Original Research

Ectomycorrhizal Status of Scots Pine Saplings Growing in Post-Agricultural Soils

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Abstract

Ectomycorrhizal communities associated with *Pinus sylvestris* L. saplings growing on abandoned post agricultural soils were studied. 1-year-old seedlings inoculated with either *Suillus luteus* Fr. or *Thelephora terrestris* (Ehrh.) Fr. had been outplanted on three sites. We investigated the long-term effect (6 and 8 years after outplanting) of inoculation on ectomycorrhizal composition and species richness. We found 17 morphotypes in total that could be identified to genus or species. In all sites the most predominant ectomycorrhizae were *Suillus luteus*, *Thelephora terrestris*, *Tomentella* sp., *Dermocybe palustris*, and *Dermocybe* sp. Species richness was higher in the case of inoculated seedlings grown at sites in Garwolin and Płońsk, whereas at the third site, Jabłonna, more diverse ectomycorrhizae possessed non-inoculated seedlings. The number of *S. luteus* mycorrhizae on seedlings inoculated with the fungus was higher than on non-inoculated ones. Dissimilar results have been found in cases of seedlings inoculated by *T. terrestris*. The latter possessed only 14% of the ectomycorrhizae. The results showed that persistence of inoculated fungi in roots of Scots pine is regulated by environmental conditions.

Keywords: Scots pine, inoculation, ectomycorrhizal fungi, agricultural lands

Introduction

Ectomycorrhizal symbiosis plays a major role in two plant processes: hydro-mineral nutrition and the portioning of C within the plant, with some ultimately being transferred to ectomycorrhizal fungi [1]. Ectomycorrhizal (ECM) fungi are practically ubiquitous in natural forests [2], whereas in areas without existing ECM inoculum (e.g. abandoned agricultural land) the situation is different [3]. Hence, colonization of roots by particular ECM fungi as the consequence of particular cultivation processes in forest nurseries [4-6] or achieved by artificial inoculation, both in the nursery or in the field [7-9], may significantly promote survival, establishment and growth of young trees in newly established forest plantations [5, 10, 11].

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One way of overcoming the problem of the absence of ectomycorrhizal fungi in soil would be the preinoculation of seedlings with selected ECM fungi. However, the seedlings should be inoculated with ECM fungi, which are best suited to the host plants, colonize their roots rapidly, and which are well adapted to the environmental conditions of the planting site [12].

In the present study, we investigated the influence of ectomycorrhizal fungi (ECM) *Suillus luteus* Fr. S. F. Gray and *Thelephora terrestris* (Ehrh.) Fr. on changes of mycorrhizal structure of Scots pine seedlings growing under field conditions. The fungi chosen for mycorrhization were considered to be suitable for colonization of conifer seedlings on farmlands [13, 14].

Most of the studies of ECM community structure have investigated mature forests [15-17] and disturbed habitats [18-20], whereas little is known about the species composi-

Table 1. Soil parameters for the examined post-agricultural lands.

Soil parameters	Forest Districts		
	Garwolin	Płońsk	Jabłonna
pH_{H_2O}	4.85	5.53	5.51
pH_{KCL}	3.98	4.49	4.48
P ($g \cdot kg^{-1}$)	0.16	0.61	0.63
Fe ($g \cdot kg^{-1}$)	1.15	2.59	3.44
Ca ($g \cdot kg^{-1}$)	0.15	0.65	1.17
Mg ($g \cdot kg^{-1}$)	0.31	0.41	0.67
K ($g \cdot kg^{-1}$)	0.45	0.74	0.75
Ca/Mg	0.48	1.5	1.7
K/Mg	1.45	1.8	1.1
Carbon (total) %	0.48	1.53	8.3
Nitrogen (total) %	0.04	0.12	0.85
C/N	12.0	12.8	9.8

tion of ECM fungi in post-agricultural lands [11, 21]. The aim of this study was to investigate the changes of the mycorrhizal structure of inoculated fungi after six and eight years of seedlings being planted out.

Materials and Methods

At the beginning of April 2002, 1-year-old seedlings of Scots pine were taken from bare root nurseries and inoclulated with vegetative fungal slurry of the fungi, viz., *Suillus luteus* (isolate 5409 IBL) in Forest Districts (FD) Garwolin and Płońsk, and *Thelephora terrestris* (isolate Tt/IBL/747) in Jabłonna. The inoculation of seedlings took place on the day of outplanting, each seedling receiving 10 mL of mycelium suspended in deionised water. The lands had been abandoned for a longer than 1-year period prior to our study. All sites were characterized by sandy soil, corresponding to the *vaccinio-myrtilliosa* forest type. Soil parameters are given in Table 1. For each treatment of seedlings (inoculated and control) six replicate plots were established. Before seedlings were planted, each plot 0.0021 ha in size (m × 7 m) was ploughed in 3 rows, ca. 12 cm deep with forestry plough and 30 seedlings were planted in beds of rows 1.5 m × 0.7 m spacing. At each site planting was carried out in the same way.

At the end of the growing season of 2009 samples of soil cores with fine roots (6 per treatment) were collected to determine the composition of the ECM communities. Samples were taken from the middle of each plot to a depth of 20 cm. The samples were rinsed on a sieve under tap water in order to remove soil particles. Roots were moved to a Petri dish with distilled water. All active tips representing mycorrhizae were counted. Ectomycorrhizal tips were identified by the presence of mantle (color, shape, and surface texture), external hyphae, a slightly swollen apex and mycelial strands [22]. Total numbers of vital mycorrhizal root tips per seedling were counted, the tips were distinguished using available identification manuals [23, 24]. Observations of root tips were conducted under a dissecting microscope at 10x to 40x magnification.

If the identity of some mycorrhizae was questionable, chosen mycorrhizal tips were taken for DNA analysis. Ectomycorrhizal fungi were identified using sequencing of the PCR amplified internal transcribed spacer of rDNA (ITS rDNA). The fungal-specific primer ITS1-F [25] and universal primer ITS 4 [26] was used for amplification by PCR as well as for sequencing. DNA sequence data were compared with sequence data in the the NCBI database (BLAST program, http://www.ncbi.nlm.nih.gov/) and deposited at the NCBI Gene Bank. Data from sequencing are presented in Table 3.

Table 2. Species richness and relative abundance (%) of mycorrhizal fungal taxa associated with Scots pine saplings from three sites.

Forest district	Treatment	Richness	*Amanita* sp	*Cenoccocum geophilum*	*Cortinarius* sp.	*Dermocybe* sp.	*Dermocybe palustris*	*Hygrophorus* sp.	*Inocybe* sp	*Laccaria* sp.	*Lactarius* sp.	*Suillus luteus*	*Suillus bovinus*	*Thelephora terrestris*	*Tomentella albomarginata*	*Tomentella submolis*	*Tomentella* sp.	*Tricholoma* sp.
Garwolin	inoculated	11	6		3	8	7		5	1		56		3	2		5	4
	non-inoculated	9	2			19	10		7			24		12	12		6	8
Płońsk	inoculated	13	5	4	11	6	8		7	8	5	15		10	6		8	7
	non-inoculated	12	4	2		10	12		7		6	7		15	9		11	5
Jabłonna	inoculated	12	8	3		11	15		5			8	4	14	10	6	7	9
	non-inoculated	14	4	5		12	6	8	6	2		6	2	23	9	3	10	5

Soil Analysis

Soil samples (cores taken to a depth of 20 cm at 5 different locations) were collected in April 2009. Soil pH_{KCl} and the quantities of the basic nutrients were determined for a one mixed sample (Table 1). The amount of N was analyzed according to the Kjeldahl method, while contents of the other nutrients were identified using the ICP method (inductively coupled argon plasma spectrometer) following mineralization in a mixture of nitric and perchloric acids.

Statistics

Data were analyzed using the statistical package Statistica 7.1. (StatSoft). Differences among means were evaluated with the t-test (P=0.05).

Results

Soil parameters- pH, P, Fe, Ca, Mg, K, C, and N, and C/N ratio differed between locations (Table 1). The pH (in H_2O and KCL) was lowest in the Garwolin site. Content of P, Fe, Ca, Mg, K, C, and N was highest in the Jabłonna site, whereas the lowest values of given nutrients occurred in Garwolin. The C/N ratio was highest Płońsk and lowest in Jabłonna.

All investigated seedlings possessed well developed ectomycorrhizae. Differences in their number between treatments were not statistically significant (Fig. 1). At the Garwolin site the ectomycorrhizae number was smaller in the case of inoculated seedlings. Opposite data were noted with respect to Płonsk and Jabłonna.

Morphological observation revealed a total of 17 fungal taxa (Table 2). A few of them have been confirmed by sequencing of DNA (Table 3). The mean relative abundance as well as species richness are shown in Table 2. Nine fungal taxa were observed in all sites and treatments. Of these, five were assigned to genus level (*Amanita* sp., *Dermocybe* sp., *Inocybe* sp., *Tomentella* sp., and *Tricholoma* sp.), and four to species level (*Dermocybe palustris*, *Suillus luteus*, *Thelephora terrestris*, and *Tomentella albomarginata*). In the case of inoculated seedlings, irrespective of the site, the frequent taxa were *S. luteus*, *D. palustris*, *D*. sp., *T. terrestris*, and *T. albomarginata*. In the case of non-inoclulated ones the most frequent taxa were *Dermocybe* sp., *T. terrestris*, and *T. albomarginata*. Greater species richness was observed for inoculated seedlings at Garwolin and Płońsk than for the non-inoculated ones. At Jabłonna higher species richness characterized non-inoculated seedlings (Table 2). Comparison of the mean relative abundance of ectomycorrhizal fungi from all sites for inoculated and non-inoculated seedlings is presented in Fig. 2.

Seedlings from both treatments were colonized by 15 ectomycorrhizal fungi. The highest mean relative abundance of *S. luteus* was observed for inoculated seedlings. *D. palustris* and *T. terrestis* ectomycorrhizae were also abundant in roots of these seedlings. The mean relative abundance of the last fungus was highest for non-inoculated seedlings. In roots of non-inoculated seedlings ectomycorrhizae of *Coritnarius* sp. were not found, nor were those of *Hygrophorus* sp. for inoculated ones.

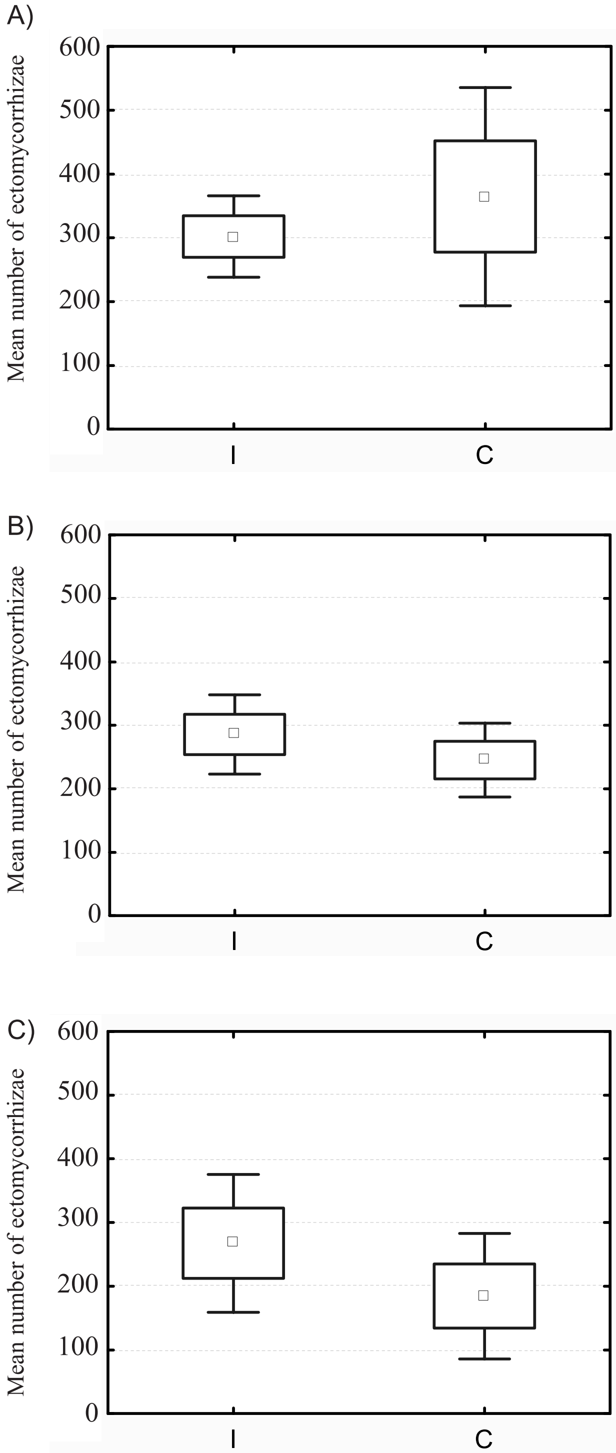

Fig. 1. Number of ectomycorrhizae of Scots pine at three sites: A) Garwolin, B) Płońsk, and C) Jabłonna. I – represents inoculated and C – non-inoculated seedlings, n=10, p=0.05, t-test.

Table 3. Molecular identification of the fungal symbiont in root tips of *Pinus sylvestris* L.

Fungus	Length of sequenced ITS (bp)	Accession No. of sequence in Gene Bank – NCBI or the sequence
Suillus luteus	587	EU379680
Suillus bovinus	695	EU379677
Thelephora terrestris	603	ATTTGAGATCGAACGTTAAAAAAGCTGTCCT CGCTGAGGAGAGACATCTGTGAGCTCCAGCA AACCTTTGTGACCAAAGGTTACCTGGCAGAC AACAGCGAGCGTAGATATTTATCACACCCGT GATGCCACCAAACACTGGGAGGCTGATTAAT TTGAGAGGAGCCGACCACAGGCCAGCAAAA CCCCCAGAGTCCAACTCATCATGGCAAACCA TGAGAGTTGAGGTGTTCATGATACTCAAACA GGCATGCCCCTCGGAATAGCCAAGGGGCGCA AGGTGCGTTCAAAGATTCGATGATTCACTGAA TTCTGCAATTCACATTACTTATCGCATTTCGCT GCGTTCTTCATCGATGCGAGAGCCAAGAGATC CGTTGCTGAAAGTTGTATTGTATTGCGTTAGA CGCGATGTACATTCCATAAAACTTTATTACAGT GTGTGTGTAAAGACGTAGAACCACAGAAGGAA GACAGGGTCCCCCAGACCATAGAACTACAGAG GGTGCACAGGTGTGAGTGGATGTGTAAACAGAG CGTGCACATGCCCCCTATGAGGGCCAGCAACA ACCCGTTTGACAATTCAGTAATGATCCTTCCGCA
		(identity of the sequence to fruit-bodies of *Thelephora terrestris* (FJ532478) is 100%)

ITS – internal transcribed spacer

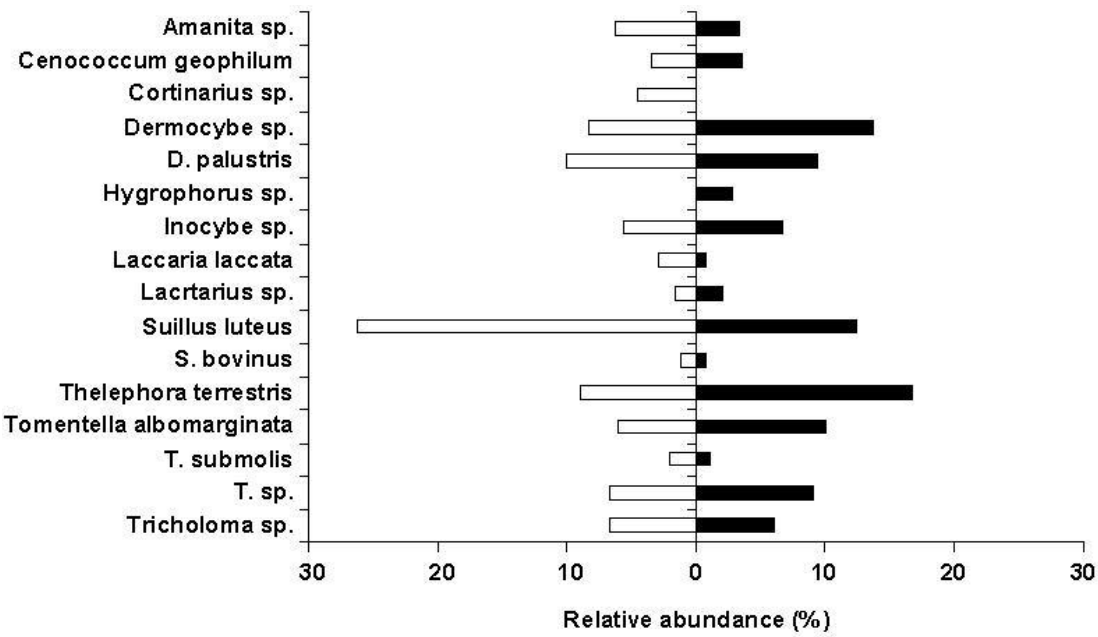

Fig. 2. Mean relative abundance of mycorrhizal taxa associated with inoculated (white columns) and non-inoculated (black columns) saplings in three sites on post-agricultural lands.

Discussion

The results of this study indicate that the success of mycorrhizal inoculation in the field largely depends on the fungal species selected and ecological conditions of the soil. Persistence of *Suillus luteus* ectomycorrhizae in roots of inoculated seedlings seems that the sites (Garwolin and Płońsk) were suitable for the fungus. Naturally, ectomycorrhizae of *S. luteus* were also observed in roots of non-inoculated seedlings, but their abundance was much greater for inoculated ones. The abundant occurrence of this fungus might be attributed to the evenness of spore distribution in a variety of habitats, spores' longevity [27], and spore resistance to abiotic factors. The ability of *Suillus* species to colonize seedlings in inoculation experiments is well known [28].

Opposite relation was observed in the case of inoculation with *Thelephora terrestris*. After six growing seasons non-inoculated seedlings had higher abundance of the fungus mycorrhizae than inoculated ones. *T. terrestris* and tomentetelloid fungi (*Tomentella* spp.) are known for their ubiquitous nature and appear to be pioneers or ruderal fungi with low host specificity, a short vegetation phase, and high dispersal rate [29]. Their abundance after outplanting seedlings usually decreases with time due to low competitiveness. However, in the case of former farmlands, where a soil's parameters are completely different than for forest soil, *T. terrestris* showed a positive role in colonization of roots [11]. *T. terrestris* was the most commonly observed ectomycorrhizal fungus in Scots pine roots two and four season after seedling outplanting. This agrees with the ability of *Thelephora* spp. to dominate under conditions of poor aeration, water-logging, and high fertilization rates [30].

Cenococcum geophilum mycorrhizae, which are very common for coniferous species, have been found on Scots pine in two sites, with low abundance not higher than 5%. This observation may indicate that soil moisture was at a proper level since *C. geophilum* often dominates roots during droughts [31].

The diversity (defined as species richness) of the ECM fungi in this study was low compared to ectomycorrhizal communities of mixed temperate forest [32]. A higher diversity of mycorrhizal fungi was observed for seedlings growing under the canopy of mature trees or in close proximity to a mature forest [4]. Limited diversity may also be expected in agricultural soils that differ from typically forest soils. Usually agricultural lands are characterized by a C-to-N ratio below or close to ten. Such a value is known to be an indication that the soil has been N-fertilized [33]. Low C-to-N ratio is likely to affect nutrient cycling because the microbial community may become carbon limited [34] and consequently limit development of mycorrhizae [35]. However, Jonsson et al. [36] found ectomycorrhizal species composition of self-regenerating Scots pine seedlings (1-10-year-old) in a forest comprised of 16-20 fungal taxa. In roots of two-year-old Scots pine seedlings growing on former farmland in Lithuania, a total of nineteen distinct mycorrhizal morphotypes was observed [9]. In this context the number of 17 taxa for seedlings investigated in the study seems to be a good result. The relatively high diversity might result from locations of mature forest in the vicinity of postagricultural lands. Taking into account the high abundance of mycorrhizae that were created by fungi belonging to the Thelephoraceae family, it seems that fungal species colonizing roots by spores are more effective than those species that disperse through growth of extrametrical mycelium.

Since inoculated fungi are in a short time replaced by indigenous fungi, a new question arises regarding the large input of costs and energy to select suitable fungi for ectomycorrhizal inoculation. The present work demonstrates that the effect of inoculation might be temporary, because it is hard to manipulate the mycorrhizal community under field conditions. Yet, the ectomycorrhizal inoculation was of great importance to seedling survival and growth [11].

Conclusions

- After the sixth and eight growing seasons, the number of mycorrhizae of inoculated seedlings did not differ significantly between the controls.
- Ectomycorrhizal species richness among investigated plantations was quite variable and in non-inoculated treatments ranged from 9 to 14, whereas for inoculated ones differences of ECM species richness ranged from 11 to 13.
- *Suillus luteus* ectomycorrhizae dominated roots of inoculated seedlings.
- In the case of non-inoculated seedlings ectomycorrhizae of *Thelephora terrestris* and *Dermocybe* sp. were most abundant.
- The results indicate that success of mycorrhization and persistence of inoculated fungi depends to a large extent on environmental factors.

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