrucosum* formed ectomycorrhizas when more than 10^5 spores per seedling were applied (Table 2). The percentages of ectomycorrhizal short roots (54–62%) produced after application of spores at 10^5–10^8 were not significantly different (Fig. 2). For all fungi tested as spore inocula, no significant growth response of *Pinus pinea* seedlings due to the inoculation was detected (Table 2). *M. ambiguus*, *Pisolithus tinctorius*, *R. luteolus* and *S. verrucosum* had a negative effect on dry biomass accumulation of seedlings when applied at the highest spore rates (Table 2). For *S. verrucosum*, the negative effect on seedling dry weight was evident at all the effective spore rates, while inoculation with *R. roseolus* significantly decreased this parameter only at the 10^3 and 10^5 spore rates. Seedling diameter was not significantly affected by inoculation with *Pisolithus tinctorius*, *M. ambiguus* and *R. roseolus* but was significantly reduced by *R. luteolus* and *S. verrucosum* at some rates of spore application (Table 2).

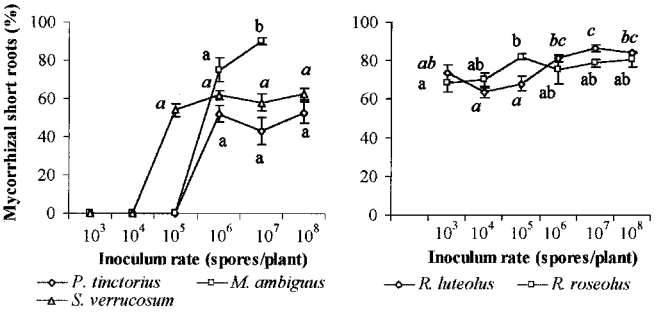

Fig. 2 Percentages of ectomycorrhizal short roots of containerized *Pinus pinea* seedlings inoculated with spores of *Melanogaster ambiguus*, *Pisolithus tinctorius*, *Rhizopogon luteolus*, *R. roseolus* or *Scleroderma verrucosum*. Means in each fungal treatment with the same letter are not significantly different by Tukey's test ($P \leq 0.05$). Non-effective inoculum rates are not included in the statistical analysis

Seedling shoot height was negatively affected by inoculation with *M ambiguus* and *Pisolithus tinctorius*, while for some rates of spore application this parameter was significantly improved by *R. luteolus*, *R. roseolus* and *S. verrucosum*.

Discussion

The use of vegetative inoculum of *H. crustuliniforme* was an effective method for obtaining containerized

ectomycorrhizal *Pinus pinea* seedlings. The isolate 126 of *H. crustuliniforme* has been tested also as vegetative inoculum for *Pseudotsuga menziesii* (Parladé 1992) with similar mycorrhization results. The slow growth of this fungal strain in pure culture is compensated by the fact that large quantities of inoculum are not required (1:64) to obtain high percentages of mycorrhization. Vegetative inocula of *H. crustuliniforme* and *H. sinapizans* already tested with *Pinus pinea* (Branzanti et al. 1985; Branzanti and Zambonelli 1988) produced high percentages of root colonization. Nevertheless, various authors have reported the low efficacy of *Hebeloma* spp. in stimulating plant development under field conditions (Stenström et al. 1990; Le Tacon et al. 1992).

When vegetative inoculum of *L. laccata* was applied here, almost all the seedlings were colonized but no more than 40% of ectomycorrhizal short roots was obtained at any inoculum rate tested. Other tests of the same isolate of *L. laccata* (127) with *Pseudotsuga menziesii* (Parladé 1992) and *Pinus pinaster* (Pera 1992) gave similar results, with percentages of ectomycorrhizas not exceeding 50–60%. Branzanti et al. (1985) reported higher (90%) percentages of *Pinus pinea* mycorrhizas with a vegetative inoculum of a different isolate of *L. laccata*. Variability between isolates of the same fungal species in colonization ability and effects on plant growth has been reported before (Trappe 1977; Wong et al. 1990; Burgess et al. 1994; De la Bastide et al. 1995). The use of vegetative inocula of *L. laccata* and *L. bicolor* included in alginate beads has produced increased mycorrhization (Hung and Trappe 1987; Villeneuve et al. 1991; Le Tacon et al. 1992; Di Battista et al. 1992). *Laccaria* appears to be poorly adapted to Mediterranean climate conditions and its sporocarps have not been found in association with *Pinus pinea* in the field. Mycorrhization results obtained in our experiment could also be a consequence of plant-fungus incompatibility.

Vegetative inoculum of *Pisolithus tinctorius* was only effective at the highest application rates (percentage of ectomycorrhizal short roots around 60%). *Pisolithus tinctorius* has been used as vegetative inoculum with different forest species in several countries (Marx 1981). This species has an ubiquitous distribution, tolerates a great variety of environmental conditions and grows quickly in pure culture (Marx 1991). However, diverse experiments carried out in field conditions around the world with different tree species have given variable results and in some cases there was no growth stimulation of the seedlings (Castellano 1994, 1996). Inoculation of *Pinus pinea* with spore inoculum of *Pisolithus tinctorius* gave percentages of ectomycorrhizas close to 50%. The minimal level of ectomycorrhizas necessary to ensure an effect on plant development in field conditions has been poorly studied. For *Pisolithus tinctorius*, this level has been established at 50% of ectomycorrhizal short roots (Marx 1980, 1991). Spore inoculum of *Pisolithus tinctorius* has been successfully used in several experiments with different *Pinus* species (Marx and Cordell 1990; Marx 1991) and also with *Quercus suber* and *Q. rubra* (Ruehle 1980). For other species, such as *Pseudotsuga menziesii* (Parladé 1992), *Abies* spp. and *Pinus ponderosa* (Alvarez and Trappe 1983), the fungus was not effective.

Rhizopogon luteolus and *R. roseolus* showed high effectiveness in forming ectomycorrhizas with *Pinus pinea* (100% of colonized seedlings at all the spore rates except the lowest). Both fungi formed more than 60% of ectomycorrhizas at all the spore rates, reaching more than 80% of ectomycorrhizal short roots at the highest rates. The wide geographic distribution of *Rhizopogon* spp., its ecological adaptation to different habitats, the specificity towards the *Pinaceae* family (Trappe 1962; Molina and Trappe 1994) and the easy application of spore inoculum make species of this genus good candidates for inoculation programs. Spore inoculum of *M. ambiguus* was effective for *Pinus pinea* mycorrhization when applied at rates higher than 10^6 spores per seedling. The optimal application rate was 10^7 spores per seedling. Spore inoculum of *M. ambiguus* has also been used for inoculation of *Pinus pinaster* and *Pseudotsuga menziesii* (Parladé et al. 1996) and maximal percentages of ectomycorrhizas were obtained at higher than 10^5 spores per seedling. To our knowledge, this is the first report of *M. ambiguus* and *R. luteolus* as *Pinus pinea* fungal symbionts (Rincón et al. 1999). Inoculation with *S. verrucosum* spores produced around 60% ectomycorrhizal short roots at an application rate of 10^5 spores per seedling. Similar results have been reported for *Pinus pinaster* inoculated with spores of *S. citrinum* (Parladé et al. 1996). Like *M. ambiguus*, *Pisolithus tinctorius*, *R. luteolus* and *R. roseolus*, *S. verrucosum* also produced abundant rhizomorphs, which are structures associated with water transport to the host plant. This could be an important selection factor for fungi in reforestation programs in the Mediterranean region.

Pisolithus tinctorius was the only fungus tested as both vegetative and spore inoculum. Most inoculation studies with *Hebeloma* or *Laccaria* have used vegetative inoculum (Branzanti and Zambonelli 1988; Le Tacon et al. 1992; Parladé 1992; Pera 1992; De la Bastide et al. 1995) since an enormous quantity of sporocarps would be required for the application of these fungi as spore inoculum in the nursery. Rincón et al. (1999) showed vegetative inocula of *R. roseolus* and *Scleroderma* sp. to be effective for *Pinus pinea* when applied at high inoculum rates, while vegetative inocula of *M. ambiguus* and *R. luteolus* did not form mycorrhizas. The difficulty in obtaining ectomycorrhizal seedlings with vegetative inoculum of *Rhizopogon* spp. has been reported previously (Molina and Trappe 1994).

In general, no significant increase in seedling growth due to inoculation with the different fungi was observed. The reserves in *Pinus pinea* cotyledons are large and seedlings probably did not depend on their mycorrhizal associates for growth during the period of the experiment. Other factors such as the use of artificial potting substrate, the confined space in the container and the constant fertilization may have suppressed the mechanisms by which the fungus influences plant develop-

ment, such as nutrient mobilization and exploration of a greater volume of soil (Molina 1980). Contrary to stimulating seedling growth, some fungi significantly decreased seedling dry weight at the highest spore rates. As suggested by Castellano et al. (1985), seedling root biomass may have been underestimated since fungal biomass (mostly represented as extramatrical mycelium and rhizomorphs) was not taken into account.

The experiments carried out in this study have allowed the selection of different ectomycorrhizal fungi for the inoculation of containerized *Pinus pinea* seedlings. The minimal application rate of inoculum ensuring adequate mycorrhization has also been established for each fungus. The use of selected ectomycorrhizal fungi and the integration of the different culture factors in the nursery are important for establishing a successful program of mycorrhizal seedling production (Cordell and Marx 1994). One of the fungi tested in this study has already been tested for inoculation of *Pinus pinea* in the nursery (Rincón et al. unpublished results) and plantations with most of the fungi used in this study are currently being developed to determine the performance of ectomycorrhizal *Pinus pinea* seedlings under field conditions.

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