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Coinoculation of containerized Douglas-fir (*Pseudotsuga menziesii*) and maritime pine (*Pinus pinaster*) seedlings with the ectomycorrhizal fungi *Laccaria bicolor* and *Rhizopogon* spp.

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Abstract Coinoculations with mycelium of *Laccaria bicolor* and spores of *Rhizopogon* spp. included in alginate gel have been carried out to determine: (1) the ability of the mixed inoculum to produce dual-colonized containerized Douglas-fir and maritime pine planting stocks and (2) the colonization pattern of the two fungi in individual root systems. For both tree species, the maximal proportion of dual-colonized seedlings obtained almost never exceeded 50%. The rest of the seedlings remained colonized by a single fungus or were non-colonized. In Douglas-fir inoculations, the relationship between the dual-colonized seedlings obtained and the initial dose of the two fungi was highly significant. The highest proportion of dual-colonized seedlings was obtained when the highest dose of *R. subareolatus* was used (10^6 spores/seedling), regardless of the dose of *L. bicolor*. Among the treatments producing 25% or more dual-colonized seedlings, differences in the proportion of *Laccaria/Rhizopogon* mycorrhizas and total root colonization percentages were not clearly related to the initial combination of doses. The proportion of dual-colonized maritime pine seedlings was not significantly related to the initial inoculation doses of the two fungi. The proportion of *Laccaria/Rhizopogon* mycorrhizas was not significantly different among treatments with 25% or more dual-colonized seedlings, whereas total colonization percentages ranged from 37% with the combination $0.08/10^4$ (g *L. bicolor* / spores *R. roseolus* per seedling) to 74% with the combination $0.08/10^6$, this difference being statistically significant.

Key words Coinoculation · Fungal colonization · Mixed inoculum · Alginate inoculum · Reforestation

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Introduction

Nursery inoculations of forest tree seedlings with ectomycorrhizal fungi have been usually carried out with only one fungal species (Castellano and Molina 1989; Marx et al. 1991). Nevertheless, the presence of several fungi forming mycorrhizas in different regions of the root system of a single plant is common in nature (Trappe 1977; Gibson and Deacon 1988). It has been hypothesized that the different fungal symbionts present in the same root system play a different role depending on the developmental stage of the plant and the environmental conditions, thus increasing potential adaptability to environmental changes (Schoeneberger and Perry 1982; Pilz and Perry 1984).

The development of methods for the production of mixed inocula would allow combination of ectomycorrhizal fungi with proven efficiency in promoting plant performance in nurseries and in the field. Among the possible benefits of coinoculations with different fungi are provision of the plant with whatever benefit each fungus provides separately and/or increase in the probability that some of the introduced symbionts survive in a range of field situations.

Mixed inocula should be easy to produce and to apply in nursery conditions to avoid increasing production costs. Among the fungal candidates for mixed inoculations are those ectomycorrhizal fungi which increase field performance of inoculated plants worldwide, such as *Laccaria bicolor* (Maire) Orton (Le Tacon et al. 1992; Castellano 1996; Pera et al. 1997). Certain *Rhizopogon* species have improved field growth of inoculated plants (Castellano 1996; Pera et al. 1997) and the recovery of plants from drought (Parke et al. 1983). Plants ectomycorrhizal with fungi that produce abundant rhizomorphs, such as *Rhizopogon* species, may be more drought tolerant because of the role of rhizomorphs in water transport (Duddridge et al. 1980). Vegetative (mycelial) inoculum of *L. bicolor* growing in peat-vermiculite has been successfully applied to Dou-

glas-fir and pine seedlings, even at low application doses (Molina 1982; Hung and Molina 1986; Hung and Trappe 1987; Le Tacon et al. 1988). Mycelium of *L. bicolor* entrapped in alginate gel has also been an effective inoculum type when applied in nurseries (Mortier et al. 1988). Spore inoculum of *Rhizopogon* species has been successfully applied to Douglas-fir and pine seedlings (Theodorou and Bowen 1973; Castellano et al. 1985; Chu-Chou and Grace 1985; Massicotte et al. 1994; Alvarez et al. 1996; Parladé et al. 1996), in contrast to the ineffectiveness of or limited results obtained with vegetative inocula (Molina 1980; Ford et al. 1985; Mousain et al. 1987).

To date, few studies have been done on multiple inoculations. Some experimental coinoculations with several ectomycorrhizal fungi have been performed under controlled conditions in vitro (Parladé and Alvarez 1993). Studies under greenhouse and nursery conditions are also scarce (Chu-Chou and Grace 1985; Reddy and Natarajan 1997). The objective of this present study was to determine: (1) the effectiveness of a mixed alginate inoculum, bearing infective propagules of two different fungi (mycelium and spores), to produce containerized Douglas-fir and maritime pine seedlings colonized by both fungi and (2) the colonization pattern of the two fungi in individual root systems.

Materials and methods

Sporocarps of *Rhizopogon subareolatus* Smith were collected in the Montseny range (province of Girona, northeastern Spain) in autumn 1995 under a 50-year-old Douglas-fir (*Pseudotsuga menziesii* (Mirb. Franco)) at 1100 m elevation in a pH 5.5 sandy soil. Sporocarps of *Rhizopogon roseolus* (Corda ex Sturm) Th. Fries were collected in a bareroot nursery located in Breda (province of Girona) in autumn 1995 under a 2-year-old *Pinus radiata* D. Don at 90 m elevation in a pH 6.5 sandy clay soil. The isolate S-238 of *L. bicolor* was obtained originally in 1976 from a sporocarp collected under *Tsuga mertensiana* (Bong.) Carr. in Oregon (USA), and transferred to the INRA Nancy fungal collection in 1980 (strain S-238-N), and then sub-cultured every 2–3 months on solid modified Pachlewski's medium (Di Battista et al. 1996).

Spores of *Rhizopogon* spp. were obtained by blending chopped sporocarps in a Waring blender at low speed in water until the spores were released as described in Parladé et al. (1996). Mycelium of *L. bicolor* S-238 was obtained by blending in water 25-day-old colonies growing in MMN agar (Marx 1969). Aliquots (10 ml) of the mycelial slurry obtained were added to flasks containing 1 l of liquid MMN medium each. The flasks were maintained stationary at 25 °C for 25 days. The actively growing mycelium was then filtered through sterilized cheesecloth, washed with sterile distilled water, let to drip and weighed.

Mixed inocula were prepared by adding spores of either *R. subareolatus* or *R. roseolus* plus fragmented mycelium of *L. bicolor* (blended in autoclaved water) at different proportions in 2-l Pyrex flasks containing 20 g/l of an autoclaved water solution of sodium alginate (SOBALG FG 100, 174). A complete factorial combination was established by mixing: 0, 2.5, 5 and 10 g/l of *L. bicolor* S-238 plus 0, 1.14×10^6, 1.14×10^7 and 1.14×10^8 spores/l of *R. subareolatus*, and 0, 2.5, 5 and 10 g/l of *L. bicolor* S-238 plus 0, 1.14×10^6, 1.14×10^7 and 1.14×10^8 spores/l of *R. roseolus*. A total of 32 flasks were prepared (16 for each dual combination). The content of each flask was gently mixed and dropped into a 0.3 M water solution of $CaCl_2$ to polymerize. The beads formed were maintained in the $CaCl_2$ solution for 1 h to complete polymerization, then washed with sterile distilled water, and kept at 4 °C in plastic bags for 1 week.

Douglas-fir and maritime pine (*Pinus pinaster* Ait.) seeds (origins OR 422 lot 422-25-88 and Aquitaine CEMAGREF lot 81245, respectively) were surface sterilized with 30% H_2O_2 for 30 min and stratified at 4 °C during 30 days. For plant production, Ray Leach Single Cell Cone-tainers (Stuewe and Sons, Inc., Corvallis, Ore) of 165 ml capacity and a mixture of Floratorf peat (Floragard, Oldenburg, Germany) and vermiculite (Termita grade 2, Asfaltex, Barcelona, Spain) in the proportion 1:1 (v:v) were used. The substrate was previously autoclaved at 120 °C for 1 h. The final pH of the mixture was 5.5.

Inoculation was made before sowing by mixing the different types of alginate beads with the substrate in the proportion 1:20 (v:v). Sixteen containers were inoculated with each of the 16 different combinations of *R. subareolatus* plus *L. bicolor* (for *P. menziesii*) and *L. bicolor* plus *R. roseolus* (for *P. pinaster*). Each container received either 0, 0.02, 0.04 or 0.08 g of *L. bicolor* and 0, 10^4, 10^5 or 10^6 spores of *Rhizopogon* spp.

Two seeds of each tree species were sown per container to ensure germination and 1 month later thinned to one per container. The treatments were established in a completely randomized design in a shadehouse covered with a 40% white mesh from March 1995 to February 1996. Mean temperatures ranged from 9.2 °C in January to 23.8 °C in August. Monthly mean relative humidity was always above 60%. Seedlings were watered regularly every other day to field capacity and fertirrigated every 15 days (from March to September) 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) (Parladé et al. 1996).

At the end of the experiment, height and root collar diameter of the seedlings were measured. For each tree species and inoculation treatment, three types of mycorrhizal assessment were made: (1) relative frequencies of seedlings with the two types of mycorrhizas present in the root system (considered even if one of the types was rare), (2) total mycorrhizal colonization in seedlings with the two types of mycorrhizas and (3) the proportion of *Laccaria/Rhizopogon* mycorrhizas in seedlings colonized by both fungi. Assessment 2 was made by counting 200 short roots per seedling as described in Parladé et al. (1996). Frequencies of seedlings with two types of mycorrhizas were analysed by multiple regression using the inoculation doses of each fungus as independent variables. Growth, mycorrhizal colonization and proportion of *Laccaria/Rhizopogon* mycorrhizas were analysed by ANOVA. Differences among means were detected by Tukey's test ($P \leq 0.05$). For assessments 1 and 2, logistic-binomial transformation was performed to normalize the data. For assessments 2 and 3, only treatments with 25% or more seedlings colonized simultaneously by the two fungi were considered representing 4 or more replicates.

Results

At the end of the experiment, both tree species reached plantable size. Douglas-fir seedlings averaged 13.8 cm in height and 3.2 mm in root collar diameter. Maritime pine seedlings averaged 16.2 cm in height and 3.4 mm in root collar diameter. No significant differences in growth were detected among the inoculation treatments.

From the 9 dual inoculation treatments applied to *P. menziesii*, 7 produced seedlings colonized simultaneously by the two fungi in a percentage ranging from 53% of the seedlings with the combination $0.08/10^6$ (g *L. bicolor* / spores *R. subareolatus* per seedling) to 15% with the combinations $0.04/10^5$ and $0.04/10^4$ (Fig. 1).

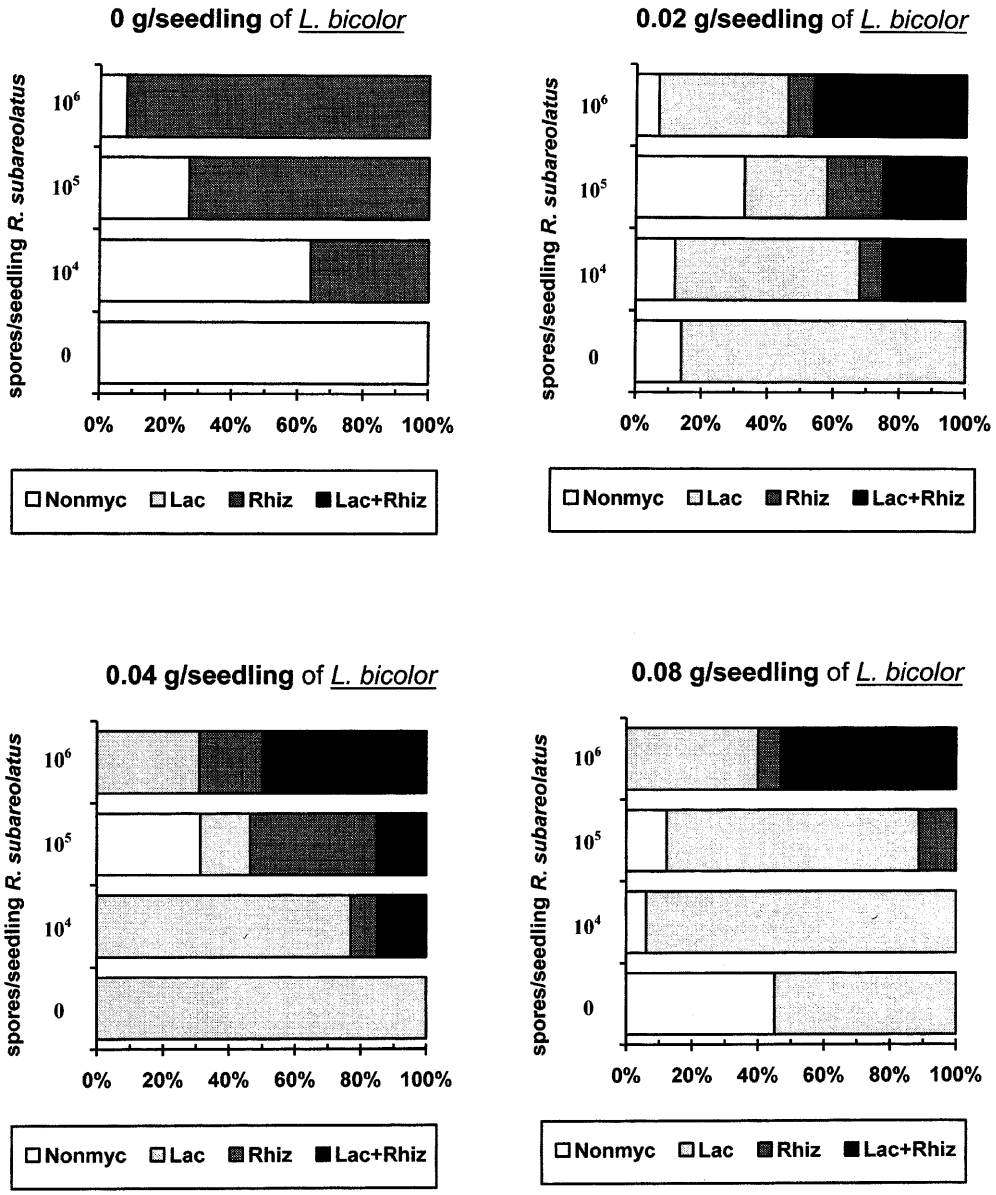

Fig. 1 Percentages of mycorrhizal and non-mycorrhizal (*Nonmyc*) Douglas-fir seedlings after inoculation with different doses of *Laccaria bicolor* (*Lac*) and/or *Rhizopogon subareolatus* (*Rhiz*)

The rest of the seedlings had either monospecific colonization or no mycorrhizas. No contaminant mycorrhizas were detected in any seedling. Multiple regression analysis between the proportion of dual-colonized seedlings obtained and the initial doses of *L. bicolor* and *R. subareolatus* revealed a significant relationship (Fig. 2). Increasing the dose of *R. subareolatus* to 10^6 spores per seedling produced the maximal amount of dual-colonized seedlings (around 50%) irrespective of the *L. bicolor* dose. At lower *R. subareolatus* doses (10^4 and 10^5 spores/seedling), doses of 0.02 and 0.04 g/seedling of *L. bicolor* produced between 15 and 25% of dual-colonized seedlings. The proportion of *Laccaria/Rhizopogon* mycorrhizas, in those treatments with 25% or more seedlings colonized by both fungi, varied from 0.19 to 2.36, although the high variability within inoculation treatments precluded statistically significant differences (Table 1). Total colonization percentages on seedlings from these treatments ranged from 43 to 75% of the short roots and were not clearly related to the initial combination of doses (Table 1).

Eight of the 9 dual inoculation treatments applied to *P. pinaster* produced seedlings with the two fungi colonizing simultaneously the same root system in a percentage ranging from 37% of the seedlings at the combination $0.08/10^6$ (g *L. bicolor* / spores *R. roseolus* per seedling) to 6% at the combination $0.02/10^4$ (Fig. 3). The rest of the seedlings had either monospecific or no mycorrhizas. Contamination by *Thelephora terrestris* Pers. ex Fr. was abundant and erratic among the different treatments. Multiple regression analysis between the proportion of dual-colonized seedlings obtained and the initial application doses of both fungi did not detect any significant relationship between them

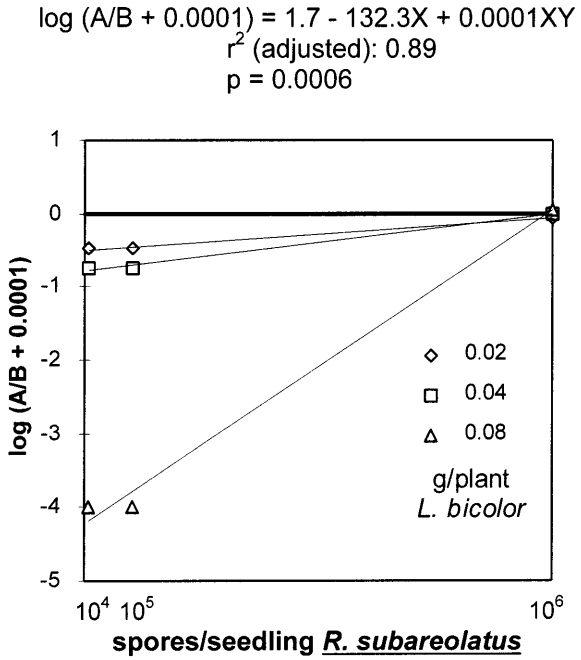

Fig. 2 Relationship (multiple regression) between the proportion of dual-colonized Douglas-fir seedlings and the initial inoculum dose of both *L. bicolor* (X) and *R. subareolatus* (Y), where A is the number of dual-colonized seedlings and B the number of single colonized plus non-colonized seedlings. Tendency lines are represented for each dose of *L. bicolor*

Table 1 Proportions of *Laccaria bicolor/Rhizopogon subareolatus* mycorrhizas and total percentages of mycorrhizal colonization on roots of Douglas-fir seedlings inoculated simultaneously with various doses of *L. bicolor* and *R. subareolatus.* Only those combinations producing equal or more than 25% dual-colonized seedlings were considered. Values followed by the same letter in the same column are not different by Duncan's multiple range test ($P \leq 0.05$). Before performing the ANOVA, total colonization data were submitted to the logistic binomial transformation

Inoculation treatment		Proportion of mycorrhizas	% Mycorrhizas
L. bicolor (g/seedling)	*R. subareolatus* (spores/seedling)	*L. bicolor/ R. subareolatus*	
0.02	10^4	2.00 ab	61 ab
0.02	10^5	2.36 b	43 a
0.02	10^6	0.46 ab	67 b
0.04	10^6	0.19 a	75 b
0.08	10^6	1.08 ab	67 b

($r^2 = 0.09$, $P = 0.7642$). Only three inoculum combinations produced 25% or more dual-colonized seedlings with a proportion of *Laccaria/Rhizopogon* mycorrhizas not significantly different among treatments, although the mean values varied from 0.17 to 4.46 (Table 2). Total colonization percentages of dual-colonized seedlings ranged from 37% with the combination $0.08/10^4$ (g *L. bicolor* / spores *R. roseolus* per seedling) to 74% with the combination $0.08/10^6$, this difference being statistically significant (Table 2).

Table 2 Proportions of *L. bicolor/R. roseolus* mycorrhizas and total percentages of mycorrhizal colonization on roots of maritime pine seedlings inoculated simultaneously with various doses of *L. bicolor* and *R. roseolus.* Only those combinations producing equal or more than 25% dual-colonized seedlings were considered. Values followed by the same letter in the same column are not different by Duncan's multiple range test ($P \leq 0.05$). Before performing the ANOVA, total colonization data were submitted to the logistic binomial transformation

Inoculation treatment		Proportion of mycorrhizas	% Mycorrhizas
L. bicolor (g/seedling)	*R. roseolus* (spores/seedling)	*L. bicolor/ R. roseolus*	
0.08	10^4	4.46 a	37 a
0.04	10^5	0.26 a	53 ab
0.08	10^6	0.17 a	74 b

Discussion

The results obtained indicate that calcium alginate is an adequate carrier of mixed inoculum containing mycelium of *L. bicolor* and spores of *Rhizopogon* spp. One-year-old dried *Rhizopogon* spores remained viable after their inclusion in alginate gel. Also, alginate inoculum of *L. bicolor* was effective in forming ectomycorrhizas as in previous tests with Douglas-fir and maritime pine under the same growing conditions (Parladé 1992; Pera 1992). The percentages of infected Douglas-fir seedlings after inoculation with spores of *R. subareolatus* included in alginate were similar to those obtained using spore suspensions in water at the same concentrations per seedling (Parladé et al. 1996). In maritime pine seedlings inoculated with spores of *R. roseolus* included in alginate, the percentages of colonized seedlings were clearly lower than those obtained using spore suspensions at the same application doses (Parladé et al. 1996).

For both tree species, the maximal proportion of dual-colonized seedlings obtained almost never exceeded 50% of the total. The rest of the seedlings remained colonized by a single fungus or were non-colonized. In Douglas-fir seedlings, the relationship between the dual-colonized seedlings obtained and the initial doses of both fungi was highly significant. High doses of *R. subareolatus* combined with any dose of *L. bicolor* maximized the proportion of dual-colonized seedlings to almost 50%. High doses of *L. bicolor* produced most of the seedlings colonized by this fungus, except when combined with the highest dose of *R. subareolatus*. These results agree with the reported competitiveness of *L. bicolor* against natural inoculum in field experiments (McAfee and Fortin 1986). The relationship between the colonizing ability of an introduced fungal strain and its inoculation intensity has also been reported in pot experiments (Garbaye 1983). Field experiments carried out in Europe demonstrated that the competitiveness of an introduced fungal strain, togeth-

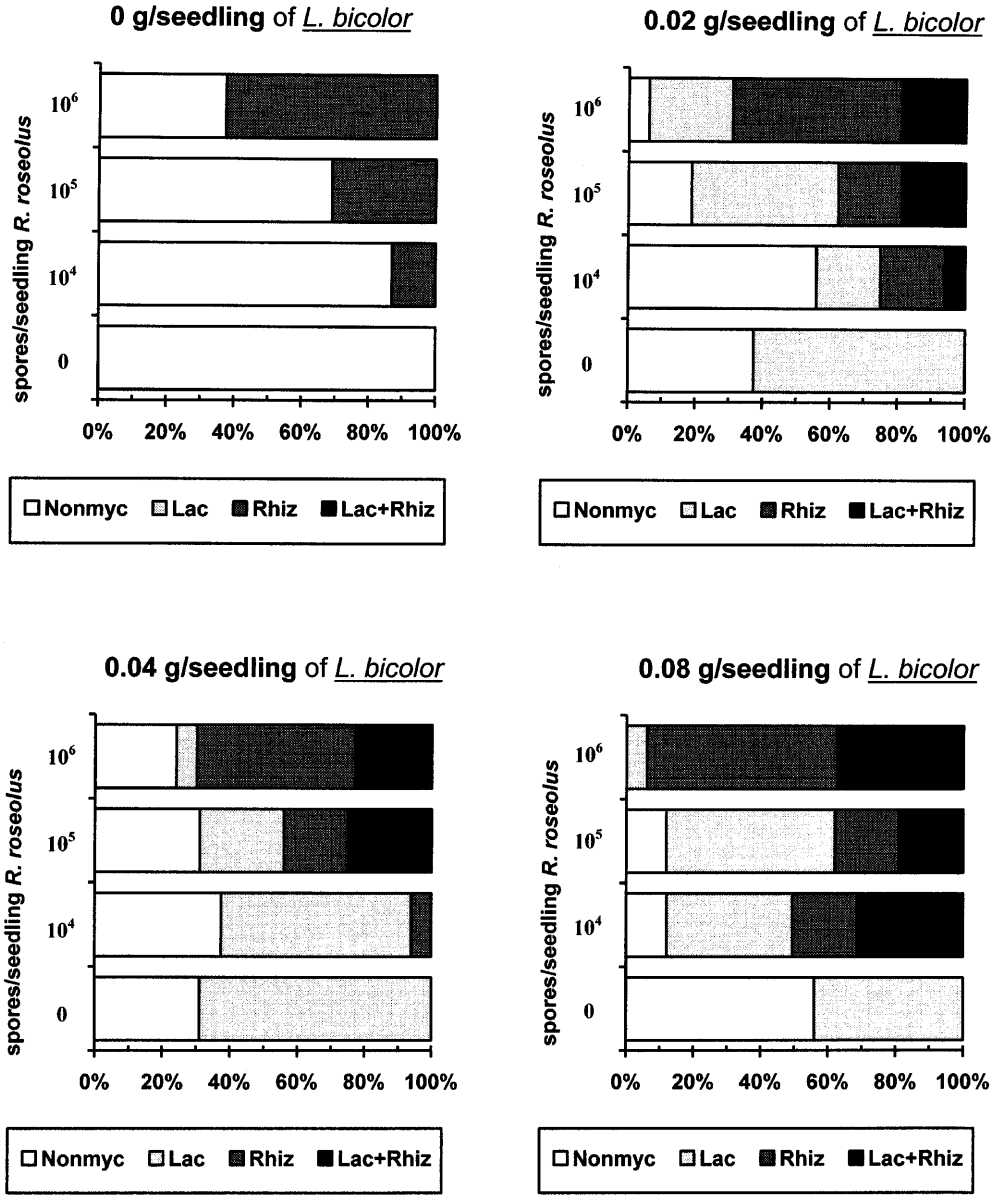

Fig. 3 Percentages of mycorrhizal and non-mycorrhizal (*Nonmyc*) maritime pine seedlings after inoculation with different doses of *L. bicolor* (*Lac*) and/or *R. roseolus* (*Rhiz*)

er with the quality of the fungal inoculum, are key factors ensuring the persistence of the introduced fungus and a long-lasting growth effect on the host plant (Le Tacon et al. 1992).

The relationship between the dual-colonized seedlings obtained and the initial fungal doses in maritime pine coinoculations was not significant. The presence of *T. terrestris* hindered assessment of the colonization ability of the introduced fungal strains, since this contaminant is a good colonizer of *Pinus* species under nursery conditions and extends rapidly and erratically (Castellano and Molina 1989).

Reports on competition between different mycorrhizal fungi introduced in single root systems are scarce and controversial. Mycelial coinoculations carried out under *in vitro* conditions demonstrated that *L. bicolor* completely displaced *R. subareolatus* from Douglas-fir roots (Parladé and Alvarez 1993). Nevertheless, Chu-Chou and Grace (1985) stated that *L. laccata*, applied as a spore inoculum from gills, was a weak competitor of *Rhizopogon* species in pot trials. Also, *T. terrestris* colonized more short roots than *L. bicolor* in nursery coinoculations using the same initial doses for both fungi (Reddy and Natarajan 1997). The different growth conditions, inoculum types and inoculum doses in these experiments, as well as the presence of native fungi as contaminants, make it difficult to compare previous results with those presented here.

Dual inoculations ought to be performed with appropriate combinations of fungi and type of inoculum. The fungi should be competitive against native fungi, have potentially complementary benefits (as may be expected for *Laccaria* and *Rhizopogon* species) and be able to coexist in the same root system. The type of ino-

culum and the application dose need to be optimized to allow simultaneous colonization of short roots by the two mycorrhizal fungi involved. Also, future economic feasibility of this inoculation method should take into account the ease of inoculation in the nursery. The method used in this study for producing and applying dual inoculum is easy to perform but, at the optimal combinations, the maximum of dual-colonized seedlings obtained was too low (50% of the coinoculated ones) to be of commercial interest. The proposed coinoculation technique may be ineffective for obtaining stable competitive equilibria in a complex dynamic relationship driven by a lot of unknown factors. More effort needs to be made to improve the inoculation methodology to obtain higher homogeneity in dual-inoculated plants. Alternative techniques, such as mixed plantations of single fungus-inoculated plants, could be considered to obtain dual-symbiotic planting stocks using the existing techniques. Hence, the benefits of dual inoculations could be elucidated in field experiments by comparison studies with monospecific-inoculated plants. This would permit evaluation of the cost-benefit relationship of dual inoculations in forestry practice and the suitability of this methodology for nursery management.

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