Ann. For. Sci. 63 (2006) 715–723
© INRA, EDP Sciences, 2006
DOI: 10.1051/forest:2006052

Original article

Mycorrhizal associations of nursery grown Scots pine (*Pinus sylvestris* L.) seedlings in Poland

Michał IWAŃSKI, Maria RUDAWSKA*, Tomasz LESKI

Institute of Dendrology, Polish Academy of Sciences, Kórnik, Poland

(Received 26 October 2005; accepted 27 January 2006)

Abstract – We investigated the species richness and composition of mycorrhizal fungi naturally colonizing one- and two-year-old *Pinus sylvestris* L. seedlings from four bare-root nurseries in Poland. We hypothesized that both edaphic factors and seedling age would affect ectomycorrhizal (ECM) community structure. We assessed the relative abundance of mycorrhizal morphotypes and distinguished ECM fungi present on root tips using RFLP (restriction fragment length polymorphism) of the ITS (internal transcribed spacer) of fungal rDNA. Most of the RFLP types were identified by comparing RFLP patterns with reference data obtained from regional sporocarps and isolates of ectendomycorrhizal species. Samples of unidentified RFLP types and representatives of suilloid RFLP patterns were sequenced and compared with available sequences. Overall, we identified 13 fungal species, with species richness varying from two to eight species among nurseries. The dominant species in each examined nursery were *Wilcoxina mikolae* and *Thelephora terrestris*. Suilloid fungi were also abundant in most of the nurseries. *S. variegatus* was the most frequent suilloid species among the nurseries. Seedling age affected the relative abundance of ECM species to some extent. Principal component analysis (PCA) revealed a lack of apparent correlations between the relative abundances of species and edaphic factors. Factors that may contribute to the maintenance of ECM community structure are discussed.

forest nursery / mycorrhiza / ITS-RFLP / Scots pine

Résumé – Associations mycorhiziennes des semis de pin sylvestre (*Pinus sylvestris* L.) en pépinière en Pologne. Nous avons étudié la richesse spécifique et la composition des champignons mycorhiziens colonisant naturellement des semis de *Pinus sylvestris* L. âgés de un et deux ans dans quatre pépinières de production de plants à racines nues en Pologne. Nous avons fait l'hypothèse que ensemble les facteurs édaphiques et l'âge des semis affecteraient la structure de la communauté ectomycorhizienne (ECM). Nous avons déterminé l'abondance relative des morphotypes mycorhiziens et distingué les champignons ECM présents dans les pointes racinaires en utilisant RFLP de ITS de l'ADNr fongique. La plupart des types RFLP ont été identifiés en comparant les modèles RFLP avec des données de référence obtenues de sporocarpes régionaux et isolés d'espèces ectoendomycorhiziennes. Des échantillons de types RFLP non identifiés et de modèles représentatifs de suilloides RFLP ont été séquencés et comparés avec les séquences disponibles. Globalement, nous avons identifié 13 espèces fongiques, avec une richesse spécifique variant de deux à huit espèces parmi les pépinières. Dans chaque pépinière étudiée, les espèces dominantes étaient *Wilcoxina mikolae* et *Thelephora terrestris*. Les champignons suilloides étaient aussi abondants dans la plupart des pépinières. *S. variegatus* était l'espèce suilloide la plus fréquente parmi les pépinières. L'âge des semis affecte dans une certaine mesure l'abondance relative des espèces ECM. Une analyse en composantes principales (PCA) révèle un manque apparent de corrélations entre l'abondance relative des espèces et les facteurs édaphiques. Les facteurs qui peuvent contribuer au maintien de la structure de la communauté des ECM sont discutés.

pépinière forestière / mycorhize / ITS-RFLP / pin sylvestre

1. INTRODUCTION

Most forest trees in coniferous boreal and temperate forests live in symbiosis with ectomycorrhizal fungi (ECM). Ectomycorrhizal associations are important in nutrient cycling, particularly in the acquisition and transfer of nutrients from heterogeneous resources [47]. Protection from soil-borne pathogens, especially in the early stages of tree development, is also considered as an important function of mycorrhizal symbiosis [12].

In contrast to host plant communities, communities of ECM fungi are characterized by high biodiversity, associated with a broad range of capabilities for uptake of organic and inorganic forms of nitrogen and the carbohydrate demands on the host [1, 4]. Consequently, the physiological diversity of ECM fungi enables niche differentiation on the basis of host age and specificity [3], soil conditions, stress factors [5, 13, 44], and propagation strategies [15, 50].

Forest nurseries in Poland produce over 1.3 billion tree seedlings per year. Scots pine seedlings constitute the majority of production and serve as a main source for reforestation of over 56 000 ha per year. Mycorrhizal fungi, naturally colonizing seedlings in nurseries, are essential for the establishment and survival of young trees for at least several years after out planting [7,9,24,49]. In afforestation of post-agricultural lands (approximately 20 000 ha per year in Poland), where no ECM inoculum is present, the importance of nursery-adapted ECM fungi is even more pronounced.

* Corresponding author: mariarud@man.poznan.pl

Table I. Description of the nurseries (P – Przymuszewo, S – Solec Kujawski, Z – Zlotow, O – Okonek).

Stand characteristics	Nursery			
	P	S	Z	O
Nursery location	53° 16' N; 17° 34' E	53° 04' N; 18°14' E	53° 21' N; 17° 03' E	53° 31' N; 16° 51' E
Mean annual temperature (°C)	7.4	7.4	8.8	7.5
Mean annual precipitation (mm)	596	500	580	608
Vegetation period (d)	210	212	216	211
Nursery age (yr)	29	30	26	11
Fertilization (kg/ha/yr)	N-150 (1-yr)*	N-120 (1-yr)	N-55 (1-yr)	N-200 (1-yr)
	N-150 (2-yr)*	N-100 (2-yr)	N-45 (2-yr)	N-100 (2-yr)
Irrigation	No	Yes	Yes	Yes
Fungicide aplication	$1 \cdot yr^{-1}$	$2 \cdot yr^{-1}$	$2 \cdot yr^{-1}$	$1 \cdot yr^{-1}$
Crop rotation	Yes	Yes	Yes	No

* First and second vegetation period respectively.

Most of the studies of ECM community structure have examined mature forests [10, 11, 14, 17, 41], and disturbed habitats [23, 29, 35, 42, 48], whereas relatively little is known about the species composition of ECM fungi in nurseries [32, 38, 46, 51].

According to Read [43], ECM community structure reflects adaptation to the level of carbohydrate supply from host and nutrient availability in the soil. Owing to fertilization, fumigation, and other techniques, nursery conditions differ significantly from those observed in natural ecosystems.

The aim of this study was to evaluate the diversity of ECM fungi in forest tree nurseries. We hypothesized that both edaphic factors and seedling age would affect ECM community structure. To test this hypothesis, we assessed the relative abundance of mycorrhizal morphotypes on one- and two-year old Scots pine seedlings from four bare-root nurseries in Poland, and identified ECM fungi present on root tips using molecular methods. We used principal component analysis to estimate the association of species composition with soil nutrient content and acidity.

2. MATERIALS AND METHODS

2.1. Site descriptions, plant material, and sampling procedure

Scots pine seedlings were collected from four bare-root nurseries: Przymuszewo (P), Solec Kujawski (S), Zlotow (Z), and Okonek (O) in northwestern Poland in October and November of 2003. Standard plant production procedures were applied during preparation of plant material. Scots pine seeds were treated with fungicides (Funaben T or Oxafun T) at the rate of 4 $g \cdot kg^{-1}$. Sowing was conducted with the single-seed seeder in rows 0.005 m apart, with spacing in a row 0.037 m in April 2001 and 2002 (two-years-old and one-year-old seedlings respectively). Soils in which seedlings were grown in all studied nurseries are fine sandy loam with poorly developed horizons and low organic matter content. Management practices, such as fertilization and fungicide applications, differed among nurseries due to differences in site conditions, site history, and response to soil properties and pathogen infections (Tab. I). Prior to the study, one-year-old (1-yr) and two-year-old (2-yr) seedlings were grown in standard nursery compartments (approximately 1 ha), for 17–18 months and 31–32 months, respectively. From each nursery five randomly selected samples composed of 3–5 seedlings from each age-class compartment were carefully excavated along with surrounding soil, packed in plastic bags, transported to the laboratory, and stored at 4 °C until further processing (no longer than 6 weeks). Sampling was avoided within 5 m of a compartment edge to avoid edge effects. A total of 40 samples (120 seedlings) from eight compartments (four nurseries × two age classes) were analyzed. In addition, three soil cores from each examined compartment were thoroughly mixed, sieved, dried and sent to the Department of Forest Soil Science of Agricultural University of Poznań Soil for chemical analyses. Total N and C contents were measured using Elemental Combustion System CHNS-O (Constech Analytical Technologies Inc., Valencia, USA). Available P_2O_5 was extracted with an acidic ammonium fluoride and the content was determined by spectrophotometry.

2.2. Mycorrhizal evaluation

Root systems of three seedlings from each sample were rinsed on a sieve under cold water in order to remove soil particles. Lateral roots were excised from the main root, cut into 5-cm fragments, and mixed in a container filled with distilled water. Twenty to 25 randomly selected fragments were examined per sample. Observations of root samples were conducted under a dissecting microscope at 10× to 60× magnification. All fine roots were examined for mycorrhizal colonization. Ectomycorrhizas were separated into morphotypes based on ramification system, color and appearance of the mantle, presence of extramatrical mycelium, rhizomorphs, and other structures (cystidia, sclerotia). Preliminary identification of morphotypes was made by comparisons with available descriptions [2, 22, 52]. One out of 50 fine roots belonging to a morphotype presumed to be ectendomycorrhizal (EECM) was cross-sectioned, stained with 0.05% tryptan blue, and examined under a microscope (400×) to verify intracellular colonization. The numbers of live mycorrhizas of each morphotype and dead fine roots were recorded. The relative abundance of each morphotype (number of root tips of each morphotype/total number of fine roots) was calculated separately for each sample. Representative subsamples (30-45 mycorrhizal systems) of each morphotype from each

Table II. Soil characteristics for each sampled compartment from the examined forest tree nurseries in Poland (P – Przymuszewo, S – Solec Kujawski, Z – Zlotow, O – Okonek), values are means ± SD (n = 3).

Soil parameter	Nursery/age class							
	P 1-yr	P 2-yr	S 1-yr	S 2-yr	Z 1-yr	Z 2-yr	O 1-yr	O 2-yr
Soil pH	5.55 b* ±0.11	5.7 b ±0.14	6.4 cd ±0.32	5.85 bc ±0.27	4.39 a ±0.21	4.31 a ±0.16	6.3 d ±0.17	6.5 d ±0.14
%C	5.08 c ±0.28	5.08 c ±0.22	7.67 d ±0.31	5.04 c ±0.24	2.65 b ±0.19	1.99 a ±0.25	1.74 a ±0.27	1.81 a ±0.22
%N	0.27 d ±0.01	0.27 d ±0.02	0.46 f ±0.03	0.32 e ±0.03	0.20 c ±0.01	0.15 b ±0.02	0.11 a ±0.01	0.10 a ±0.02
C/N ratio	18.9 d ±0.5	19 d ±0.3	16.9 c ±0.4	15.6 b ±0.4	13.1 a ±0.4	12.9 a ±0.5	16.1 bc ±0.4	17.1 c ±0.2
$\%P_2O_5$	9.9 b ±0.35	7.8 a ±0.36	18.3 d ±0.38	7.3 a ±0.32	12.3 c ±0.39	11.5 c ±0.4	11.7c ±0.34	12.3 c ±0.36

* Letters indicate significant differences between compartments at $p < 0.05$ (Tukey's test).

compartment were photographed and stored at –20 °C for subsequent molecular identification.

2.3. Identification of mycorrhizal species present on fine roots

Ectomycorrhizal fungi present on fine roots were identified using RFLP (restriction fragment length polymorphism) of the ITS (internal transcribed spacer) of fungal rDNA and amplified with PCR (polymerase chain reaction). A standard pair of primers ITS1/ITS4, described by White et al. [53], was used to amplify the ITS region. The fungal DNA extraction method followed those of White et al. [53] and Gardes et al. [18] with minor modifications. Each sample consisted of a single mycorrhizal tip. The PCR protocol followed that of Kåren et al. [30]. To optimize PCR amplification 1:10, 1:20, and 1:40 dilutions of extracted templates were tested before the sample was presumed to be not amplified. Controls with ultra pure water were run to check for contamination. The amplified product was digested with three enzymes: *Hinf* I, *Mbo* I, and *Taq* I (Eurx). Restriction fragments were separated using 2% agarose gel electrophoresis (10V/cm), stained with 0.5% ethidium bromide, and recorded on black and white Polaroid™ film. Morphotypes were identified by comparing RFLP patterns of the ITS with reference RFLP patterns obtained from a regional collection of sporocarps and isolates of EECM fungi using the Taxotron® software package (Pasteur Institute, Paris, France). Samples of unidentified RFLP types and representatives of suilloid RFLP patterns were sequenced with ITS1/4 primers in the Laboratory of Molecular Biology of Adam Mickiewicz University and compared with available sequences from GenBank using Blastn.

2.4. Data analysis

The diversity of the ectomycorrhizas on the seedlings was expressed as the number of identified ECM species (species richness). The relative abundance of individual ECM fungal species was calculated as a proportion of the total number of ectomycorrhizal roots averaged over each sample. One way analysis of variance with Tukey's test was used to compare the soil parameters, degree of mycorrhizal colonization, the extent of ECM and EECM colonization and relative abundances of mycorrhizal species between nurseries and age classes. Principle Components Analysis (PCA) was used to analyze the relationship between the mycorrhizal relative abundance of ECM fungal species and tested variables (soil parameters and age of seedlings). Relative abundance values were transformed ($\log_{10}+1$) before analysis, and the PCA was performed with CANOCO 4.51 software.

3. RESULTS

3.1. Soil factors

Soil properties of all examined nurseries and age classes are summarized in Table II. The measured soil factors (pH, C, N and P contents, C/N ratio,) exhibited a high degree of variation among the examined nurseries and age classes. The pH (H_2O) was highest in Okonek (O 1-yr, O 2-yr) and Solec Kujawski nurseries (S 1-yr), intermediate in Solec Kujawski (S 2-yr) and Przymuszewo (P 1-yr and P 2-yr) nurseries, and lowest in the Zlotow nursery (Z 1-yr, Z 2-yr) (p = 0.05). C, N and P contents were highest in S 1-yr reaching 7.67%, 0.46% and 18.3% respectively, whereas lowest values of C and N contents occurred in O nursery on both age classes (1.74% C, 0.10% N) and lowest P content in S 2-yr (7.3%).

3.2. Mycorrhizal root colonization of ectomycorrhizal (ECM) and ectendomycorrhizal (EECM) fungi

Overall values of mycorrhizal colonization were high in each nursery and age class, with little variation among seedlings of the different nurseries and age classes (Fig. 1a). Colonization was significantly lower than all other nurseries and age classes (p = 0.05) only in the Okonek nursery on two-year-old seedlings (O 2-yr). Mean colonization of seedlings was 78.6%, ranging from 67.8% in the O 2-yr compartment to 90.6% in the P 2-yr compartment.

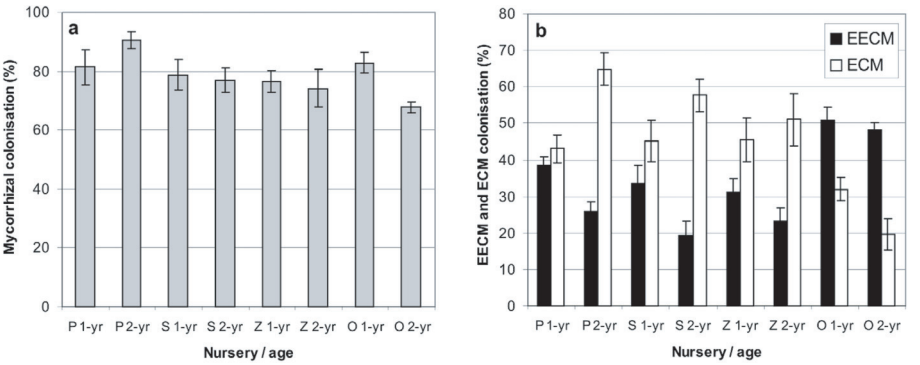

Figure 1. Mean values and standard deviations of (a) overall mycorrhizal colonization and (b) relative abundance of ectendo-, and ectomycorrhizas of 1-, and 2-year-old Scots pine seedlings from four nurseries: P: Przymuszewo; S: Solec Kujawski; Z: Zlotow; O: Okonek.

EECM colonization was significantly greater than ECM colonization only in O nursery, in all other nurseries ECM colonization surpassed EECM colonization, nevertheless not significantly in P 1-yr (Fig. 1b). The relative abundance of ectomycorrhizas increased with seedling age from approximately 45% in one-year- old seedlings to 64%, 58%, and 51% in two-year-old seedlings in the P, S, and Z nurseries, respectively, however, the differences were statistically significant only for the P ($p = 0.0014$) and S ($p = 0.009$) nurseries. The relative abundance of ECM in the O nursery was considerably lower than in the remaining nurseries ($p < 0.03$) and decreased with seedling age from 32% to 20%. In contrast, the highest EECM colonization occurred in the O nursery (50% on both one- and two-year-old seedlings). Mean EECM colonization in the P, S, and Z nurseries was 34% in one-year-old seedlings and 23% in two-year-old seedlings with the lowest value in the S 2-yr compartment (19%).

3.3. Morphotype assessment

Morphological assessment was carried out on approximately 8000 mycorrhizas from 120 seedlings. Based on gross morphology, eight mycorrhizal morphotypes were distinguished (listed and described in Tab. III). We found seven morphotypes in the Z nursery, six in P, four morphotypes in S, and only two in the O nursery.

The most common morphotypes, EECM and Tter, were present in each examined nursery and seedling age class. Despite a decreased abundance with increased seedling age, they dominated and comprised on average 38% and 25% of the root tips of the one-year-old seedlings, whereas on two-year-old seedlings they occurred on 27% and 13% of the roots (pooled data from four nurseries). Two morphotypes belonging to the suilloid group (suilloid white – SW, and suilloid pink – SP) were present on seedlings from the P, S, and Z nurseries. Their relative abundance was significantly higher on two-year than one-year-old seedlings. The suilloid brown morphotype (SB) was found on root systems of seedlings from the P nursery. The *Hebeloma*-type morphotype (Heb) was present in the P and Z nurseries, but its relative abundance was much greater in the Z nursery on both one- and two-year-old seedlings (3% P 1-yr; 7% P 2-yr; 20% Z 1-yr; 22% Z 2-yr). Tub and Black morphotypes were detected only in the Z nursery on two-years-old seedlings at low relative abundances at 0.5% for Tub and 6% for Black.

3.4. Molecular identification

In total, 228 out of 352 mycorrhizal tips representing all morphotypes, yielded PCR product and was subjected to ITS-RFLP analysis. Overall successful amplification rate reached 65%. Root tips from the suilloid group (morphotypes SW, SP, and SB) were the most difficult to amplify (54%)

Most of the morphotypes in this study consisted of more than one RFLP type. The RFLP types identified as *Suillus* species were detected in more than one morphotype (Tab. IV). Since the suilloid morphotypes turned out to be species mixtures of three *Suillus* and two *Rhizopogon* species, we decided to treat this group in quantitative estimations as a combined suilloid group. We are cautious of drawing conclusions concerning the relative abundances of suilloid species in the P and S nurseries based on the frequency of suilloid RLFP patterns obtained from mycorrhizas, because the successful amplification rate was quite variable between the suilloid morphotypes and samples, indicating a possible bias in the estimations (Tab. IV).

We obtained 13 distinct RFLP patterns, nine of which were matched with our reference data bank containing 94 fungal taxa. The RFLP type *P. fortinii*-like was very similar to the RFLP pattern of the *Phialocephala fortinii* isolate, but the 120 bp band in the *Taq*I digestion was missing. This might be explained either as an intraspecific polymorphism of the ITS sequence or a failure to detect a faint band on the gel. The remaining RFLP types were identified as *Wilcoxina mikolae, Rhizopogon roseolus,* and *Tuber* sp. by comparing the ITS sequences with GenBank accessions. The ITS sequence analysis of 12 suilloid mycorrhizas confirmed the adequacy of the RFLP typing (97–99% homology with reference sequences from GenBank).

Table III. Description of mycorrhizal morphotypes.

Morphotype	Description	Site[a]
EECM	Ectendomycorrhizal, light to dark brown, with pale tip, single or dichotomously branched, fairly long, thin, reticulate, and shiny mantle, extramatrical hyphae (EMH) and strands absent, *Wilcoxina mikolae*-type	All
Black	Single mycorrhizas, black, shiny mantle, often as a compact patch, loose black hyphae extending towards the pale tip, in Ingleby et al. [22] described as ITE.3, *Phialocephala fortinii* in [28]	Z 2-yr
Tub	Orange, single, club-shaped mycorrhizas with a smooth mantle texture and small cystydia emanating from mantle, EMH and rhizomorphs absent, *Tuber*-type [52]	Z 2-yr
Tter	Yellow-brown to dark brown, single, dichotomously or irregularly branched, smooth, thick mantle, cystydia infrequently found, EMH sparse or absent; *Thelephora terrestris*-type	All
SW	Suilloid – white, single to coralloid, white, thick, gelatinous mantle, abundant fluffy EMH, colored as the mantle, differentiated and interconnected strands, also white, soil particles closely associated with mycorrhizas	All except O 1-yr, O 2-yr
SP	Suilloid – pink, dichotomous or cluster-forming mycorrhizas, thick, pink mantle, EMH and differentiated strands present, concolorous with mantle	All except O 1-yr,O 2-yr
SB	Suilloid – brown, dichotomously branched, light brown mycorrhizal systems, at the base covered by white, abundant EMH, strands brown	P 1-yr, P 2-yr
Heb	Long, dichotomous mycorrhizas, white to light brown, patchy mantle, covered by cottony EMH, strands absent, *Hebeloma* – type	P 1-yr, P 2-yr Z 1yr, Z 2-yr

[a] Refers to nurseries and seedling age classes were the morphotype was observed, P – Przymuszewo, Z- Zlotow, O – Okonek nursery.

3.5. Mycorrhizal community composition

The highest diversity of mycorrhizal fungi was found in nursery P (eight species) and Z (seven species), intermediate levels in nursery S (five species) and the lowest diversity in nursery O (two species) (Tab. V). No differences in species richness between the two age classes were detected in the P, S, and O nurseries, whereas in the Z nursery, three additional species (*Tricharina ochroleuca, Phialocephala fortinii,* and *Tuber* sp.) were identified on two-year- old compared to one-year-old seedlings. Since these species were present at low abundances in the community (6% in Z 2-yr), we conclude that at least in nursery conditions, seedling age has a negligible influence on qualitative community structure.

The most common and abundant mycorrhizal symbionts were *Wilcoxina mikolae* and *Thelephora terrestris,* which were both present in each examined nursery and seedling age class. The relative abundances of these species generally decreased with seedling age from approximately 63% on one-year old seedlings to 40% in rootlets of two-year old seedlings (data pooled for both species and all nurseries; $r^2 = 0.35$, $p < 0.0001$, for details see Tab. V).

The group of suilloid fungi had the richest representation in the P nursery where three *Suillus* and two *Rhizopogon* species were present in both seedling age classes. Among the nurseries, *S. variegatus* was the most frequent and was detected on seedlings from the P, S, and Z nurseries. *R. luteolus* and *R. roseolus* occurred only in the P and S nurseries, whereas *S. bovinus* and *S. luteus* were present only in the P nursery. The relative abundance of the suilloid group increases with seedling age from 12% to 40% in nursery P, from 18% to 41% in S, and from 14% to 26% in Z. Tukey's test indicates that seedling age class had a significant effect on the abundance of the suilloid group in these nurseries ($p < 0.001$ for P and S; $p = 0.0117$ for Z).

Likewise for *H. crustuliniforme* in nursery P, we found a small, but significant ($p = 0.009$) increase in colonization with age class (3% in P 1-yr; 7% in P 2-yr), whereas *H. longicaudum* in nursery Z had similar colonization percentages in both age classes (20% in Z 1-yr; 22% in Z 2-yr).

Principal component analysis (PCA) based on pooled data from all examined nurseries revealed a lack of apparent correlations between the relative abundances of species (or species group in case of the suilloid taxa) and edaphic factors (Fig. 2) with the exception of a weak correlation between *T. terrestris* abundance and soil pH ($r^2 = 0.519$; $p = 0.0436$).

4. DISCUSSION

Molecular approaches (RFLP and sequencing of rDNA ITS) combined with classical methods, such as morphotyping, enabled reliable assessment of fungal species richness and allowed us to track morphological variability

Table IV. Molecular identification with morphotype assignment of mycorrhizas, total number of mycorrhizas identified by ITS-RFLP, and total number of RFLP-taxa identified for each nursery and age class.

Species / RFLP type	Morphotype	No. of root tips with specific RFLP pattern							
		Przymuszewo		Solec Kujawski		Zlotow		Okonek	
		P-1yr	P-2yr	S-1yr	S-2yr	Z-1yr	Z-2yr	O-1yr	O-2yr
Wilcoxina mikolae	EECM[b]	9	13	6	16	4	4		
Wilcoxina mikolae RFLP type II*	EECM							6	5
Thelephora terrestris	Tter	5	8	4	9	3	4	3	4
Suillus variegatus[a]	SW[b], SP[b]	4	10	14	13	5	7		
Suillus bovinus[a]	SW, SP	4	7						
Suillus luteus[a]	SW, SP, SB	5	12						
*Rhizopogon luteolus**	SP	3	6	3	5				
Rhizopogon roseolus	SW	1	2	2	2				
Hebeloma crustuliniforme	Heb[b]	2	3						
Hebeloma longicaudum	Heb					4	6		
Tricharina ochroleuca	Black[b]						2		
Phialocephala fortinii-like	Black						1		
Tuber sp.*	Tub						1		
No. of mycorrhizal tips used in ITS-RFLP		48	96	48	72	24	32	12	12
Unamplified		15	36	19	27	8	7	3	3
Total no. of RFLP types		8	8	5	5	4	7	2	2

[a] RFLP type found in more than one morphotype, [b] morphotype with more than one RFLP pattern, * RFLP type identified by ITS sequence analysis of ITS.

Table V. Relative abundance of mycorrhizal species on roots of Scots pine seedlings from forest tree nurseries, counted as a percent of the total number of fine roots, standard deviations in parentheses (n = 5).

Nursery/ age class	*Wilcoxina mikolae*	*Thelephora terrestris*	Suilloid group*	*Hebeloma crustuliniforme*	*Hebeloma longicaudum*	*Tricharina ochroleuca*	*Tuber* sp.	*Phialocephala fortinii*-like	Species richness
P 1-yr	38.4 (± 2.4)	27.8 (± 2.7)	12.4 (± 2.4); 5[a]	2.8 (± 1.9)	–	–	–	–	8
P 2-yr	25.8 (± 2.2)	9.0 (± 1.6)	49.0 (± 4.9); 5	6.8 (± 4.1)	–	–	–	–	8
S 1-yr	33.6 (± 3.9)	26.8 (± 3.5)	18.4 (± 4.2); 3	–	–	–	–	–	5
S 2-yr	19.2 (± 3.3)	16.6 (± 2.4)	41.0 (± 4.9); 3	–	–	–	–	–	5
Z 1-yr	31.2 (± 3.5)	12.0 (± 3)	13.6 (± 2.0); 1	–	19.8 (± 2.7)	–	–	–	4
Z 2-yr	16.8 (± 2.7)	7.0 (± 2.1)	21.6 (± 3.2); 1	–	22.4 (± 3.2)	4	0.5	2	7
O 1-yr	50.8 (± 3.7)	32.0 (± 3.2)	–	–	–	–		–	2
O 2-yr	48.2 (± 4.4)	19.6 (± 4.2)	–	–	–	–		–	2

* *Suillus* and *Rhizopogon* spp. treated as a combined group, [a] indicates number of suilloid species.

amongst mycorrhizas formed by individual fungal species. The number of RFLP types was greater than the number of morphotypes recorded (13 vs. 8). Three of the morphotypes corresponded well with specific RFLP patterns, whereas five other morphotypes consisted of more than one RFLP type. On the other hand, specific RFLP types could be found in mycorrhizas assigned to different morphotypes. All three *Suillus* species were detected in at least two morphotypes (Tab. IV). Although the SB morphotype showed a distinct RFLP type in this study, our ongoing study of young Scots pine plantations revealed that this suilloid morphotype might also consist of several suilloid species (unpublished). This type of intermingled correlations between morphotypes and RFLP types has been reported previously [13, 30].

Our investigation revealed high morphological variability of mycorrhizas formed by suilloid species. This fact, together with inconsistent amplification rates of their ITS, prevented us from an assessment of their exact abundances in nurseries. Since all three morphotypes classified as "suilloid" turned out to be *Suillus* or *Rhizopogon* species, we believe that despite apparent differences, classifying the suilloid mycorrhizas as a single morphotype would be a more appropriate approach in future research. The morphological variability of suilloid fungi might be attributed to phenotypic variation of individuals and differences in environmental conditions (Mleczko, pers. comm.). Conversely, a close evolutionary relationship between the *Suillus* and *Rhizopogon* genera [19] probably contribute to the resemblance of mycorrhizas formed by different suilloid species.

Two RFLP types were found within the one EECM morphotype, both identified as *W. mikolae*, either by RFLP typing (P, S, Z nurseries) or sequencing of ITS region (unknown

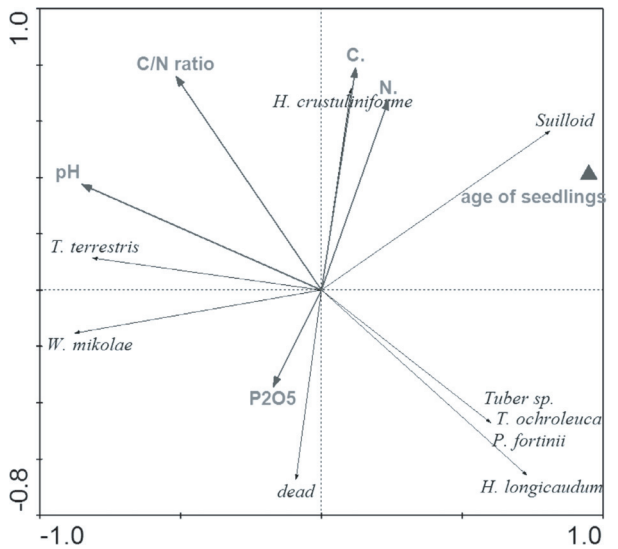

Figure 2. Principal component analysis (PCA) of ECM community structure in forest tree nurseries. Eigenvalues for the first and second axis are 0.53 and 0.338, respectively.

RFLP type 1 from the O nursery). Intraspecific polymorphisms of ITS sequence have been previously reported for many mycorrhizal fungi [20, 30], including *W. rehmii* [16] and unidentified morphotype described as an E-strain [36].

The species richness in the examined nurseries varied between two and eight fungal taxa on both seedling age classes (Tab. V). Similar results were obtained in other surveys of mycorrhizal communities in forest nurseries. On the basis of morphological and anatomical methods, Ursic et al. [51] characterized six mycorrhizal species present on two- and three-year-old *Pinus strobus* seedlings from a bare-root nursery. In a broad survey of the mycorrhizal status of Scots pine seedlings in Polish nurseries, Rudawska et al. [46] found between one and nine mycorrhizal types per nursery, whereas four to nine mycorrhizal taxa were identified on *Picea glauca* seedlings from four containerized seedling nurseries [32]. Similarly, Menkis et al. [38] found nine mycorrhizal species colonizing Scots pine seedlings in bare-root nurseries.

As shown previously, seedlings growing under the canopy of mature trees or in close proximity to a mature forest seem to maintain a higher diversity of mycorrhizal fungi. Jonsson et al. [25] demonstrate that the ectomycorrhizal species composition of self-regenerating Scots pine seedlings (1 to 10 year-old cohorts) in a forest is similar to that of surrounding trees and is comprised of 16–21 fungal taxa. Kranabetter and Wylie [34] found an ECM species richness gradient on naturally regenerated western hemlock seedlings in forest openings, ranging from 13.1 morphotypes under the canopy to 7.8 in 15 m gap positions. Similarly, a comparison of ECM communities of young *P. muricata* seedlings after a stand-replacing fire in shrub and forest sites showed marked differences in species richness (3 and 13 ECM species, respectively) [21].

The discrepancy between ECM species richness and composition of even-aged seedlings growing in nurseries (likewise in settings where no mature trees are present) and under the canopy of trees might be explained in terms of different life history strategies of ECM species. It seems that fungal species richness at the tree seedling stage is a function of the inoculum potential of different fungal species and their ability to colonize roots from spores [50]. ECM species which disperse effectively via resistant propagules would appear first in disturbed habitats and nurseries, whereas those species which colonize host roots mainly through growth of the extramatrical mycelium (EM) would be unable to persist, but would occur under a forest canopy. In mature forest stands, a carbohydrate supply from established trees might facilitate ECM colonization of young seedlings [25]. The roots of seedlings are incorporated into an established mycelial network connected to mycorrhizas of trees, and consequently fungi with higher carbohydrate demands (so-called "late-stage" fungi) may colonize seedlings.

Most of the ECM fungi identified on Scots pine seedlings in the examined nurseries are typical colonizers of nursery-grown conifers. *W. mikolae* and *T. terrestris* have been reported repeatedly on nursery seedlings [22, 32, 38, 51, 52] and are known for their ubiquitous nature. They appear to be pioneer or ruderal fungi with low host specificity, a short vegetation phase, and high dispersal rate [6]. Due to low competitiveness their abundance after outplanting typically decreases with time. Similarly, *Hebeloma* spp. are classified as pioneer species, occurring in young forest plantations with a low humus content and in disturbed habitats [37]. The abundant occurrence of suilloid fungi, especially *Rhizopogon* spp. might be attributed to the evenness of spore distribution in a variety of habitats, longevity of spores [33], and spore resistance to abiotic factors. Rincón et al. [45] showed that *Rhizopogon* spp. are efficient colonizers of *Pinus pinea* seedlings and significantly increase N and P concentrations of inoculated plants. Also performance and survival of the *Pinus pinea* seedlings outplanted in formerly arable land, is improved after inoculation with *Rhizopogon* spp., suggesting their considerable competitive abilities [40] The ability of *Suillus* species to colonize seedlings in laboratory conditions and in inoculation experiments is well documented [47]. *Suillus* species occur in nurseries [8, 31, 39] and on naturally regenerating seedlings [21, 25]. Three ascomycetes occurred in the Z nursery, namely *T. ochroleuca*, *Tuber* sp., and *P. fortinii*. *Phialocephala fortinii*, a dark-septate fungus, is a reported associate of pines at early successional stages [25, 27]. The mycorrhizal status of this species is unclear, although it forms inter- and intracellular exchange interfaces in roots, it can be mutualistic, neutral, or pathogenic under different conditions and with different host species [26]. As was demonstrated by Jumpponen et al. [27], *P. fortinii* significantly improved P uptake and growth of *Pinus contorta* seedlings and increased N uptake and total seedling biomass when N was added. In light of the fact that N amendments are a common nursery practice, it is likely that *P. fortinii* is beneficial for pine seedlings in nursery conditions.

Acknowledgements: We thank Dr Mark Tjoelker for critical reading of the manuscript and English correction and Dr Marek Kasprowicz for statistical advice. We also wish to thank two anonymous reviewers for helpful comments on this study.

REFERENCES

[1] Abuzindah R.A., Read D.J., The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. utilization of peptides and proteins by ectomycorrhizal fungi, New Phytol. 103 (1986) 481–493.

[2] Agarer R., Colour atlas of ectomycorrhizae, Einhorn-Verlag, Schwäbisch Gmünd, Germany, 1987-1996.

[3] Bruns T.D., Bidartondo M.I., Taylor D.L., Host specifity in ectomycorrhizal communities: what do the exceptions tell us? Integ. Comp. Biol. 42 (2002) 352-359.

[4] Buscot F., Munch J.C., Charcosset J.Y., Gardes M., Nehls U., Hampp R., Recent advances in exploring physiology and biodiversity of Ectomycorrhizas highlight the functioning of these symbioses in ecosystems, FEMS Microbiol. Rev. 24 (2000) 601-614.

[5] Cairney J.W.G., Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi, Mycorrhiza 9 (1999) 125-135.

[6] Colpaert J.V., *Thelephora,* in: Cairney J.W.G., Chambers S.M. (Eds.) Ectomycorrhizal fungi: key genera in profile, Springer-Verlag Berlin Heidelberg, 1999, pp. 325–246.

[7] Dahlberg A., Effect of soil humus cover on the establishment and development of mycorrhizae on containerized *Pinus sylvestris* L. and *Pinus contorta* ssp. *latifolia* Engelm. after outplanting, Scand. J. For. Res. 5 (1990) 103–112.

[8] Dahlberg A., Finlay R.D., *Suillus*, in: Cairney J.W.G., Chambers S.M. (Eds.), Ectomycorrhizal fungi: key genera in profile, Springer-Verlag Berlin Heidelberg, 1999, pp. 33–64.

[9] Dahlberg A., Stenström E., Dynamic changes in nursery and indigenous mycorrhiza of *Pinus sylvestris* seedlings planted out in forest and clearcuts, Plant Soil 136 (1991) 73–86.

[10] Dahlberg A., Jonsson L., Nylund J.-E., Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden, Can. J. Bot. 75 (1997) 1323–1335.

[11] Dickie I.A., Reich P.B., Ectomycorrhizal fungal communities at forest edges, J. Ecol. 93 (2005) 244–255.

[12] Duchesne L.C., Role of ectomycorrhizal fungi in biocontrol, in: Pfleger F.L. Linderman R.G. (Eds.), Mycorrhizae in plant health, APS Press, St. Paul, Minn., 1994, pp. 27–45.

[13] Erland S., Taylor A.F.S., Diversity of ecto-mycorrhizal fungal communities in relation to the abiotic factors, in: van der Heijden M.G.A., Sanders I. (Eds.), Mycorrhizal ecology, Springer-Verlag Berlin Heidelberg, 2002, pp. 163–195.

[14] Erland S., Jonsson T., Mahmood S., Finlay R.D., Below-ground ectomycorrhizal community structure in two *Picea abies* forest in southern Sweden, Scand. J. For. Res. 14 (1999) 209–217.

[15] Fleming L.V. Effects of soil trenching and coring on the formation of ectomycorrhizas on birch seedlings grown around mature trees, New Phytol. 98 (1983) pp. 143–153.

[16] Fujimara K.E., Smith J.E., Horton T.R., Weber N.S., Spatafora J.W., Pezizalean mycorrhizas and sporocarps in ponderosa pine (*Pinus ponderosa*) after prescribed fires in eastern Oregon, USA, Mycorrhiza 15 (2005) 79–86.

[17] Gardes M., Bruns T.D., Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views, Can. J. Bot. 74 (1996) 1572–1583.

[18] Gardes M., White T.J., Fortin J.A., Bruns T.D., Taylor J.W., Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA, Can. J. Bot. 69 (1991) 180–190.

[19] Grubisha L.C., Trappe J.M., Molina R., Spatafora J.W., Biology of ectomycorrhizal genus *Rhizopogon*. VI. Re-examination of infrageneric relationships inferred from phylogenetic analyses of ITS sequences, Mycologia 94 (2002) 607–619.

[20] Horton T.R., Bruns T.D., The molecular revolution in ectomycorrhizal ecology: peeking into the black-box, Mol. Ecol. 10 (2001) 1855–1871.

[21] Horton T.R., Cázares E., Bruns T.D., Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire, Mycorrhiza 8 (1998) 11–18.

[22] Ingleby K., Mason P.A., Last F.T., Fleming L.V., Identification of ectomycorrhizas, HMSO, London, 1990.

[23] Jansen A.E., Dighton J., Effects of air pollutants on ectomycorrhizas, Air Pollution Research Report 30, Brussels, 1990.

[24] Jones M.D., Durall D.M., Cairney J.W.G., Ectomycorrhizal fungal communities in young forest stands regenerating after clearcut logging, New Phytol. 157 (2003) 399–422.

[25] Jonsson L., Dahlberg A., Nilsson M.-C., Kåren O., Zackrisson O., Continuity of ectomycorrhizal fungi in self-regenerating boreal *Pinus sylvestris* forests studied by comparing mycobiont diversity on seedlings and mature trees, New Phytol. 142 (1999) 151–162.

[26] Jumpponen A., Dark septate endophytes – are they mycorrhizal? Mycorrhiza 11 (2001) 207–211.

[27] Jumpponen A., Mattson K.G., Trappe J.M., Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter, Mycorrhiza 7 (1998) 261–265.

[28] Kaldorf M., Renker C., Fladung M., Buscot F., Characterization and spatial distribution of ectomycorrhizas colonizing aspen clones released in an experimental field, Mycorrhiza 14 (2004) 295–306.

[29] Kåren O., Nylund J.-E., Effects of ammonium sulphate on the community structure and biomass of ectomycorrhizal fungi in a Norway spruce stand in southwestern Sweden, Can. J. Bot. 75 (1997) 1628–1642.

[30] Kåren O., Högberg N., Dahlberg A., Jonsson L., Nylund J.-E., Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis, New Phytol. 136 (1997) 313–325.

[31] Karkouri K.E., Martin F., Douzery J.P.E., Mousain D., Diversity of ectomycorrhizal fungi naturally established on containerised *Pinus* seedlings in nursery conditions, Microbiol. Res. 160 (2005) 47–52.

[32] Kernaghan G., Sigler L., Khasa D., Mycorrhizal and root endophytic fungi of containerized *Picea glauca* seedlings assesed by rDNA sequence analysis, Microb. Ecol. 45 (2003) 128–136.

[33] Kjøller R., Bruns T.D., *Rhizopogon* spore bank communities within and among California pine forests, Mycologia 95 (2003) 603–613.

[34] Kranabetter J.M., Wylie T., Ectomycorrhizal community structure across forest openings on naturally regenerated western hemlock seedlings, Can. J. Bot. 76 (1998) 189–196.

[35] Lilleskov E.A., Fahey T.J., Horton T.R., Lovett G.M., Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska, Ecology 83 (2002) 104–115.

[36] Mah K., Tackaberry L.E., Egger K.N., Massicotte H.B., The impacts of broadcast burning after clear-cutting on the diversity of ectomycorrhizal fungi associated with hybrid spruce seedlings in central British Columbia, Can J. For. Res. 31 (2001) 224–235.

[37] Marmeisse R., Guidot A., Gay G., Lambilliotte R., Sentenac H., Combier J.-P., Melayah D., Fraissinet-Tachet L., Debaud J.C., *Hebeloma cylindrosporum* – a model species to study ectomycorrhizal symbiosis from gene to ecosystem, New Phytol. 163 (2004) 481–498.

[38] Menkis A., Vasiliauskas R., Taylor A.F.S., Stenlid J., Finlay R., Fungal communities in mycorrhizal roots of conifer seedlings in forest nurseries under different cultivation systems, assessed by morphotyping, direct sequencing and mycelial isolation, Mycorrhiza 16 (2005) 33–41.

[39] Molina R., Trappe J.M., Mycorrhiza management in bareroot nurseries, in: Mary L., Landis T.D. (Eds.), Forest nursery manual: Production of bareroot seedlings, martinus Nijhoff/Dr W. Junk Publishers, The Hague/Boston/Lancaster, 1984, pp. 211–223.

[40] Parladé J., Luque J., Pera J., Rincón A.M., Field performance of *Pinus pinea* and *P. halepensis* seedlings inoculated with *Rhizopogon* spp. and outplanted in formerly arable land, Ann. For. Sci. 61 (2004) 507–514.

[41] Peter M., Ayer F., Egli S., Honegger R., Above- and below-ground community structure of ectomycorrhizal fungi in three Norway spruce (*Picea abies*) stands in Switzerland, Can. J. Bot. 79 (2001) 1134–1151.

[42] Peter M., Ayer F., Egli S., Nitrogen addition in a Norway spruce stand altered macromycete sporocarp production and below-ground ectomyccorrhizal species composition, New Phytol. 149 (2001) 311–325.

[43] Read D.J., Mycorrhizas in ecosystems, Experientia 47 (1991) 376–391.

[44] Read D.J., Perez-Moreno J., Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? New Phytol. 157 (2003) 475–492.

[45] Rincón A., Parladé J., Pera J., Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerised *Pinus pinea* L. seedlings produced in a commercial nursery, Ann. For. Sci. 62 (2005) 817–822.

[46] Rudawska M., Leski T., Gornowicz R., Mycorrhizal status of *Pinus sylvestris* L. nursery stock in Poland as influenced by nitrogen fertilization, Dendrobiology 46 (2001) 49–58.

[47] Smith S.E., Read D.J., Mycorrhizal symbiosis, 2nd ed., San Diego, CA, USA Academic Press, 1997.

[48] Stendell E.R., Horton T.R., Bruns T.D., Early effects of prescribed fire on the structure of the ectomycorrhizal fungus community in a Sierra Nevada ponderosa pine forest, Mycol. Res. 103 (1999) 1353–1359.

[49] Stenström E., Ek M., Field growth of *Pinus sylvestris* following nursery inoculation with mycorrhizal fungi, Can. J. For. Res. 20 (1990) 914–918.

[50] Taylor D.L., Bruns T.D., Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagules communities, Mol. Ecol. 8 (1999) 1837–1850.

[51] Ursic M., Peterson R.L., Husband B., Relative abundance of mycorrhizal fungi and frequency of root rot on *Pinus strobus* seedlings in a southern Ontario nursery, Can J. For. Res. 27 (1997) 54–62.

[52] Ursic M., Peterson R.L., Morphological and anatomical characterization of ectomycorrhizas and ectendomycorrhizas on *Pinus strobus* seedlings in a southern Ontario nursery, Can. J. Bot. 75 (1997) 2057–2072.

[53] White T.J., Bruns T.D., Lee S.B., Taylor J.W., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications, Academic Press, London, 1990, pp. 315–322.

To access this journal online:
www.edpsciences.org/forest