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Afforestation of abandoned farmland with conifer seedlings inoculated with three ectomycorrhizal fungi—impact on plant performance and ectomycorrhizal community

A. Menkis · R. Vasiliauskas · A. F. S. Taylor · J. Stenlid · R. Finlay

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Abstract The aim of a 3-year study was to investigate whether inoculation of *Pinus sylvestris* L. and *Picea abies* (L.) Karst. seedlings with mycorrhizas of *Cenococcum geophilum* Fr., *Piceirhiza bicolorata*, and *Hebeloma crustuliniforme* (Bull.) Quel. has any impact on: 1) survival and growth of outplanted seedlings on abandoned agricultural land, and 2) subsequent mycorrhizal community development. For inoculation, the root system of each plant was wrapped in a filter paper containing mycelium, overlaid with damp peat–sand mixture and wrapped in a paper towel. In total, 8,000 pine and 8,000 spruce seedlings were planted on 4-ha of poor sandy soil in randomized blocks. Already after the first year natural mycorrhizal infections prevailed in the inoculated root systems, and introduced mycorrhizas were seldom found. Yet, the seedlings that had been pre-inoculated with *C. geophilum* and the *P. bicolorata* during the whole 3-year period showed significantly higher survival and growth as compared to controls. Moreover, the independent colonization of roots by *C. geophilum* and the *P. bicolorata* from natural sources was also observed. A diverse mycorrhizal community was detected over two growing seasons in all treatments, showing low impact of inoculation on subsequent fungal community development. A total of 19 additional ectomycorrhizal morphotypes was observed, which clustered into two well-separated groups, according to host tree species (pine and spruce). In conclusion, the results showed limited ability to increase tree survival and growth, and to manipulate the mycorrhizal community even by extensive pre-inoculations, indicating that fungal community formation in root systems is governed mainly by environmental factors.

Keywords *Cenococcum* · *Piceirhiza* · *Hebeloma* · *Pinus sylvestris* · *Picea abies*

A. Menkis (✉) · R. Vasiliauskas · A. F. S. Taylor · J. Stenlid · R. Finlay
Department of Forest Mycology and Pathology,
Swedish University of Agricultural Sciences,
P.O. Box 7026, SE-750 07 Uppsala, Sweden
e-mail: Audrius.Menkis@mykopat.slu.se

Introduction

The establishment and performance of outplanted seedlings has often been reported to depend on ectomycorrhizal (ECM) fungi (Perry et al. 1987; Kropp and Langlois 1990; Stenström et al. 1990; Le Tacon et al. 1994; Pera et al. 1999; Baum et al. 2002; Dunabeitia et al. 2004), which may enhance uptake of water and nutrients (Smith and Read 1997) and lengthen the life and increase the growth of roots (Chilvers and Gust 1982; Wilcox 1996) by protecting them against drought, pathogens, and heavy metal pollution (Chakravarty and Unestam 1985; Colpaert and Vanassche 1992; Morin et al. 1999; Van Tichelen et al. 2001; Ortega et al. 2004). Because of the absence of host trees, a farmland is often deficient in natural ectomycorrhizal inoculum (Hacskaylo 1973), thus mycorrhization of seedling roots at outplanting might be beneficial. However, seedlings used in afforestation are often cultivated as bare root seedlings in forest nurseries and reportedly are only colonized by ECM fungi to a lesser extent (Dunabeitia et al. 2004; Menkis et al. 2005). Consequently, failure in afforestation has been hypothesized to be caused by the absence of mycorrhizas (Bjorkman 1970; Mikola 1970; Marx 1980). One way of overcoming this problem would be preinoculation of seedlings with selected ECM fungi. As this is a highly resource-consuming effort, the seedlings should be inoculated with ECM fungi best suited to host tree species and that rapidly colonize their roots, and which are well adapted

to the environmental conditions of the planting site (Perry et al. 1987).

The present work was carried out with three ECM fungi: *Cenococcum geophilum* Fr., *Piceirhiza bicolorata* (Brand et al. 1992), and *Hebeloma crustuliniforme* (Bull.) Quel., which possess characteristics that might be of importance for successful establishment of conifer seedlings on a farmland.

P. bicolorata is a distinct ECM morphotype formed by an ascomycete of the *Hymenoscyphus ericae* aggregate (Vrålstad et al. 2000), which occurs on a variety of conifers and hardwoods. The ability of this fungus to form mycorrhizas with both ectomycorrhizal and ericoid hosts (Villarreal-Ruiz et al. 2004) may provide access to a broad belowground network and consequently larger nutrient sources. Association of *P. bicolorata* with *P. sylvestris* and *P. abies* seedlings in numerous forest nurseries (Menkis et al. 2005) indicate its suitability to the managed environment, e.g., arable land. This fungus has also been found in association with pioneer tree species inhabiting heavy metal polluted soils, and thus might be involved in nutrient mobilization and substrate detoxification processes (Vrålstad et al. 2000). Although it was hypothesized that *P. bicolorata* aggregate might be beneficial for plants in marginal habitats (Vrålstad et al. 2000), this was not tested in the field inoculation experiments.

C. geophilum is known as an extremely drought-tolerant ECM ascomycete (Mexal and Reid 1973; Trappe 1977; Pigott 1982), which also grows and forms mycorrhizas over the pH range 2.4–7.5 and at high salt concentrations (Mikola 1948; Saleh-Rastin 1976). *C. geophilum* is associated with a wide range of tree species in forest nurseries and in forest ecosystems (Trappe 1988; Dahlberg and Stenström 1991; Menkis et al. 2005). It was found as the dominant species at the boundary of forest and agricultural land both near and distant (20 m) from the trees, and thus may play an important role in natural establishment of tree seedlings (Dickie and Reich 2005).

The basidiomycete *H. crustuliniforme* is an efficient root colonizer of young trees (Dighton and Mason 1985). Its survival and function on alkaline-saline substrates (Senior et al. 1993; Kernaghan et al. 2002; Muhsin and Zwiazek 2002) and the ability to protect plants against root pathogens (Perrin and Garbaye 1983) might be important for performance of outplanted seedlings. However, the reports on its effect on seedling growth are contradictory, as both the stimulation and retarding of growth have been observed (Theodorou and Bowen 1970; Bledsoe et al. 1982; Le Tacon and Bouchard 1986).

The main aim of the present work was to determine whether artificial inoculation of root systems with the three ECM fungi has any effect on survival and growth of the seedlings after outplanting under field conditions of abandoned farmland. At the same time, we investigated the impact the treatment of seedling roots with fungal inocula might have on subsequent mycorrhizal community development.

Materials and methods

Study sites and experimental design

The study area comprised 4 ha of abandoned farmland at Pocelonys in southern Lithuania (54°20′ N, 24°14′ E, 150–160 m above sea level). Within the area, mean annual precipitation is about 620 mm and the length of the growing season is ca. 190 days. Average temperature during the growth season is ca. 15°C. The site is characterized as low-productivity agricultural land and had been cultivated for many decades until the beginning of the 1990s. Large applications of fertilizers were repeatedly applied during the last three decades of cultivation, which have likely resulted in low background levels of ECM fungi. Before our study, the land was abandoned for more than 10 years and in the meantime turned to natural grassland lacking woody vegetation. The site is characterized by sandy soils, corresponding to *vaccinio-myrtilliosa* forest type.

Planting was carried out in April 2003 with 1-year-old pine (*Pinus sylvestris*) and 2-year-old spruce (*Picea abies*) seedlings, which were greenhouse-cultivated as bare root seedlings in a local forest nursery. A previous study had shown that in similarly cultivated seedlings from this nursery only ca. 20% of seedling roots were mycorrhizal (Menkis et al. 2005). Within tree species, seedlings were of similar height—ca. 11 cm for pine and 15 cm for spruce. For each tree species, three different ECM treatments and the non-inoculated control treatment were established in a randomized block design with four replicate blocks containing each of the four treatments. Before seedling outplanting, each 0.125-ha plot (25×50 m) was ploughed in 16 rows to ca. 12 cm deep with a forestry plough and 500 trees per plot were planted in rows at 1.6×1.5 m spacing. In total, 8,000 *P. sylvestris* and 8,000 *P. abies* seedlings were planted at an initial density of 4,000 seedlings per hectare. The distance between the pine and spruce sites was ca. 150 m.

Soil characteristics

Soil samples were collected in June 2003. Each of the 16 pine and 16 spruce plots was divided into two subplots from which soil cores were taken in the bed of ploughed rows to a depth of 20 cm at five different locations. Within each subplot, all sampled cores were pooled and analyzed as a bulk sample. Soil analyses were carried out at the Forest Soils Testing Laboratory, Lithuanian Forest Tree

Breeding and Seed Farming Centre, Girionys, Kaunas reg., Lithuania. Soil pH in H_2O and the basic nutrients (mg/100 g of soil) as mineralizable N, P_2O_5, and K_2O were determined. The results are summarized in Table 1. Among the plots with the same tree species differences in tested soil properties were, in most cases, not significant; however, significant differences in soil pH, P_2O_5, and K_2O content were found between the pine and spruce plots (Table 1). As the soil samples were taken a mere 1.5 months after the seedlings were planted, we assume that the estimated soil properties of the site were preexisting before the plantation was established.

Inoculum preparation and inoculation

Cenococcum geophilum (isolate UP162), a *Piceirhiza bicolorata* fungal isolate (isolate aurim678) and *Hebeloma-crustuliniforme* (isolate UP184) isolates were obtained from the culture collection of the Department of Forest Mycology & Pathology, Swedish University of Agricultural Sciences, Uppsala. Fungal stock cultures were maintained in darkness at 21°C on half-strength modified Melin–Norkrans (MMN) medium (Marx 1969).

Initially, the ability of selected fungi to form ectomycorrhizas was determined on sterile, 2-week-old *P. sylvestris* and *P. abies* seedlings, which were aseptically inoculated with agar plugs from the fungal stock cultures in Petri dishes with growth substrate of fine sphagnum peat, vermiculate, 1/10 strength liquid MMN mixture in the ratio 1:4:2 (Rosling et al. 2004). After 6 weeks incubation in a growth chamber, the fungi colonized the majority of seedling fine roots producing distinctive ectomycorrhizas. All fungi were re-isolated from the inoculation trials onto modified MMN media to maintain high vigor of stock cultures.

Vegetative inoculum was produced using a modification of the paper-sandwich technique (Chilvers et al. 1986). Initially, 14–16 agar plugs from the actively growing fungal stock cultures were aseptically placed in empty 14-cm-diameter Petri dishes. These were overlaid with five sheets of sterile, 13-cm-diameter filter paper (Munktell Analytical Filter Paper, Grycksbo, Sweden), which were moistened with 30 ml of liquid MMN media. The layering procedure was repeatedly carried out until the Petri dishes were filled. The final layer was mycelial plugs on the uppermost filter papers. The filter papers were situated in between the mycelial plugs, which allowed mycelial growth from both directions and consequently better establishment. Filter papers used in the control treatment were inoculated in the same manner, but only with sterile MMN agar plugs. The inoculated Petri dishes were incubated in darkness at 21°C for 30 days.

Filter papers with established ECM mycelia (Fig. 1a) and sterile control treatment sheets were soaked three times in sterile deionized water to remove any remaining nutrients. The filter papers were then transported submerged in sterile water to the afforestation site (Marx and Daniel 1976). Standardized inoculations (one filter paper per seedling containing identical fungal genotype) were carried out immediately before planting. The root system of each seedling was shortened to a size similar to that of the

Table 1 Soil chemical properties from different treatments in studied plantations of *Pinus sylvestris* and *Picea abies*

Treatment		Basic nutrients (mg/100 g)		
	pH (H_2O)	Mineralizable N	P_2O_5	K_2O
Pinus sylvestris				
Cenococcum geophilum	7.31a±0.07	6.05a±0.49	24.95a±1.59	5.89a±0.67
Piceirhiza bicolorata	7.33a±0.06	5.98a±0.56	31.75b±1.91	5.81a±0.66
Hebeloma crustuliniforme	6.96b±0.11	6.29a±0.45	24.19a±1.51	6.53a±0.96
Control	7.21ab±0.11	5.86a±0.24	22.58a±1.66	5.45a±0.69
p	0.02	0.42	0.003	0.38
Picea abies				
Cenococcum geophilum	6.44a±0.22	5.96a±0.29	16.96a±2.86	10.56a±0.74
Piceirhiza bicolorata	6.31ab±0.29	5.85a±0.57	13.75a±2.78	10.70a±1.45
Hebeloma crustuliniforme	5.79b±0.20	5.64a±0.32	17.93a±3.54	11.61a±0.84
Control	5.98ab±0.18	6.08a±0.47	15.85a±3.80	10.49a±1.22
p	0.04	0.46	0.37	0.46
All				
P. sylvestris	7.20a±0.05	6.04a±0.22	25.87a±1.01	5.92a±0.36
P. abies	6.13b±0.12	5.88a±0.20	16.12b±1.58	10.84b±0.53
p	<0.001	0.59	<0.001	<0.001
All plants	6.67±0.09	5.96±0.15	20.99±1.12	8.38±0.45

Values are for mineral soils (mean ± S.E., n=8). Means in the same column followed by the same letter are not significantly different.

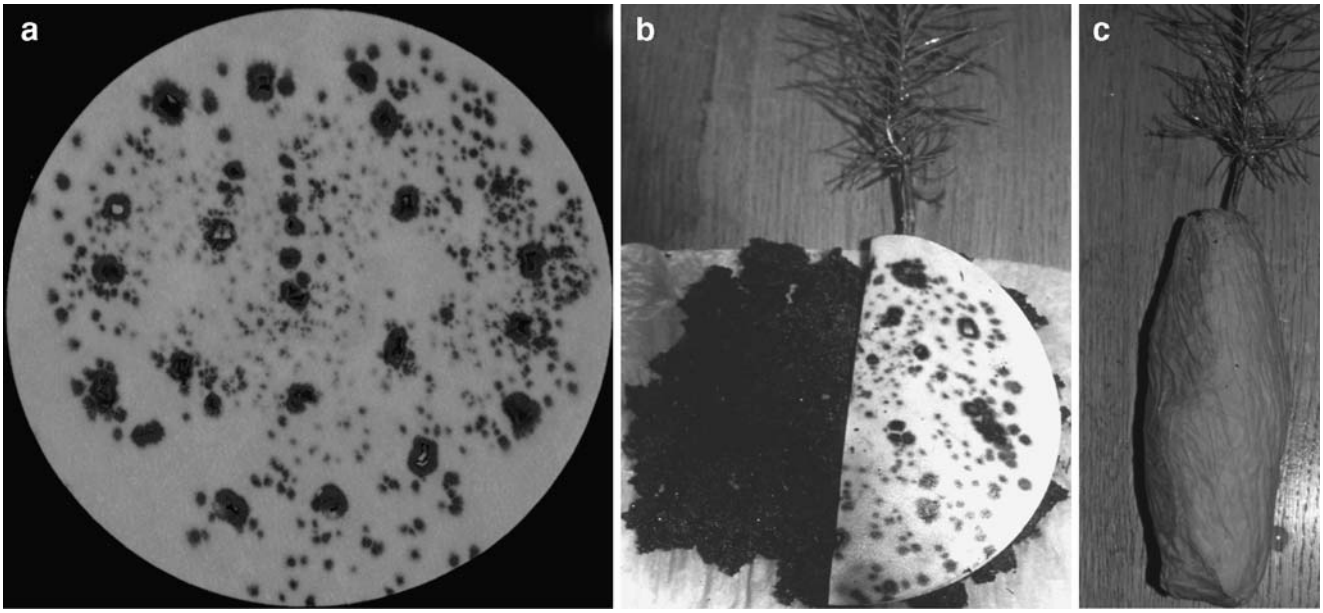

Fig. 1 Method used for inoculation of *Pinus sylvestris* and *Picea abies* seedlings: **a** vegetative inoculum—mycelium of *Piceirhiza bicolorata* growing on MMN moistened filter paper; **b** components and inoculation procedure—roots wrapped in filter paper containing ectomycorrhizal mycelium are covered with damp sand–peat mixture and everything is wrapped in paper towel; **c** inoculated seedling before outplanting

filter paper (ca. 13 cm diameter) and wrapped in the filter paper containing ECM mycelium (Fig. 1b) or in the filter paper of the control treatment. In addition, a layer of damp sphagnum peat–sand mixture 3:1 was placed on the top of filter paper, and this was then wrapped in a double layer of paper towel (Tork, SCA, Sweden) (Fig. 1c). In total, 12,000 of seedlings inoculated with three ECM fungi and 4,000 control seedlings were immediately outplanted after inoculation. To check vitality of the inocula, after the planting was completed remaining inoculated filter papers were brought back to the laboratory and from each treatment the respective fungus was re-isolated into pure culture. Moreover, to check that mycorrhization of roots is not restricted by the inoculation system, inoculated seedlings of each treatment in triplicate were planted in plastic pots with non-sterile sand and grown for 8 weeks in the growth chamber at 21°C and photoperiodicity 16 h light and 8 h dark.

Morphotyping and measurements

In the field, inoculation success and the composition of the ECM communities were studied twice, at the end of the growing seasons of 2003 and 2004. The number of investigated plants and roots are shown in Table 2. After random collection of seedlings in each plot, the root systems were excised from the stems, individually packed into plastic bags, transported to the laboratory, and kept at 4°C for a maximum of 4 weeks. Before investigation, each root system was washed in tap water and 20 single root tips from each plant were randomly collected from different parts of the root system using forceps. Mycorrhizal tips were morphotyped as described by Menkis et al. (2005). For identification, these were compared with published illustrations (Agerer 1986–1988; Agerer et al. 1996–1998). Only morphotypes that matched the descriptions were given taxonomic names. The morphotypes that did not match any of those from available publications were classed as unidentified, grouped accordingly to morphological characters, and given a descriptive name (e.g., Unidentified no. 1 [white reddish]), as presented in Table 3.

Survival of plants was determined three times, at the end of growing seasons of 2003, 2004, and 2005, by counting all living trees. The height increment was measured for all living trees at the end of the second and third growth seasons (2004 and 2005).

Statistical analyses

The impact of treatment on seedling survival and mycorrhizal colonization was analyzed using chi-square (χ^2) tests (Conover 1980; Mead and Curnow 1983). The aim was to answer the question: how strong is the evidence that a given treatment has an impact on seedling survival, and on seedling mycorrhization, when compared to control or another treatment? The comparisons were calculated from the actual number of observations in respective treatment pairs (living vs dead trees, and mycorrhizal vs non-mycorrhizal plants/root tips).

The survival of seedlings was evaluated separately for each respective year (2003, 2004 and 2005) and tree species

Table 2 Mycorrhizal colonization of plants/roots in studied plantations during two growing seasons

Treatment	Total no. of planted seedlings	No. of plants/root tips examined each year	Mycorrhizal colonization (%)			
			Year 2003		Year 2004	
			Plants[a]	Roots[b]	Plants[a]	Roots[b]
Pinus sylvestris						
Cenococcum geophilum	2,000	20/400	95.0a	66.0a	95.0a	67.2a
Piceirhiza bicolorata	2,000	20/400	100a	66.7a	100a	78.5b
Hebeloma crustuliniforme	2,000	20/400	95.0a	69.2a	100a	77.5b
Control	2,000	20/400	75.0a	52.0b	100a	63.5a
Picea abies						
Cenococcum geophilum	2,000	20/400	85.0a	34.5a	100a	70.5a
Piceirhiza bicolorata	2,000	20/400	75.0a	30.0a	100a	70.0a
Hebeloma crustuliniforme	2,000	20/400	85.0a	20.7b	95.0a	63.0ab
Control	2,000	20/400	65.0a	29.0a	100a	54.2b
All						
P. sylvestris	8,000	80/1,600	91.2a	63.5a	98.7a	71.7a
P. abies	8,000	80/1,600	77.5b	28.6b	98.7a	64.4b
All plants	16,000	160/3,200	84.4	46.0	98.7	68.1

Within columns of respective tree species, values followed by the same letter are not significantly different.
[a] Plants with at least one mycorrhizal root tip were classed as mycorrhizal.
[b] Root tips with characteristic structures of mycorrhizal fungi were classed as mycorrhizal.

(pine and spruce). The proportions of living and dead plants were pairwise compared to the treatments in all possible combinations (e.g., pine, year 2003: *C. geophilum* vs *P. bicolorata*; *C. geophilum* vs *H. crustuliniforme*; *C. geophilum* vs control; *P. bicolorata* vs *H. crustuliniforme*; *P. bicolorata* vs control; *H. crustuliniforme* vs control; similar for years 2004 and 2005; and then the same for spruce). This allowed us to estimate the relative impact of each treatment on the survival of each tree species during the 3-year period. As each of the datasets was subjected to three comparisons, confidence limits for p values of the chi-squared tests were reduced three times ($p/3$), as required by the Bonferroni correction (Sokal and Rohlf 1995).

The extent of mycorrhizal colonization of pine and spruce seedlings was at first evaluated separately for the years 2003 and 2004. At this stage, the proportions of mycorrhizal and non-mycorrhizal plants/root tips were also compared between the treatment pairs in all possible combinations (e.g. pine, year 2003: *C. geophilum* vs *P. bicolorata*; *C.g.* vs *H. crustuliniforme*; *C. g.* vs control; *P. b.* vs *H.c.*; *P. b.* vs control; *H.c.* vs control; similar for year 2004; and then the same for spruce). This allowed to estimate the relative impact of each treatment on mycorrhization of plants during the 2-year period. Furthermore, the values from the same treatment were compared between different years (e.g., pine, *C. geophilum* for 2003 vs 2004; similar for *P. bicolorata*, *H. crustuliniforme* and control; and then the same for spruce). This provided the information on the possible influence of each treatment on the subsequent development of mycorrhizal communities in the field. Finally, the comparisons of mycorrhizal colonization were done between pine and spruce, within the same respective treatment and year (e.g., *C. geophilum*, year 2003: pine vs spruce; *C. geophilum*, year 2004: pine vs spruce; similar for *P. bicolorata*, *H. crustuliniforme*, and control). Here, the respective susceptibility of pine and spruce to colonization by both inoculated and natural mycorrhizas was evaluated. As the five comparisons were made with each dataset, confidence limits for p values of the chi-squared tests were reduced five times ($p/5$), as required by the Bonferroni correction.

Height increment of the seedlings was analyzed by one-way analysis of variance (ANOVA) (Chalmers and Parker 1989; Fowler et al. 2001). Pearson correlation coefficients were calculated to examine the relationship between soil chemical properties and survival and height increment of plants ($p<0.05$). The statistics were computed using Minitab® Statistical Software (Minitab® 2003). Ectomycorrhizal community structures were analyzed using principal component analysis (PCA) (Fowler et al. 2001) in CAN OCO 4.5 (Ter Braak and Smilauer 1998).

Results

Survival and growth of plants

The establishment of planted stock in our experimental plantations was rather high, and, after the first growing

Table 3 Frequency of mycorrhizal morphotypes (percent of plants colonized/percent of mycorrhizal roots colonized) on root tips of *Pinus sylvestris* and *Picea abies* seedlings under different treatments in 2003 and 2004

Morphotypes	*Pinus sylvestris*					*Picea abies*				
	Cg[a]	Pb[a]	Hc[a]	Co[a]	All	Cg[a]	Pb[a]	Hc[a]	Co[a]	All
Year 2003										
Amphinema byssoides	–/–	–/–	–/–	–/–	–/–	25.0/9.8	20.0/9.3	–/–	35.0/21.0	20.0/10.0
Cenococcum geophilum	45.0/14.8	–/–	5.0/0.3	10.0/0.8	15.0/3.9	50.0/11.3	–/–	35.0/2.5	25.0/3.8	27.5/4.4
Hebeloma crustuliniforme	–/–	–/–	25.0/3.0	–/–	6.3/0.8	–/–	–/–	–/–	–/–	–/–
Piceirhiza bicolorata	–/–	60.0/28.3	–/–	–/–	15.0/7.1	5.0/0.3	35.0/8.3	5.0/0.5	10.0/0.8	13.8/2.4
Rhizopogon sp.	25.0/8.3	30.0/11.3	65.0/45.5	40.0/10.3	40.0/18.8	–/–	–/–	–/–	–/–	–/–
Thelephora terrestris	25.0/11.0	15.0/5.3	30.0/8.0	35.0/16.3	26.3/10.1	25.0/8.3	30.0/10.0	30.0/7.5	5.0/0.5	22.5/6.6
Suillus luteus	55.0/30.0	40.0/15.5	25.0/9.8	45.0/21.8	41.3/19.3	–/–	–/–	–/–	–/–	–/–
Suillus sp.	5.0/1.0	–/–	5.0/2.8	–/–	2.5/0.9	–/–	–/–	–/–	–/–	–/–
Unidentified no. 1 (white reddish)	5.0/1.0	5.0/3.0	–/–	–/–	2.5/1.0	–/–	–/–	–/–	–/–	–/–
Unidentified no. 2 (yellow brown)	–/–	15.0/3.5	–/–	10.0/3.0	6.3/1.6	30.0/5.0	15.0/2.5	55.0/10.3	20.0/3.0	30.0/5.2
Year 2004										
Amphinema byssoides	–/–	–/–	–/–	–/–	–/–	80.0/49.8	55.0/37.0	60.0/43.3	80.0/39.5	68.8/42.4
Cenococcum geophilum	25.0/6.5	5.0/0.3	20.0/1.8	5.0/0.3	13.8/2.2	40.0/10.5	25.0/4.8	30.0/3.8	10.0/3.5	26.3/5.6
Paxillus sp.	–/–	–/–	–/–	5.0/0.8	1.3/0.2	–/–	–/–	–/–	–/–	–/–
Piceirhiza bicolorata	5.0/0.8	55.0/23.0	10.0/0.5	–/–	17.5/6.1	25.0/6.5	50.0/20.0	35.0/6.0	50.0/6.0	40.0/9.6
Rhizopogon sp.	30.0/11.0	50.0/13.0	30.0/15.3	40.0/16.5	37.5/13.9	–/–	–/–	–/–	–/–	–/–
Suillus luteus	45.0/11.0	55.0/16.3	50.0/24.3	45.0/23.3	48.8/18.7	–/–	–/–	–/–	–/–	–/–
Suillus sp.	5.0/1.3	–/–	–/–	–/–	1.3/0.3	–/–	–/–	–/–	–/–	–/–
Thelephora terrestris	50.0/23.0	15.0/4.5	25.0/14.3	20.0/7.8	27.5/12.4	–/–	–/–	5.0/2.3	–/–	1.3/0.6
Unidentified no. 1 (white reddish)	–/–	5.0/0.8	–/–	–/–	1.3/0.2	–/–	–/–	–/–	–/–	–/–
Unidentified no. 2 (yellow brown)	20.0/4.8	30.0/6.3	25.0/7.3	30.0/2.3	26.3/5.1	10.0/2.5	20.0/5.0	20.0/2.8	20.0/2.5	17.5/3.2
Unidentified no. 3 (light carroty)	35.0/8.3	40.0/14.0	35.0/13.5	25.0/11.0	33.8/11.7	–/–	–/–	–/–	–/–	–/–
Unidentified no. 4 (brownish)	–/–	–/–	–/–	5.0/0.3	1.3/0.1	–/–	–/–	–/–	–/–	–/–
Unidentified no. 5 (light yellow)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	10.0/1.3	2.5/0.3
Unidentified no. 6 (matt yellowish)	–/–	5.0/0.3	–/–	–/–	1.3/0.1	–/–	–/–	–/–	–/–	–/–
Unidentified no. 7 (pale reddish)	–/–	–/–	5.0/0.8	–/–	1.3/0.2	–/–	–/–	5.0/3.3	–/–	1.3/0.8
Unidentified no. 8 (dark reddish)	10.0/0.8	–/–	–/–	10.0/1.5	5.0/0.6	–/–	–/–	–/–	–/–	–/–
Unidentified no. 9 (reddish)	–/–	–/–	–/–	–/–	–/–	–/–	10.0/3.3	5.0/1.8	5.0/1.5	5.0/1.6
Unidentified no. 10 (white yellow)	–/–	5.0/0.3	–/–	–/–	1.3/0.1	–/–	–/–	–/–	–/–	–/–
Unidentified no. 11 (grey whitish)	–/–	–/–	–/–	–/–	–/–	5.0/1.3	–/–	–/–	–/–	1.3/0.3

[a] Treatments: Cg *Cenococcum geophilum*, Pb *Piceirhiza bicolorata*, Hc *Hebeloma crustuliniforme*, Co non-inoculated control

season, consisted of 67–78% and 77–88% for pine and spruce, respectively (Fig. 2). Yet, its dynamics during study period in both tree species and in all treatments has revealed a similar trend of slightly increasing mortality. During the first three growing seasons, pine seedlings inoculated with *C. geophilum* and *P. bicolorata* showed survival rates 10–13% higher than control seedlings (chi-squared test, $p<0.0001$). By contrast, the survival of seedlings inoculated with *H. crustuliniforme* did not differ significantly from the control ($p>0.065$; Fig. 2a). In spruce, during the first three growing seasons only *C. geophilum* inoculated seedlings had significantly higher survival than the control seedlings (ca. 9%; $p<0.0001$). *P. bicolorata* had no effect on survival, while the seedlings treated with *H. crustuliniforme* had significantly lower survival (ca. 7%; $p<0.0001$; Fig. 2b) than the control seedlings.

At outplanting, seedlings of each species were similar in height and in root system size; however, one-way ANOVA revealed certain differences in height increment among some treatments after second and third growing seasons (Fig. 3). After the third season, pine seedlings inoculated with *C. geophilum* had significantly greater height increment compared to the control ($p<0.03$), while those inoculated with *H. crustuliniforme* were not significantly different ($p>0.09$), and seedlings inoculated with *P. bicolorata* were significantly lower that the control ($p<0.0001$).

By contrast, spruce seedlings inoculated with the *P. bicolorata* had significantly greater height increment than that of non-inoculated plants ($p<0.001$) after the second growing season, and this was also observed for both *P. bicolorata* and *H. crustuliniforme* after the third growing season. The inoculation of spruce with *C. geophilum* did

not enhance height increment of trees to a significant extent (Fig. 3). For both tree species, a significant increase in height increment was observed in corresponding treatments compared to the second and third growing seasons ($p<0.0001$). Generally, pine seedlings in each corresponding year and treatment exhibited significantly greater height increment than spruce seedlings ($p<0.001$) (Fig. 3). No significant correlation was found between tested soil chemical properties and the survival and height increment of seedlings of either pine or spruce ($p>0.095$).

Ectomycorrhizal colonisation

Table 2 shows that after the first growing season, mycorrhizal colonization was observed on 95–100% of inoculated pine seedlings and on 75% of control pines, and on 75–85% of inoculated spruce and 65% of control spruce. However, the differences in colonization of inoculated and control plants in each tree species were not statistically significant. After the first season, the pooled mycorrhizal colonization of all pine seedlings appeared to be significantly higher than that of all spruce (by 14%; $p<0.02$). After the second

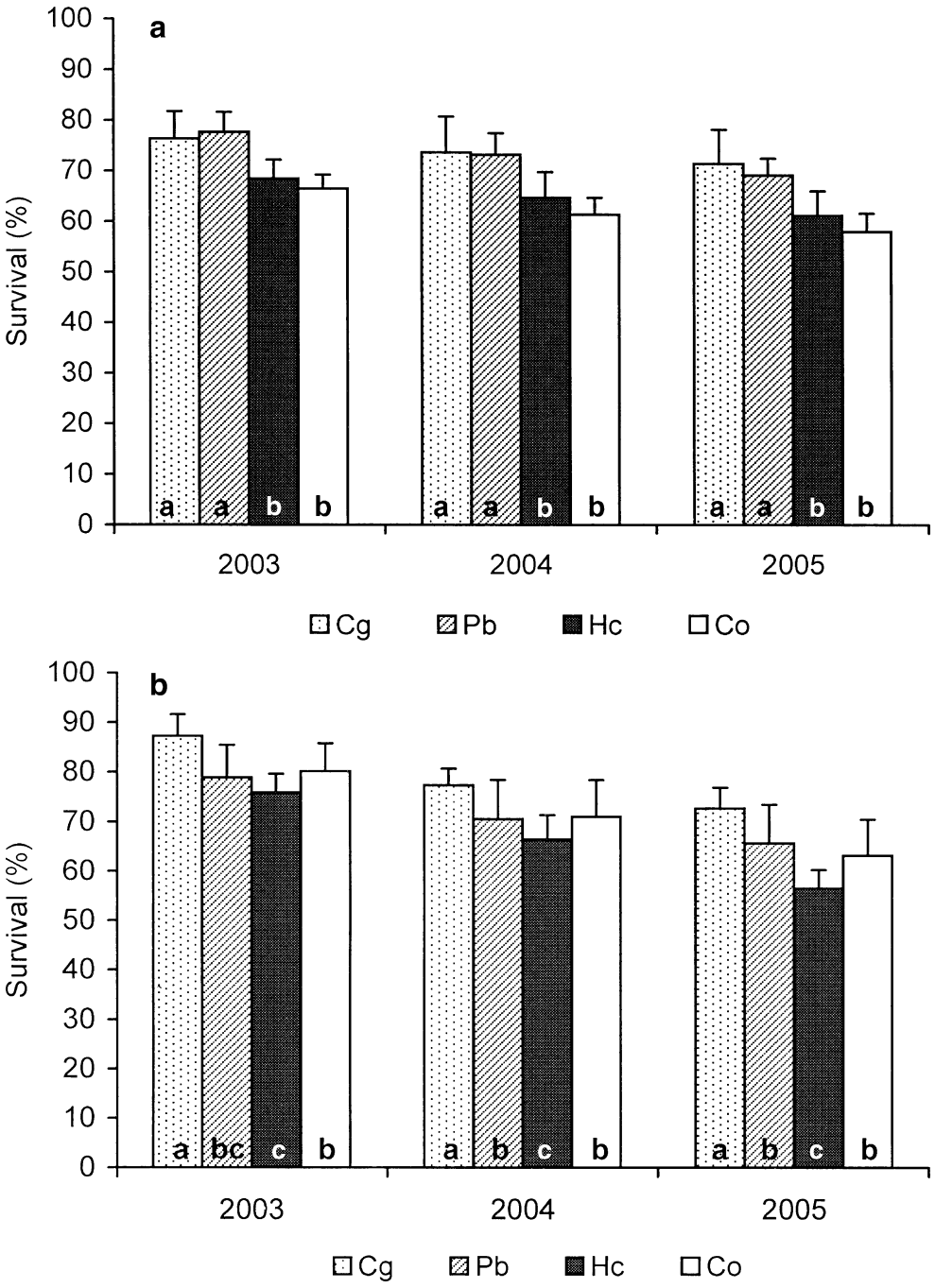

Fig. 2 **a** Survival of *Pinus sylvestris* seedlings during three growing seasons. Within the same year, statistically significant differences between the treatments (Cg—*Cenococcum geophilum*, Pb—*Piceirhiza bicolorata*, Hc—*Hebeloma crustuliniforme*, Co—non-inoculated control) in chi-squared tests are designated by different *letters*. *Error bars* indicate standard error of the mean. **b** Survival of *Picea abies* seedlings during three growing seasons. Within the same year, statistically significant differences between the treatments (Cg—*Cenococcum geophilum*, Pb—*Piceirhiza bicolorata*, Hc—*Hebeloma crustuliniforme*, Co—non-inoculated control) in chi-squared tests are designated by different *letters*. *Error bars* indicate standard error of the mean

growing season, mycorrhizal colonization of inoculated pines remained ca. 95–100%, but the mycorrhization of control plants increased to 100% (Table 2). For spruce seedlings, the second growing season resulted in a sharp increase in mycorrhization: up to 95–100% in each treatment and the control. Consequently, after two growing seasons no significant differences in the proportion of mycorrhizal plants were observed either among different treatments of spruce (including control) or between the two tree species (Table 2).

After the first season, the proportion of colonized roots (66–69%) of inoculated pine seedlings did not differ between the different mycorrhizal treatments. In non-inoculated controls, the mycorrhization was 14–17% lower, and in the comparisons with inoculation treatments the differences were statistically significant ($p<0.0003$; Table 2). After the second year, mycorrhizal colonisation of root systems in *P. bicolorata* and *H. crustuliniforme* treatments, and in the control increased by 8–12%, while in *C. geophilum* treatment it remained about the same (+1%). Consequently, at this stage pine seedlings inoculated with *H. crustuliniforme* and *P. bicolorata* showed significantly higher (14–15%) colonization of roots than non-inoculated seedlings ($p<0.0001$), but the difference between the *C. geophilum* treatment and the control was not significant (Table 2).

By contrast, root systems of spruce seedlings after the first season showed comparatively low ECM colonization: in each treatment and the control only 21–35% of root tips were mycorrhizal; less than half the level on pine (when pooled, 29% vs 64%; $p<0.0001$; Table 2). Moreover, *C. geophilum* and *P. bicolorata* treatments were not significantly different from the control, and in the *H. crustuliniforme* treatment root colonization was significantly lower than in the control and in the other two treatments (by 8–14%; $p<0.035$). However, the second year resulted in a sharp increase in mycorrhization of root systems of spruce, and the gap between the two tree species narrowed considerably. Thus, in non-inoculated control seedlings the extent of root system colonization by ECM fungi increased almost twofold (up to 54% of roots), in *C. geophilum* and *P. bicolorata* treatments increased by more than twice (to 70%), and in *H. crustuliniforme* treatment increased more than three times (to 63%; Table 2). However, despite these increases, a significant difference remained after the second year between mycorrhization of pine and spruce root systems (when pooled, 72 vs 64%; $p<0.0001$). At this stage, in *C. geophilum* and *P. bicolorata* treatments ECM colonization of spruce root systems was significantly higher than in the control (by 16%; $p<0.0001$). In *H. crustuliniforme* treatment mycorrhization was higher than in control, but the difference was not statistically significant (Table 2).

After the first growing season, mycorrhizal colonization of roots was in all treatments (including control) significantly higher for pine than for spruce ($p<0.0001$). After the second season, colonization of roots remained higher for pine ($p<0.03$), with the exception of the *C. geophilum* treatment where mycorrhization did not significantly differ between the tree species ($p>0.32$).

Ectomycorrhizal community structure

Examination of the inoculated systems incubated in the growth chamber showed that the majority of fine roots of pine and spruce seedlings were colonized by the target fungi. *H. crustuliniforme* was the most efficient colonizer inhabiting ca. 75% of fine roots, while *C. geophilum* and *P. bicolorata* mycobiont colonized ca. 50% of fine roots. It was also observed that roots readily grew out through the

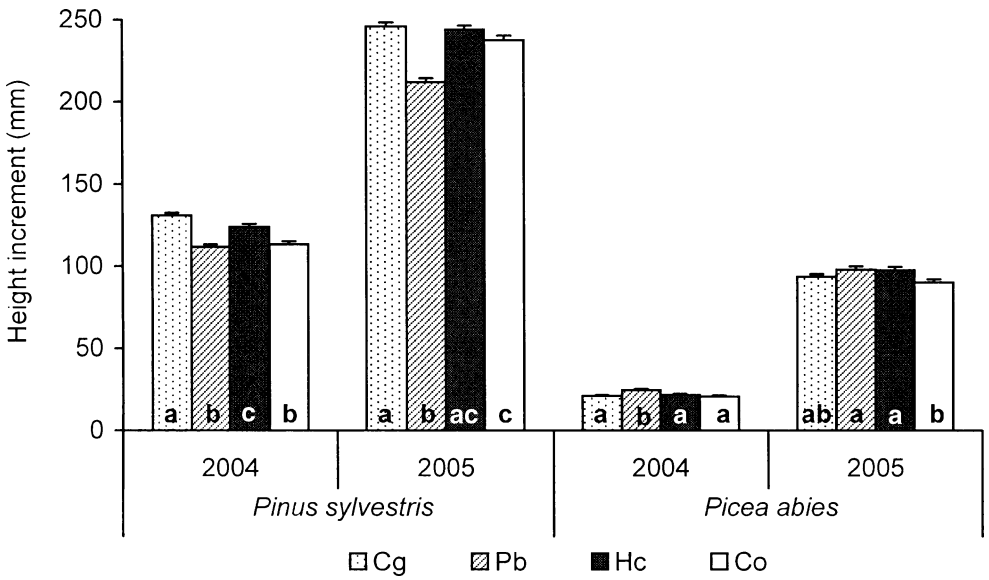

Fig. 3 Height increment of *Pinus sylvestris* and *Picea abies* seedlings after the second and third growing seasons. Within tree species and the same year, statistically significant differences between the treatments (Cg—*Cenococcum geophilum*, Pb—*Piceirhiza bicolorata*, Hc—*Hebeloma crustuliniforme*, Co—non-inoculated control) in one-way ANOVA are designated by different *letters*. *Error bars* indicate standard error of the mean

layer of paper towel, and these were already colonized by the introduced fungi.

By contrast, after the first growing season the majority of observed mycorrhizas in the field were not the ones that had been inoculated (Table 3). Moreover, at this stage morphotypes of *C. geophilum* and *P. bicolorata* were also observed on roots of control and *H. crustuliniforme*-inoculated seedlings, but in all cases at significantly lower frequencies ($p<0.0001$). After the second year, *C. geophilum* and *P. bicolorata* morphotypes were recorded at variable frequencies throughout all treatments, except for the pine control, but *H. crustuliniforme* disappeared completely from both tree species (Table 3).

Many other mycorrhizas were recorded on our study sites both after the first and the second growing seasons. A total of ten distinct morphotypes were observed after the first growing season and 19, including ten new, after the second season. When pooled, 20 morphotypes were detected, 11 of which were unique for pine, four unique for spruce, and five common to both tree species. Eleven morphotypes (55%) could not be matched to published descriptions and remained unidentified (Table 3).

The PCA of ectomycorrhizal communities clustered the morphotypes observed on pine and spruce into two well-separated groups (Fig. 4). The first axis explained 45.2% of the variation, while axis 2 explained a further 30.0%. A tight cluster of *P. sylvestris* indicated that there were no obvious differences in fungal community structure either between different treatments or growing seasons. The most common ECM morphotypes in different treatments/seasons were largely the same and included *Suillus luteus* (L.) Gray, *Rhizopogon* sp., *Thelephora terrestris* Ehrh. ex Fr., *P. bicolorata*, and Unidentified no. 3 (Fig. 4; Table 3). On the other hand, the elongated cluster of *P. abies* indicated the dynamic variation in ECM community structure between the two growing seasons (Fig. 4). One of the main reasons for this was sharply increased frequency of roots colonized by *Amphinema byssoides* (Pers.: Fr.) J. Erikss. and *P. bicolorata* (Table 3).

The differences in ECM community structure of *P. abies* were also observed between the different treatments after the first growing season. However, after the second season, all treatments were more or less similar to each other and clustered together (Fig. 4). In most cases, the most common taxa on spruce were *A. byssoides*, *T. terrestris*, *P. bicolorata*, *C. geophilum*, and Unidentified no. 2. However, the frequencies of these morphotypes differed considerably between different treatments and growing seasons (Table 3).

Discussion

The results of the present work showed that: 1) the persistence of inoculated mycorrhiza in the field and its effect on tree host depended on the fungus; 2) in two out of three fungal treatments (*C. geophilum* and *P. bicolorata*), inoculation resulted in positive effects on seedling survival and growth; 3) although significant, such effects were rather limited, but persisted at least for 3 years (Figs. 2 and 3); 4) the persistence of inoculated strains was generally low and natural colonization by indigenous ectomycorrhizas was abundant (Table 3).

There are a number of studies in which high persistence of inoculated mycorrhizal symbionts has been reported in forest plantations, but those involved different fungal and tree species than those used in the present work, and were carried out under different ecological conditions. For example, introduced *Pisolithus* spp. was shown to persist for up to 3 years in Chinese eucalypt plantations (Dell et al. 2002), and inoculated *Laccaria bicolor* (Maire) Orton persisted for 3–10 years in Douglas fir plantations in France (Selosse et al. 1998a,b; Di Battista et al. 2002). In Mediterranean pine plantations, inoculated *Suillus collinitus* persisted for 4 years (El Karkouri et al. 2006), and in Australia inoculated *Amanita muscaria* strains persisted for more than 30 years (Sawyer et al. 2001). By contrast, Heinonsalo et al. (2004) did not detect the inoculated *L. bicolor* strains in a forest plantation after 4 years and no treatment-related impact was observed in soil microbial communities. Despite that, they recorded significantly

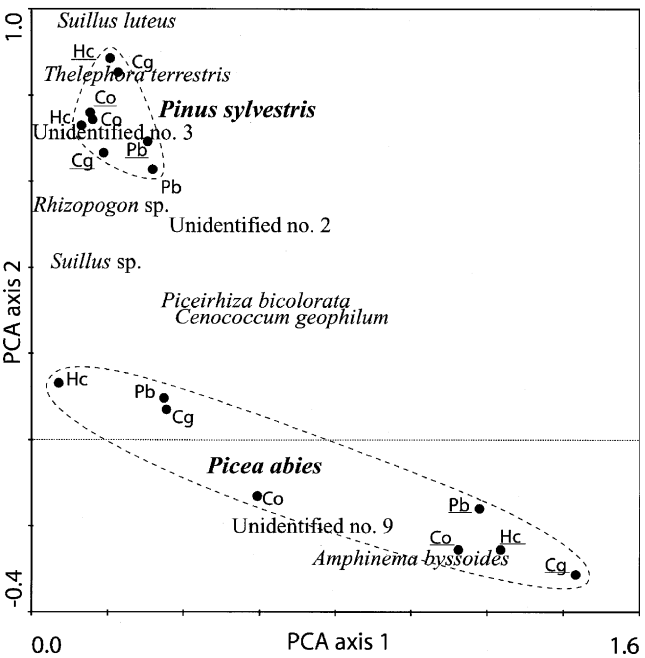

Fig. 4 First and second axes of a principal component analysis of ectomycorrhizal communities in roots of *Pinus sylvestris* and *Picea abies* seedlings from different inoculation treatments (Cg—*Cenococcum geophilum*, Pb—*Piceirhiza bicolorata*, Hc—*Hebeloma crustuliniforme*, Co—non-inoculated control) and different growing seasons (Cg—2003; Cg—2004). Taxonomic names correspond to a position in the ordination (centred), and show the ten most common ectomycorrhizal fungi detected by mycorrhizal morphotyping

increased shoot growth in the mycorrhiza-inoculated plots compared with the non-inoculated plots, which corresponds well to the results of the present work. Yet, such cases when significant growth effects have been observed in spite of the fact that the introduced fungi did not persist are hard to explain, but, on the other hand, the positive impact might be initiated during more successful establishment of mycorrhiza-inoculated seedlings immediately after outplanting. Therefore, the possibility cannot be excluded that the growth conditions at the very early stages after planting have persisting effects for subsequent development of a tree seedling in the field.

It is known that mycorrhizal colonization under field conditions is to a large extent governed by ecological factors such as soil characteristics, tree species, and fungi (McAfee and Fortin 1985; Hedlund and Gormsen 2002). Thus, as our sites were apparently suitable for *C. geophilum* and *P. bicolorata*, a number of independent colonizations of seedlings by these fungi were observed in non-treated sets even after the first growing season, and substantially increased after the second (Table 3). Simultaneously, there seemingly was a high number of well-adapted indigenous mycorrhizas in the area, and after 2 years 19 additional morphotypes were observed on our seedlings (Table 3). Consequently, the formation of natural ectomycorrhizal communities was to a large extent observed in our plots, which proceeded much faster in pine than in spruce plantations. After the first growing season, in all treatments, the pine ECM community was far more diverse and showed a higher proportion of colonized roots than did the spruce ECM community, while during the second year the mycorrhization of spruce increased sharply (Tables 2 and 3). Moreover, the ectomycorrhizal communities of pine and spruce were clearly different (Fig. 4). Besides the tree species, an additional contributing factor could be the soil properties that differed markedly between the plantations (Table 1).

Therefore, the different rates of survival and growth of trees pre-inoculated with mycorrhizas observed in preceding studies (Baum et al. 2002; Beckjord and McIntosh 1984; Cram et al. 1999; Danielson and Visser 1989; Dunabeitia et al. 2004; Le Tacon et al. 1994; Loopstra 1988; Pera et al. 1999; Riffle and Tinus 1982; Stenström and Ek 1990; Stenström et al. 1990; Teste et al. 2004) could probably be explained by the diversity of factors (fungus, soil, tree species) involved in mycorrhizal establishment. In our recent work, we also demonstrated that ECM colonization rates and community structure differ significantly in forest nurseries under different cultivation systems (Menkis et al. 2005), and that different pathogens and endophytes attack conifer seedling roots planted in nurseries, forest clear-cuts, and abandoned farmland (Menkis et al. 2006), which might also be of importance to seedling survival and growth.

Finally, although significantly positive effects of mycorrhizal inoculations on tree performance and growth are reported in the present work, these were not dramatic and were associated with large investment of resources. One reason for the rather slight impact could be the good survival and growth, and an extensive natural mycorrhization of our trees on control plots. The artificial mycorrhization of seedlings, therefore, might be more useful for afforestation of marginal sites with harsh conditions where the first months or even weeks are crucial for tree establishment. Indeed, the improved seedling survival is encouraging because it can prove decisive in, e.g., dry years and, when cumulated over a long period of time, could result in a significant difference in terms of silviculture. More observations and measurements are, therefore, necessary to elucidate this, and will be done in our experimental plantations in the future.

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