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M. Sudhakara Reddy · K. Natarajan

Coinoculation efficacy of ectomycorrhizal fungi on *Pinus patula* seedlings in a nursery

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Abstract The coinoculation efficacy of the ectomycorrhizal fungi *Laccaria laccata* and *Thelephora terrestris* on the growth and mycorrhizal development of *Pinus patula* seedlings was studied and compared to individual inoculation of these fungi in a nursery. The total number of mycorrhizas was higher in seedlings inoculated with the combined inoculum than with the individual inocula. The colonization by *T. terrestris* was higher than *L. laccata* when the seedlings were inoculated with the two fungi simultaneously. Coinoculation significantly increased the height and dry weight of the seedlings compared with individual inoculation, both in steam-sterilized and unsterilized soil.

Key words Combined inoculum · *Laccaria laccata* · *Thelephora terrestris* · *Pinus patula* · Mycorrhizal development

Introduction

Colonization of several fungi in the same root system is a common phenomenon in nature (Borchres and Perry 1990; Gibson and Deacon 1988; Trappe 1977). It has been shown that the proportions of several ectomycorrhizal types change in root systems after site disturbance (Pilz and Perry 1984) because of differences in fungal response to environmental changes. Parlade and Alvarez (1993) suggested that forest tree seedlings with multiple mycorrhizas can withstand a wider range of plantation site conditions than plants with only one species of ectomycorrhizal fungus. Most fungal inoculations are designed to produce seedlings infected with only one species of mycorrhizal fungus (Marx et al. 1991) and little information is available on the effects of coinoculation of different fungi on seedling growth and mycorrhizal development. Chu-Chou and Grace (1985) inoculated *Pinus radiata* seedlings with spores of three different fungi simultaneously and obtained plants showing intermediate growth characteristics. Parlade and Alvarez (1993) inoculated mycorrhizal fungi in pairs to aseptically grown Douglas-fir seedlings in a peat-vermiculite substrate and found that total plant biomass was significantly increased by the presence of any fungal combination. Hung and Trappe (1987) suggested using mixed inoculation of various fungi to improve field performance of Douglas-fir seedlings. In the present study, the ectomycorrhizal fungi *Laccaria laccata* (Scop.) Berk & Br. and *Thelephora terrestris* (Ehrh.) Fr. were used to inoculate *Pinus patula* Schlecht. & Cham. seedlings in the nursery and their effect compared to individual inoculation of the fungi on growth and mycorrhizal development.

Materials and methods

Laccaria laccata (KN1) was isolated from the basidiomata collected from *P. patula* plantations and *T. terrestris* (KN2) from the basidiomata associated with *P. patula* seedlings in a nursery in the Nilgiri hills (2300 m amsl), Tamilnadu, South India. The cultures were deposited at the Centre for Mycorrhizal Culture Collection, Tata Energy Research Institute, New Delhi, India and maintained at 25 °C on modified Melin-Norkrans medium (MMN) (Marx 1969).

Mycelial inoculum was prepared in a vermiculite carrier according to Marx and Bryan (1975). *L. laccata* or *T. terrestris* was grown aseptically 1-l Erlenmeyer flasks containing 750 ml of vermiculite moistened with 375 ml of MMN liquid medium. The flasks were incubated at 25 °C in the dark. After 12 weeks incubation, inoculum was removed from the flasks and leached with cool tap water to remove unused nutrients. Excess free water was removed by gently squeezing the inoculum wrapped in cheese cloth. The inoculum for coinoculation was prepared by combining equal volumes of these two inocula. Fungus-free vermiculite served as the control. The inoculum was stored at 4 °C until use.

M. Sudhakara Reddy (✉)[1] · K. Natarajan
Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Madras 600 025, India

Present address:
[1] Biotechnology Division, Thapan Corporate R&D Centre, Post Box #68, Patiala-147001, India
Fax: +91-175-212002; e-mail: btd@tcrdcpt.ren.nic.in

Soil used was collected under natural vegetation on the upper region of the Nilgiri hills and had a pH of 5.3, an organic matter content of 4.8%, and total N,P,K levels of 245, 5.2 and 35 kg/acre, respectively. The soil was sterilized by autoclaving at 121 °C for 1 h. The nursery experiment was conducted at the Forest Department Nursery, Ootacamund, in the Nilgiri hills. The soil were placed in plastic bags (2 kg/bag) and 30 ml inoculum per bag of the two fungi were added separately or in combination and mixed into the upper 8–10 cm manually. The total volume of inoculum was the same in the combined and single fungus treatments. *P. patula* seeds collected from the plantations were surface sterilized in 30% hydrogen peroxide for 30 min, washed thoroughly with sterile water and sown at 5–6 seeds per bag. After germination, seedlings were thinned to 1 per bag. No fertilizers were applied throughout the study. The inoculated and uninoculated seedlings were arranged in a completely randomized block design of 10 blocks and each block represented 10 replicates of each treatment with all the treatments in a nursery.

Ten seedlings from each treatment were harvested at 1-month intervals from month 5 to 8 and at 2-month intervals from month 10 to 12 to study growth and mycorrhizal development. The harvested seedlings were carefully brought to the laboratory and root samples freed from soil by gentle washing in water. Entire root systems were examined under a stereomicroscope to count the number of mycorrhizal short roots (Richards and Wilson 1963). The percent mycorrhizas was calculated as total number of mycorrhizal short roots formed by inoculated fungi/total number of mycorrhizal short roots $\times 100$. The short roots colonized by *T. terrestris* either by inoculated or naturally occurring fungus was taken as the total number of mycorrhizal short roots in *T. terrestris*-inoculated treatments. Plant height was measured and the shoots and roots were dried at 85 °C for 48 h. The mycorrhizal types produced by different fungi were identified according to Mohan et al. (1993 a–c) and Natarajan et al. (1988).

A statistical analysis was performed for each harvest and soil separately by one way analysis of variance. The mycorrhizal percentages were log transformed to give a normal distribution. The significance of differences ($P<0.05$) were determined by Duncan's Multiple Range Test. Two-way analysis was used to compare the results from sterilized and unsterilized soil.

Results

Generally, coinoculation of *L. laccata* and *T. terrestris* increased the total number of mycorrhizal short roots compared with individual inoculation in both sterilized and unsterilized soil. After 12 months, the combined treatment of *L. laccata* and *T. terrestris* had significantly increased the total number of mycorrhizal short roots compared with individual inoculum in unsterilized soil (Table 2). The percentage of short roots colonized by *T. terrestris* was higher than *L. laccata* at 12 months, when the seedlings were inoculated separately in unsterilized soil (Fig. 1A), whereas in sterilized soil the colonization did not differ significantly (Fig. 2A). In the combined treatment, the percentage colonization by *L. laccata* increased up to 8 months and gradually declined by 10 and 12 months whereas the percentage colonization by *T. terrestris* was reduced up to 8 months and later increased at 10 and 12 months in sterilized soil (Fig. 2B). In unsterilized soil, the colonization by *T. ter-*

Table 1 Effect of *Laccaria laccata* (*Ll*) and *Thelephora terrestris* (*Tt*) and combined inoculum (*Ll*+*Tt*) on the growth and mycorrhizal development of *Pinus patula* seedlings grown in sterilized soil. Means sharing a common letter in the same column at each time point are not significantly different at $P<0.05$. Each value is the mean of 10 replicates

Time after infection (months)	Treatment	Height (cm)	Shoot dry wt. (mg)	Root dry wt. (mg)	Shoot/root ratio	Number of mycorrhizal short roots
5	Ll+Tt	7.3 a	93 a	41 a	2.2 a	163 a
	Ll	6.8 a	66 a	19 c	3.4 a	18 c
	Tt	6.2 a	74 a	34 ab	2.1 b	70 b
	Control	6.1 a	71 a	26 bc	2.7 ab	7 c
6	Ll+Tt	8.7 a	235 a	103 a	2.2 a	339 a
	Ll	6.8 bc	67 b	30 b	2.2 a	128 b
	Tt	8.0 ab	105 b	44 b	2.3 a	171 b
	Control	6.2 c	85 b	30 b	2.8 a	61 b
7	Ll+Tt	13.1 a	392 a	162 a	2.4 b	476 a
	Ll	7.8 b	100 b	45 b	2.2 b	71 c
	Tt	10.0 b	186 b	70 b	2.6 b	291 b
	Control	8.8 b	217 b	55 b	3.9 a	171 bc
8	Ll+Tt	14.3 a	560 ab	175 b	3.2 a	572 ab
	Ll	12.7 ab	705 a	291 a	2.4 b	634 a
	Tt	11.3 bc	522 b	201 b	2.5 b	450 b
	Control	9.2 c	246 c	80 c	3.0 a	103 c
10	Ll+Tt	19.2 a	1222 a	436 a	2.8 a	797 a
	Ll	14.2 b	576 b	366 b	1.5 b	641 a
	Tt	11.3 c	467 bc	232 bc	2.0 b	651 a
	Control	9.7 c	334 c	128 c	2.6 a	187 b
12	Ll+Tt	20.3 a	1698 a	490 a	3.4 a	1452 ab
	Ll	14.9 b	881 b	415 a	2.1 b	1688 a
	Tt	12.9 b	702 bc	462 a	1.5 b	975 b
	Control	9.8 c	403 c	227 b	1.7 b	232 c

Table 2 Effect of *L. laccata* (*Ll*) and *T. terrestris* (*Tt*) and combined inoculum (*Ll*+*Tt*) on the growth and mycorrhizal development of *P. patula* seedlings grown in unsterilized soil. Means sharing a common letter in the same column at each time point are not significantly different at $P<0.05$. Each values is the mean of 10 replicates

Time after infection (months)	Treatment	Height (cm)	Shoot dry wt. (mg)	Root dry wt. (mg)	Shoot/root ratio	Number of mycorrhizal short roots
5	Ll+Tt	6.1 b	61 b	33 a	1.8 a	94 ab
	Ll	6.4 b	51 b	22 b	2.3 a	92 ab
	Tt	8.7 a	92 a	35 a	2.6 a	135 a
	Control	5.9 b	57 b	24 b	2.3 a	75 b
6	Ll+Tt	7.5 a	116 a	68 a	1.7 b	277 a
	Ll	7.3 a	107 a	52 b	2.0 b	123 b
	Tt	8.5 a	108 a	38 bc	2.8 a	144 b
	Control	8.8 a	97 a	34 c	2.8 a	175 b
7	Ll+Tt	7.6 b	95 c	67 b	1.4 b	126 bc
	Ll	7.4 b	87 c	53 b	1.6 b	70 c
	Tt	9.2 a	287 a	115 a	2.4 a	327 a
	Control	9.1 a	200 b	72 b	2.7 a	165 b
8	Ll+Tt	9.9 a	171 b	84 a	2.0 b	197 ab
	Ll	8.9 a	204 ab	111 a	1.8 b	211 ab
	Tt	9.5 a	280 ab	107 a	2.6 ab	260 a
	Control	9.2 a	298 a	91 a	3.2 a	143 b
10	Ll+Tt	14.8 a	486 a	183 a	2.5 a	446 a
	Ll	9.7 b	313 a	195 a	1.6 a	356 a
	Tt	10.7 b	399 a	200 a	1.9 a	302 a
	Control	9.1 b	375 a	176 a	2.1 a	265 a
12	Ll+Tt	18.6 a	1044 a	577 a	1.8 b	892 a
	Ll	13.6 b	665 ab	237 b	2.8 a	445 b
	Tt	11.5 b	584 b	340 b	1.7 b	565 b
	Control	10.3 b	488 b	303 b	1.5 b	454 b

restris was higher than *L. laccata* (Fig. 1B). The production of *T. terrestris* fruit bodies was observed from 7 months onwards in seedlings inoculated with *T. terrestris* and also in the combined treatment, both in sterilized and unsterilized soil, whereas *L. laccata* fruit bodies were observed from 10 months onwards only in *L. laccata*-inoculated seedlings, in both sterilized and unsterilized soil.

The uninoculated seedlings were mostly colonized by the naturally occurring ectomycorrhizal fungi in the nursery. The percentage colonization of indigenous mycorrhizal fungi was higher in uninoculated seedlings than in inoculated seedlings (Fig. 3). The seedlings were first colonized by unidentified mycorrhizas mostly belonging to the Ascomycetes. Later, *T. terrestris*, *Rhizopogon* sp. and *Inocybe* sp. colonization was observed in both inoculated and uninoculated seedlings. Among the indigenous fungi, *T. terrestris* colonization was higher than other fungi in uninoculated seedlings. Fruit bodies of *T. terrestris* were observed in uninoculated seedlings in unsterilized soil from 8 months onwards. In the combined treatment, the percent colonization of indigenous mycorrhizal fungi was lower than after individual inoculation, both in sterilized and unsterilized soil up to 10 months. After 12 months, no significant difference was observed between combined inoculation and *T. terrestris* inoculation in either soil (Fig. 3). Fruit bodies of *Inocybe* sp. were observed at 12 months in seedlings grown in unsterilized soil inoculated with both fungi.

Mycelial inocula of the two fungi separately or in combination produced different growth responses by the seedlings. Seedlings inoculated with the combined fungi showed significantly higher shoot height than after individual fungal inoculation, both in sterilized and unsterilized soil at 10 and 12 months (Tables 1, 2). Coinoculation of the two fungi produced significantly higher shoot dry weights than individual inoculation in sterilized soil at 10 and 12 months (Table 1). In unsterilized soil, seedlings inoculated with the combined inoculum had a significantly higher shoot dry weight than *T. terrestris*-inoculated seedlings at 12 months, but did not differ significantly from seedlings inoculated with *L. laccata* (Table 2). Root dry weight was significantly higher in seedlings inoculated with both fungi than individual inoculation in unsterilized soil (Table 2). The shoot/root ratio was significantly higher in seedlings inoculated with the combined inoculum than individual inoculation in sterilized soil (Table 1).

Generally seedlings grown in sterilized soil showed higher growth and mycorrhizal development than in unsterilized soil when the fungi were inoculated separately or in combination (Tables 1,2). Two-way analysis of variance revealed a significant differences between the soils and among the treatments with respect to total number of mycorrhizal short roots, seedling height and

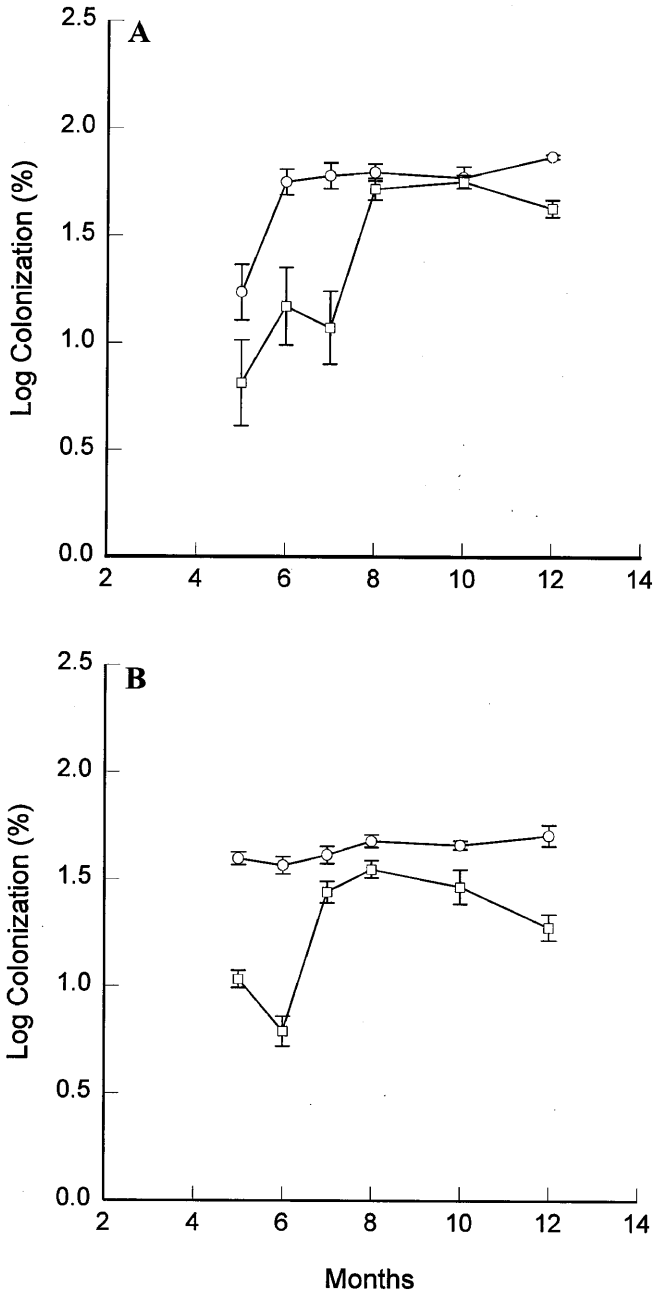

Fig. 1 Colonization of *Laccaria laccata* (□) and *Thelephora terrestris* (○) in *Pinus patula* seedlings inoculated individually (**A**) or in combination (**B**) in unsterilized soil. *Vertical bars* represent standard errors ($n = 10$)

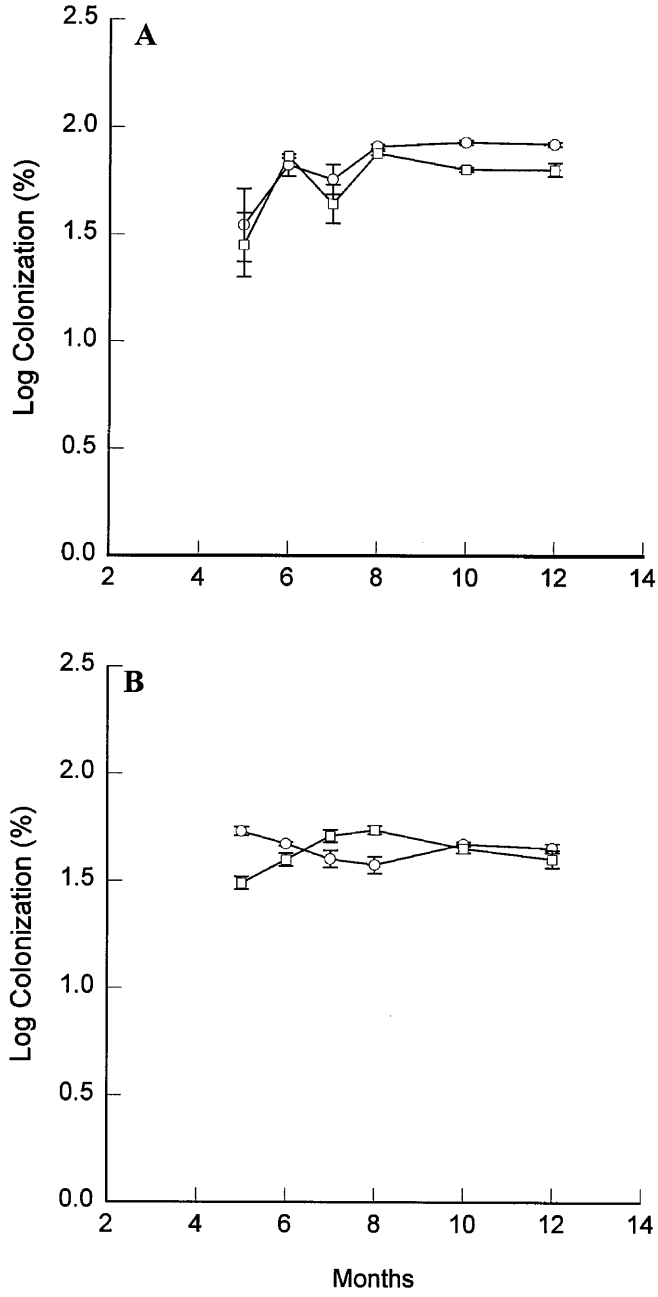

Fig. 2 Colonization of *L. laccata* (□) and *T. terrestris* (○) in *P. patula* seedlings inoculated individually (**A**) or in combination (**B**) in sterilized soil. *Vertical bars* represent standard errors ($n = 10$)

weight. The percent colonization by *T. terrestris* varied significantly between the soils where as *L. laccata* colonization did not vary with the soil, either in individual or combined inoculation.

Discussion

Coinoculation of *L. laccata* and *T. terrestris* produced more biomass and short root formation in *P. patula* seedlings than uninoculated seedlings both in sterilized and unsterilized soil. It has been suggested that for inoculation at the nursery stage an "early stage" fungus will be more successful than a "late stage" fungus (Mason et al. 1983). A very limited number of ectomycorrhizal fungi are found in *P. patula* plantations in the Nilgiri hills and there seems to be a distinct pattern of succession among these fungi (Natarajan et al. 1992). *Laccaria laccata* and *T. terrestris* were selected because of their "early-stage" nature (Natarajan et al. 1992), high

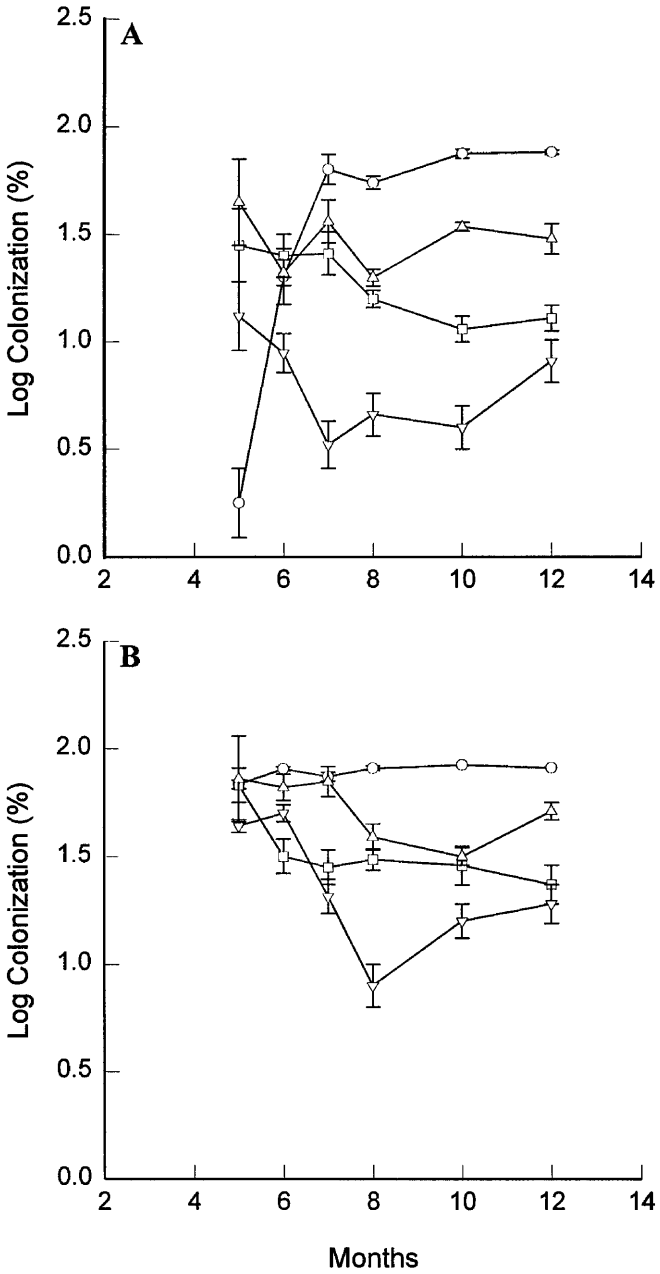

Fig. 3 Colonization of indigenous mycorrhizal fungi in *P. patula* seedlings in sterilized (**A**) and unsterilized soil (**B**) (○ control, □ *T. terrestris*, △ *L. laccata*, and ▽ combined inoculum). *Vertical bars* represent standard errors ($n = 10$)

colonization ability (Reddy and Natarajan 1996) and greater beneficial effects on seedlings than other fungi, such as *Amanita muscaria, Rhizopogon luteolus, Scleroderma citrinum* and *Suillus brevipes,* associated with *P. patula* (Mohan 1991). Other studies also showed that inoculation with *L. laccata* and *T. terrestris* improved the growth and mycorrhizal development of various tree species in the nursery (Browning and Whitney 1991; Holden et al. 1983; Hung and Molina 1986; Mason et al. 1983; Riffle and Tinus 1982; Shaw et al. 1982; Theodorou and Bowen 1970; Thomas and Jackson 1983). Although the effects of induced *T. terrestris* colonization are difficult to establish because of its natural occurrence under experimental conditions, this species has been selected for the improvement of seedling growth. Marx et al. (1991) reported that *T. terrestris* is well adapted to nursery conditions because it is ecologically adapted to the excellent tilth, fertility and moisture conditions of nursery soils and often fails at outplanting sites. In the Nilgiri hills, Natarajan et al. (1992) reported that *T. terrestris* disappears after 7 years in *P. patula* plantations.

In the present study, seedlings inoculated with the combined treatment received only 50% of the fungus volume provided by individual inoculation. Parlade and Alvarez (1993) inoculated four pairs of ectomycorrhizal fungi to estimate the relative inoculum doses needed to establish two mycorrhizal fungi simultaneously in the same root system and found that the level of root colonization depended on the aggressiveness of the fungus rather than the size of the inoculum. Marx et al. (1991) reported that only one fungus of a mixture applied as a vegetative inoculum prevailed and successfully colonized the root system of plants. However, in the present study, both fungi colonized the root system, suggesting also that colonization ability depends mainly on aggressiveness, irrespective of whether inoculation is individual or combinated.

The percent colonization of inoculated fungi, either individually or in combination, varied from 5 to 12 months in both sterilized and unsterilized soils. Wong and Fortin (1990) reported that the formation of ectomycorrhizas by a fungus depends on temperature, pH, nutrients, moisture, aeration, external carbohydrates and other abiotic factors, giving ample reasons for variation in colonization of inoculated fungi. The percent colonization of indigenous fungi also varied among the treatments, both in sterilized and unsterilized soil. In the present study, sterilized soil was used to determine the colonization ability of the inoculated and indigenous fungi. Comparing results from sterilized and unsterilized soils,it was found that the total number of mycorrhizas and seedling heights and weights were much higher in sterilized soil than in unsterilized soil, particularly for the coinoculation treatments. Ruehle (1983) also showed that seedling growth and mycorrhizal development were significantly higher in fumigated soil than in nonfumigated soil when the seedlings were inoculated with *Pisolithus tinctorius*. This may be due to elimination of native symbionts, pathogenic fungi and other competitive soil microbial associates by the sterilization process, which allows inoculated fungi to colonize more and improve seedling growth. The total number of mycorrhizas produced by indigenous fungi was not counted separately in this study, but observed the colonization pattern of indigenous fungi in plants of different age. Among the indigenous colonizers, the ascotype fungi colonized first followed by *T. terrestris*, *Rhizopogon* sp. and *Inocybe* sp., both in uninoculated

and inoculated seedlings. Though the indigenous fungi are major competitors of introduced fungi on seedling roots, the introduced fungi colonized strongly when inoculated either singly or in combination in unsterilized soil.

High colonization ability in monospecific inoculations is one of the desirable characteristics used to select strains of ectomycorrhizal fungi for wide-scale nursery inoculations with vegetative inoculum (Kropp and Langlois 1990; Trappe 1977). As suggested by Parlade and Alvarez (1993), this trait needs 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. From our results, we conclude that coinoculation of ectomycorrhizal fungi significantly improves the growth and mycorrhizal development of *P. patula* seedlings in the nursery compared with individual inoculation. Further studies using different combinations of ectomycorrhizal fungi in different proportions may reveal the importance of coinoculation in improving the growth and mycorrhizal development of tree seedlings.

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