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Coinoculation of aseptically grown Douglas fir with pairs of ectomycorrhizal fungi

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Abstract. Douglas fir seedlings grown under aseptic conditions in a peat-vermiculite substrate were inoculated with four pairs of ectomycorrhizal fungi to assess the relative inoculum dosages needed to establish two mycorrhizal fungi simultaneously in the same root system. The dual fungal combinations tested were: *Pisolithus arhizus* + *Rhizopogon subareolatus*, *P. arhizus* + *R. roseolus*, *Laccaria bicolor* + *P. arhizus* and *L. bicolor* + *R. subareolatus*. A total of 12 ml of inocula per plant was applied at the rates: 0+12, 3+9, 6+6, 9+3, 12+0, and 0+0 (v+v) for each combination. After 3 months growth, the number of mycorrhizas and uninfected short roots as well as the total plant biomass produced were recorded. Inoculations were successful with the fungal combinations *P. arhizus* + *R. subareolatus* and *L. bicolor* + *P. arhizus*. Plants developed *P. arhizus* and *R. subareolatus* mycorrhizas only at the rate 9Pa+3Rs; at other rates tested, only monospecific mycorrhizas were formed. Plants developed *L. bicolor* and *P. arhizus* mycorrhizas at the three rates containing both fungi. *L. bicolor* behaved as an aggressive root colonizer and its level of root colonization remained constant at increasing rates of *P. arhizus* inoculum. *L. bicolor* displaced *R. subareolatus* at all inocula rates. *P. arhizus* displaced *R. roseolus* except at the rate 3Pa+9Rr, with only a low number of mycorrhizas formed by either fungus. Total plant biomass was significantly increased by the presence of any fungal combination up to four times the values for uninoculated controls. *P. arhizus* and *R. subareolatus* were more effective in promoting plant growth and stimulating short root formation than either *L. bicolor* or *R. roseolus*.

Key words: Mycorrhiza – *Pseudotsuga menziesii* – *Pisolithus arhizus* – *Laccaria bicolor* – *Rhizopogon*

Introduction

The establishment of several mycorrhizal fungi in the same root system is a common phenomenon occurring in nature (Benecke and Göbl 1974; Borchers and Perry 1990; Gibson and Deacon 1988; Laiho 1970; Trappe 1977). It has been demonstrated that proportions of several types of ectomycorrhizas in the root system change after site disturbance (Perry and Rose 1983; Pilz and Perry 1984; Schoeneberger and Perry 1982), probably due to differences in the fungal response to environmental changes. Thus, mycorrhizal diversity probably modifies the ecological adaptability of trees. Forest seedlings with multiple mycorrhizas can presumably withstand a wider range of plantation site conditions than plants having only one species of ectomycorrhizal fungi. Available information on multiple inoculations under controlled conditions is scarce. Chu-Chou and Grace (1985) successfully inoculated *Pinus radiata* seedlings with spores of three fungi simultaneously and obtained plants showing intermediate growth characteristics. However, when a mixture of fungi was applied as vegetative inoculum, only one fungus prevailed and successfully colonized the root systems of the plants (Marx et al. 1991).

The production of plants inoculated with mycelial inocula of more than one species of ectomycorrhizal fungi requires previous studies on the relative colonization ability of the fungi involved. Wong and Fortin (1990) point out that the aggressiveness of an ectomycorrhizal fungus may be assessed by the number of ectomycorrhizas formed on inoculated root systems. The formation of ectomycorrhizas by a fungus depends on temperature, pH, nutrients, moisture, aeration, external carbohydrates and other abiotic factors (Wong and Fortin 1990). Host-fungus specificity also affects root colonization by the fungus (Malajczuk et al. 1990). The presence of two fungi in the same root system may result in interactions that complicate the assessment of the relative colonization ability of each fungus.

The present research was done to estimate the relative root colonizing ability of four fungi applied simultaneously in pairs at various rates of inoculum to aseptically grown Douglas fir seedlings. Two host-generalist fungi, *Pisolithus arhizus* and *Laccaria bicolor*, and two host-specific species of *Rhizopogon* (Ho and Trappe 1987; Molina and Trappe 1982) were selected for the studies. Both host-generalist fungi are known to increase seedling performance on routine reforestation sites worldwide (Kropp and Langlois 1990; Marx 1980), and *P. arhizus* is also effective in adverse sites (Marx and

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Ruehle 1989). It has ben suggested that inoculations using specific host-fungus combinations with different species of *Rhizopogon* would be suitable for reforestation of droughty sites (Castellano and Trappe 1985) common in Spain. The identification of compatible fungal pairs capable of coexisting in the same root system is desirable for future attempts at multiple inoculations of container-grown Douglas fir seedlings.

Materials and methods

Douglas fir seeds (origin OR 261, lot 313-1980) were surface-sterilized with 30% H_2O_2 and germinated aseptically in glass vials containing 2% malt agar to detect possible contaminants (Molina and Palmer 1982). Seedlings with 2-cm-long radicles were transferred into sterilized glass synthesis tubes containing a mixture of peat-vermiculite (1:10, v:v) moistened with modified Melin Norkrans (MMN) liquid medium (Marx 1969; Molina and Palmer 1982). The fungal isolates used were *L. bicolor* (Maire) Orton S-238 [formerly identified as *L. laccata* before its reclassification by Armstrong et al. (1989)] collected under *Larix occidentalis* Nutt. in Oregon, USA (Molina 1982), *P. arhizus* (Scop: Pers.) Rausch (syn. *P. tinctorius* (Pers.) Coker & Couch) A-93 collected under *Quercus suber* L. in Girona (Spain), *Rhizopogon roseolus* (Corda ex Sturm) Th. Fries A-96 collected under *Pinus sylvestris* L. in Tarragona (Spain) and *R. subareolatus* Smith A-116 collected under *Pseudotsuga menziesii* (Mirb.) Franco in Girona. Ectomycorrhizal fungi which had been grown in MMN liquid medium for 18 days were prepared for experimental inoculations by aseptically filtering mycelium, washing, and resuspending in 100 ml of sterile distilled water. Each fungal suspension was then homogenized in a Waring blender at low speed for 10 s and adjusted to a density of 10 mg (dry weight) of mycelium per ml by adding sterile distilled water. Aliquots of the mycelial slurries were pipetted into replicated pure culture synthesis tubes containing 1-month-old seedlings. Each plant was inoculated with a total of 12 ml of mycelial suspension for each dual combination of fungi applied at the rates: 12+0, 9+3, 6+6, 3+9, and 0+12 (v+v). Uninoculated control plants received 12 ml of distilled water without fungi. The fungal combinations studied were: *P. arhizus* + *R. subareolatus*, *P. arhizus* + *R. roseolus*, *L. bicolor* + *P. arhizus*, and *L. bicolor* + *R. subareolatus*.

All the treatments were maintained for three months in a pure culture synthesis apparatus with 16-h photoperiod and a light intensity of 150 $\mu mol\ s^{-1}\ m^{-2}$ (400–700 nm). Synthesis tubes were partially inmersed in a running water bath to keep the root temperature in the range of 20–25° C. A total of five replicates were prepared for each inocula rate within each fungal combination.

At the end of the growing period, plants were harvested and root systems examined under the stereomicroscope for the formation of ectomycorrhizas and uninfected short roots. Differences in colour facilitated counting of the mycorrhizas formed by each fungal species. Plants were oven-dried at 80° C for 48 h and weighed. Data on total plant biomass were tested for normality and variance homogeneity to perform an analysis of variance. Differences among means were determined by Turkey's multiple range test ($P \leq 0.05$).

Results

The inoculated plants grew better than the uninoculated controls (Table 1). Growth and total number of short roots were directly related. Plants inoculated with either *P. arhizus* or *R. subareolatus* were significantly larger (Table 1) and developed more short roots than did plants inoculated with *R. roseolus* or *L. bicolor* (Fig. 1).

Table 1. Relative total dry weights of axenic Douglas fir seedlings inoculated with 12 ml of mycelial suspensions of four pairs of ectomycorrhizal fungi applied at different rates. The values are expressed relative to the controls (0+0). Values followed by the same letter in each column are not significantly different by Tukey's test ($P \leq 0.05$). Lb, *L. bicolor*; Pa, *P. arhizus*; Rr, *R. roseolus*; Rs, *R. subareolatus*

Inoculum rate (ml)	Dual fungal combinations			
	Pa+Rs	Pa+Rr	Lb+Rs	Lb+Pa
0+0	1.0a	1.0a	1.0a	1.0a
12+0	3.9b	4.6d	2.0b	2.0b
9+3	4.1b	4.2cd	2.6bc	2.9c
6+6	3.9b	4.4cd	3.4cd	3.0c
3+9	4.1b	3.6bc	3.2cd	2.6c
0+12	3.9b	3.0b	3.6d	2.6c

Plants inoculated with the combination *P. arhizus* + *R. subareolatus* formed a similar total number of short roots regardless of inocula rate (Fig. 1a). *R. subareolatus* formed mycorrhizas in all treatments whereas *P. arhizus* formed mycorrhizas only when inoculated alone, or at the rate 9Pa+3Rs. Only at the rate 9Pa+3Rs were both types of mycorrhiza present in the root system, with levels of 20% and 45% for *P. arhizus* and *R. subareolatus*, respectively.

Plants inoculated with the combination *P. arhizus* + *R. roseolus* showed a decrease in the total number of short roots formed as the rate of *R. roseolus* increased (Fig. 1b). The two types of mycorrhiza never formed simultaneously in the same root system. *R. roseolus* was unable to form mycorrhizas at the rates 9Pa+3Rr and 6Pa+6Rr, and although capable of forming mycorrhizas alone and at the rate 3Pa+9Rr, the level of mycorrhizal formation was always low (<26%).

Plants inoculated with the combination *L. bicolor* + *P. arhizus* formed the two types of mycorrhiza at the three rates which included both fungi. When the proportion of *L. bicolor* mycelia was reduced by two thirds, from 9Lb+3Pa to 3Lb+9Pa, the level of mycorrhizas formed remained constant (49–50%) indicating that *L. bicolor* had a higher root colonization capability than *P. arhizus* (Fig. 1c). Increasing concentrations of *P. arhizus* mycelia appeared to stimulate short root formation, as was observed in other dual combinations (Figs. 1a, b).

Plants inoculated with the combination *L. bicolor* + *R. subareolatus* showed an increase in the total number of short roots as the proportion of *R. subareolatus* increased (Fig. 1d). The number of *L. bicolor* mycorrhizas was directly related to the number of short roots, indicating that *L. bicolor* was a better colonizer than *R. subareolatus*, which itself was incapable of forming mycorrhizas in the presence of *L. bicolor*.

Discussion

Total plant biomass and short root formation were stimulated by the presence of all ectomycorrhizal fungi

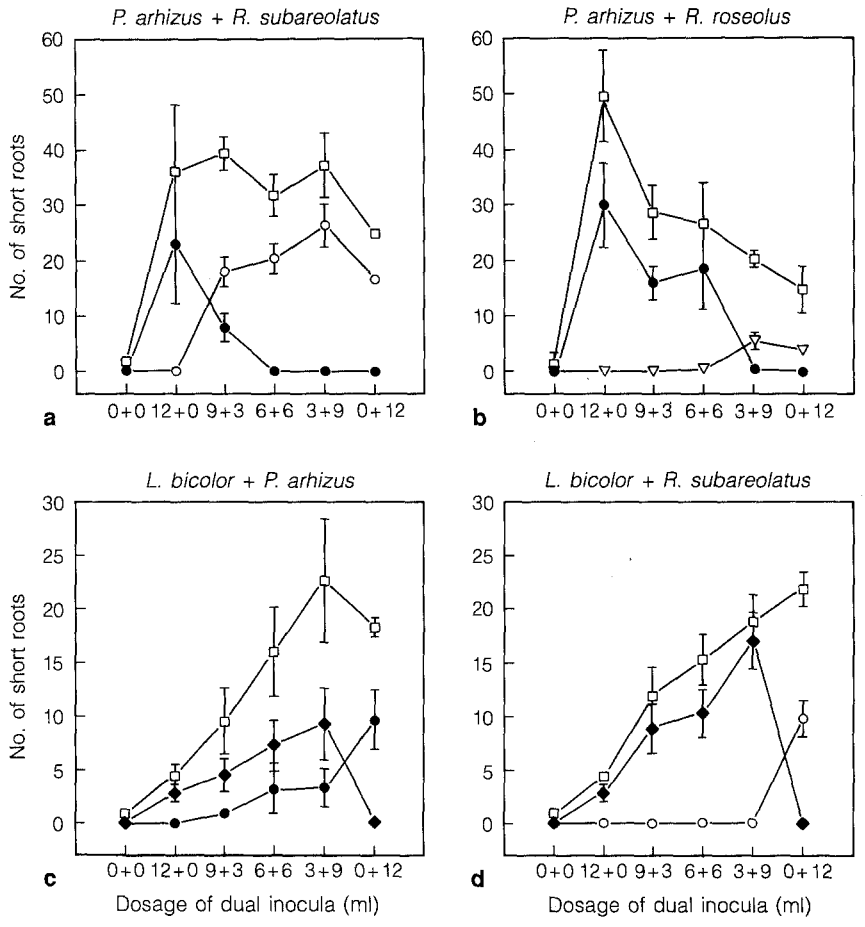

Fig. 1a–d. Effect of dual inoculations on the total number of short roots and the number of mycorrhizas formed by each fungus. *Vertical bars* represent the standard errors of the means of five replicates. □, Total short roots; ◆, *L. bicolor*; ●, *P. arhizus*; ▽, *R. roseolus*; ○, *R. subareolatus*

tested, in agreement with other results obtained under similar experimental conditions (Marx and Zak 1965; Marx et al. 1970). *P. arhizus* and *R. subareolatus* were more effective in promoting seedling growth and root production than either *L. bicolor* or *R. roseolus*. Graham and Linderman (1980) found *L. laccata* to stimulate and *P. arhizus* to inhibit lateral root formation. Root production was paralleled by ethylene production, with *L. laccata* and several *Rhizopogon* species being producers and *P. arhizus* being a non-producer. Later research confirmed *L. laccata* and *L. bicolor* as ethylene producers (Livingston 1991). Whether the fungal isolates used in the present study produce ethylene or other plant growth regulators (Ho and Trappe 1987; Slankis 1973) is unknown.

Successful dual inoculations were obtained with the pairs: *P. arhizus* + *R. subareolatus* and *L. bicolor* + *P. arhizus*. The number of mycorrhizas formed by *P. arhizus* was always lower than its companion fungus, indicating a poorer root colonization capacity than either *R. subareolatus* or *L. bicolor*. The aggressive *L. bicolor* appeared to restrain colonization by *P. arhizus* and to displace *R. subareolatus* completely. The strain *L. bicolor* S-238 has been selected, among other beneficial characteristics, for its excellent root-colonizing capacity of Douglas fir and other conifers (Hung and Trappe 1987; Molina and Chamard 1983). In studies of outplanted inoculated Douglas fir seedlings, *L. bicolor* S-238 precluded the development of possible competitors and was capable of colonizing all new roots formed to a depth of 50 cm (Villeneuve et al. 1991). *R. subareolatus,* as a member of the section *Villosuli* (Smith and Zeller 1966), is considered host specific to Douglas fir (Ho and Trappe 1987; Molina and Trappe 1982). Its presence in Spain is undoubtly due to an earlier introduction of Douglas fir seedlings. It has become adapted to the edaphic-climatic conditions of northeastern Spain where the sporocarps may be found readily in some Douglas fir plantations. Although not as aggressive as *L. bicolor, R. subareolatus* is a good root colonizer under aseptic conditions. However, when applied as vegetative inoculum to Douglas fir seedlings reared in environment-controlled greenhouse conditions, it failed to form mycorrhizas (unpublished work) similar to the behaviour of other *Rhizopogon* species (Molina 1980). Like *R. colossus* and *R. vinicolor* (Castellano and Trappe 1985), *R. subareolatus* forms ectomycorrhizas readily with container-grown Douglas fir when applied as spores (unpublished work).

Although it was known to form few mycorrhizas with Douglas fir (<26%) *R. roseolus* was included in the study for comparison with good root colonizers. Chu-Chou and Grace (1985) considered this species a slow but thorough colonizer, and an aggressive competitor once established in radiata pine roots. Our results, confirmed by later greenhouse assays (Parladé 1992), in-

dicate that *R. roseolus* is not a suitable ectomycorrhizal fungus of Douglas fir.

High colonization ability in monospecific inoculations is one of the desirable charcteristics used to select strains of ectomycorrhizal fungi for wide scale nursery inoculations with vegetative inoculum (Kropp and Langlois 1990; Trappe 1977). This trait may need to be moderated in the selection process if the objective of the inoculation is to have two ectomycorrhizal fungi coexisting in the same root system. Otherwise, the presence of the selected strain may preclude the formation of mycorrhizas by another possible fungal partner when both fungi are present in mycelial form. Dual inoculation of Douglas fir seedlings with a host-generalist and a host-specific fungus, perhaps the most desirable combination to improve plantation performance, may be best achieved by inoculating the two fungi in different (vegetative versus spore) inoculum forms.

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