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ORIGINAL PAPER

Succession of ectomycorrhizal fungi in naturally regenerated silver birch *Betula pendula* Roth stands on post-agricultural land

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ABSTRACT

Silver birch *Betula pendula* is a pioneer tree species that often naturally regenerates on post-agricultural land. The succession of ectomycorrhizal fungi depends on many factors including local environmental conditions and in particular soil conditions. Therefore, the objective of this study was to analyse the mycorrhizal fungal communities in four young silver birch stands regenerated by self-sowing on post-agricultural land. The stand ages were the following: 1-2 years old (class I), 2-7 years old (class II), 6-11 years old (class III), and 11-16 years old (class IV). Soil samples were collected from each stand for soil and mycorrhizal analysis. Soil properties in all age classes differed from forest soils. The high proportion of non-mycorrhizal root tips in age classes I and II (10.8% and 20.4%, respectively) may result from limited inoculum availability. The high proportion of non-vital root tips, up to 55.7% in age class IV, indicates that soil conditions were not conducive to mycorrhizal functioning. A total of 23 taxa of mycorrhizal fungi were detected with *Inocybe moravica* being the most abundant and frequently occurring species (relative abundance – 34.6%). Another species present in all age classes was *Paxillus involutus*. However, the majority of the recognised taxa (14) were found exclusively in a single age class. The youngest stand had only three ectomycorrhizal fungal species, while a higher number of fungal taxa was observed in the three older stands (12, 11 and 13, respectively). Dominance, Shannon-Wiener and Simpson indices showed similarity of biological diversity among fungal communities of all stands, while they differed in terms of species composition, as confirmed by the low Jaccard similarity index. The greatest influence on the dissimilarities among investigated age classes can be attributed to *Paxillus involutus* 17.14%, *Amanita muscaria* 17.07% and *Inocybe moravica* 14.18% The results of this study suggest that soil properties and mycorrhizal inoculum availability influenced the results more than stand age.

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Introduction

There are two birch species of economic importance in Europe: silver *Betula pendula* Roth and downy *B. pubescens* Ehrh. birch. Both have a wide natural range of occurrence in Eurasia, extending from the Atlantic to eastern Siberia (Hynynen *et al.*, 2010). In Poland, they are widespread throughout the country, however, downy birch is not as common as silver birch and plays a smaller role in forest management (Zarzycki, 1979). Silver birch is a typical pioneer species that appears in unforested and degraded areas and is often self-sown on former agricultural land (Cameron, 1996; Uri *et al.*, 2007). Birch is an ectomycorrhizal (ECM) tree species that forms symbiotic relationships with Basidiomycota and Ascomycota fungi. EMC fungi provide trees with nutrients, mainly nitrogen and phosphorus compounds, from otherwise unavailable recalcitrant inorganic and organic sources. Other functions of mycorrhizal symbiosis include increased protection of trees against the following: pathogens, drought, temperature extremes, metal toxicity, and stress conditions (Smith and Read, 2008). ECM fungi are crucial for the ability of their plant partners to colonize novel sites, help start successional processes, and develop plant communities driving primary and secondary succession (Smith and Read, 2008; Kałucka and Jagodziński, 2016).

Studies of ectomycorrhizal fungal succession were started in the 1980s in a young *B. pendula* stands in Scotland (*e.g.* Fox, 1983; Mason *et al.*, 1983; Last *et al.*, 1987). Till now such research has been carried out for many different tree species, *e.g.*, Douglas fir *Pseudotsuga menziesii* (Mirb.) Franco (Twieg *et al.*, 2007), Jack pine *Pinus banksiana* Lamb. (Visser, 1995), or common oak *Quercus robur* L. (Keizer and Arnolds, 1994).

The quantitative and qualitative composition of mycorrhizal fungal communities changes with stand age (Kranabetter *et al.*, 2005; Twieg *et al.*, 2007; Rudawska *et al.*, 2018). While describing ECM fungal succession, the following terms are often used: 'early-stage' and 'late-stage' fungi (Visser, 1995; Twieg *et al.*, 2007; Kałucka and Jagodziński, 2016). The former include such fungi species groups such as *Laccaria*, *Thelephora*, *Inocybe* and *Hebeloma* which colonize seedlings effectively through spores (*e.g.* Deacon *et al.*, 1983; Mason *et al.*, 1984). They are also considered r-strategists. They have a relatively low carbon demand, rapid mycelial growth, and occurrence in soils with nutrients mainly in the inorganic pool (Dighton and Mason, 1985). In turn, the 'late-stage' fungi such as *Lactarius*, *Cortinarius* and *Russula* are not able to colonize the roots of seedlings from spores under unsterile conditions. They are K-strategists with higher carbohydrate demand, slower mycelial growth, and occurrence in the soil where nutrients are mainly in the organic pool (Dighton and Mason, 1985). Fungi that occur at various ages are called 'multi-stage' (Visser, 1995; Twieg *et al.*, 2007). These terms, however, are generally descriptive and do not adequately reflect the complexity of fungal succession patterns in ecosystems (Twieg *et al.*, 2007).

In general, in successional sequences, the number of ECM species increases with tree age up to approximately 30-40 years and then decreases to an intermediate and relatively constant level (Keizer and Arnolds, 1994; Visser, 1995). Species richness of ECM fungi increases until canopy closure (Last *et al.*, 1987; Twieg *et al.*, 2007) as changes in the soil conditions, especially organic matter accumulation, might create new niches for ECM fungi (Hashimoto and Hyakumachi, 2000). During canopy closure tree growth rates are rapid and leaf area maximal with correspondingly high potential for carbon allocation to roots and mycobionts. The host

roots are also more abundant and uniformly distributed corresponding with a higher diversity of ECM fungi (Visser, 1995; Kranabetter *et al*., 2005; Twieg *et al*., 2007).

Apart from stand age, many other factors affect the rate of succession and the composition of ECM fungal communities including the following *inter alia*: host plant species, plant vigor and genotype, inoculum availability, competition among ECM fungi, non-ECM plant community structure as well as soil attributes (Keizer and Arnolds, 1994; Twieg *et al*., 2009).

The limited availability of inoculum (Kałucka and Jagodziński, 2016) and differences in the chemical and physical properties of post-agricultural soils compared to forest ones (*e.g*., higher nitrogen content and lower C/N ratio) (Smal and Olszewska, 2008; Kałucka and Jagodziński, 2016) might be a problem for the formation of ectomycorrhizal relationships by the trees regenerating on post-agricultural land. This has an impact on the formation of ECM fungal communities as they strongly depend on environmental conditions, especially soil conditions and nutrient availability (Keizer and Arnolds, 1994; Visser, 1995).

Silver birch is an important tree species ecologically and economically. Although there have been quite a few studies on the ectomycorrhizal fungal communities of this species to date, knowledge is still not complete. Also, taking into account that the succession of ectomycorrhizal fungi depends on many factors including local environmental conditions, in particular soil conditions, this study aimed to analyse the mycorrhizal fungal communities in four young, differently aged silver birch stands that regenerated by self-sowing on post-agricultural land. We hypothesize that the ECM fungal community changes along the age gradient. As the diversity of ECM fungi increases with stand age, the community composition of each stand age class comprises age-appropriate groups of ECM fungi.

Materials and methods

Study site. The research was carried out in four silver birch stands, created from natural regeneration on former farmland in Dobieszyn, Poland. (51°35'N, 21°10'E). The soil in the study area developed on glacio-fluvial meltwater sands, and glacial tills and was classified as podzols and luvisols. The annual mean temperature in the study area is 6-7°C. The warmest month is July (16-17°C) and the coldest is January (–2°C). The growing season (mean daily air temperature above +5°C) begins in early April and lasts on average 200-210 days. The long-term mean annual precipitation amounts to 550-600 mm (Martyn, 2000).

The area where the study plots were established used to be cropland. The history of the site as well as the cessation of use for agriculture and the natural character of silver birch succession were confirmed with the local forest authorities. The analysed birch stands grew on adjacent strips approximately 20 m wide and about 200 m long of arable land abandoned 16, 11, 7 and 2 years earlier, respectively (Fig. 1). The age of the trees in each stand was determined by counting the annual rings and equalled the following: 1-2 years in age class I, 2-7 years in class II, 6-11 years in class III and 11-16 years in class IV. The density of trees (number of trees per hectare) of the analysed age classes equalled 1,555,556, 165,000, 12,500, and 12,644, respectively. On the north side of the study area (the shorter sides of the birch stands) there was a pine stand that was approximately 50 years old.

Roots sampling and analysis. Soil cores (3.6 cm diameter × 15 cm depth) for mycorrhizal study were collected at the end of September 2013. Twelve samples from each stand (in total 48) were taken along a transect running through the middle of each stand (approximately 10 m from the ends of the stand), along the longer side. The distances between the sampling points were approximately 10-15 m.

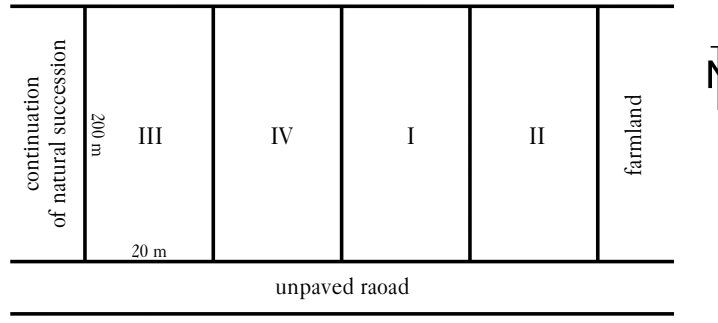

Fig. 1.
Experimental design

The dry weight and length of roots in a single sample were determined. The length of the roots was determined by the cutting method (Böhm, 1985). This method involves randomly arranging the roots on a grid of squares of an arbitrary side length (in the research presented here the sides of the squares were 0.5 cm) and determining the number of lines that were intersected by roots. Root lengths were calculated using the following formula:

$$L = 11/14 \cdot n \cdot a$$

where:

L – length of the roots in cm,
n – number of intersections of the grid of squares,
a – length of the square's side (a=0.5 cm).

Ectomycorrhizal assessment. The washed roots were arranged on a Petri dish with distilled water and analysed (at 10-40 × magnification) using a Delta IPOS-808 dissecting microscope coupled with a camera. Mycorrhizal root tips were identified by the presence (along with colour, shape and surface texture) of the mycelial mantle, as well as the presence of extramatrical mycelium and rhizomorphs. Based on the above features and referenced graphical materials (Agerer, 1987-2008), vital mycorrhizal root tips (VM) were counted and assigned to the corresponding morphotypes. The non-mycorrhizal (NM) root tips which lacked the characteristics of ectomycorrhizae and had root hairs were also counted along with the non-vital (NV) root tips that were heavily wrinkled and dead.

Each mycorrhizal morphotype was photographed. Three representative mycorrhizal root tips of each observed morphotype were placed in eppendorfs filled with 70% ethanol, labelled and stored at –20°C until further molecular analysis was conducted. The final grouping and assignment of the classified morphotypes previously based on the morphological characteristics of mycorrhizal root tips was achieved through the analysis of fungal rDNA.

Molecular identification of mycorrhizal fungi. We amplified the internal transcribed spacer (ITS) region of the rDNA using ITS1F and ITS4 primers (White *et al.*, 1990; Gardes and Bruns, 1993) before going on to sequence the product of the polymerase chain reaction (PCR). Before the DNA extraction, root tips were dried and then ground in an Eppendorf tube with a plastic mortar in the presence of liquid nitrogen. Genomic DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) in line with the manufacturer's instructions. Reactions were performed in a 25 µl mixture containing 1 ng of genomic DNA, 0.5 µM of each

primer, 0.2 µM of each dNTP, 2.5 µl of 10×PCR reaction buffer and 1 U of DreamTaq™ DNA Polymerase (Thermo Fisher Scientific). The amplification reaction was performed in a PTC-200™ Programmable Thermal Controller thermocycler (MJ Research, Inc.) under the following conditions: initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation, annealing and elongation for 25 s at 95°C, 25 s at 56°C and 50 s at 72°C, respectively, with a final extension step at 72°C for 10 min. Directly after the reaction, 1 µl of PCR product was electrophoresed on a 1% TAE agarose gel with 1 kb DNA Ladder Plus (Invitrogen, Carlsbad, CA, USA) as a molecular weight marker. The amplified PCR fragments of DNA were purified with CleanUp Kit (A&A Biotechnology, Gdynia, Poland). Both strands of the PCR products were sequenced with a 3730XL DNA Analyser (Applied Biosystems, Foster City, CA, USA) at *Genomed* Company (Warsaw, Poland). The nucleotide sequences were read and manually edited using FinchTV v. 1.4.0 (Geospiza Inc., Seattle, WA, U.S.A.) and aligned with sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov) using the BLASTn algorithm to confirm the taxonomy of the fungi studied. Fungal species were defined based on a 97% sequence similarity level which is within the range of intraspecific ITS sequence similarity. The taxonomy of the fungi was confirmed in the UNITE database (https://unite.ut.ee). Obtained sequences were submitted to the GenBank database (Table 1).

No attempt was made to analyze molecular data for *Cenococcum geophilum s.l.* as the taxonomy of this species complex is not well delineated by variations in the ITS region alone (Jany *et al*., 2002; Douhan and Rizzo, 2005). Two taxons were identified only on morphological features and were named *Lactarius*-like and *Tomentella*-like.

Soil analysis. For soil analysis three soil samples were taken from two topsoil layers: 0-5 cm and 5-15 cm of each stand which formed a combined sample. The following soil chemical properties were determined: pH (potentiometrically, in 1 M KCl and H_2O solution), nitrogen and organic carbon content (with a LECO CNS True Mac Analyser: Leco, St. Joseph, MI, USA), C/N ratio and basic cation content (in 1 M ammonium acetate, using a Thermo Scientific iCAP 6000 ICP OES analyser, Thermo Fisher Scientific, Cambridge, UK).

Data analysis. Communities of mycorrhizal fungi in each age class were described according to the following measures: (i) species/taxon richness, *i.e.* number of taxa, (ii) abundance (degree of mycorrhizal colonisation) a ratio of vital mycorrhizal (VM) root-tips to all root tips in the given soil sample including non-mycorrhizal (NM) and non-vital (NV) tips expressed in percentage terms, (iii) relative abundance as the ratio of the number of each mycorrhizal species/taxon root--tips to the total number of vital mycorrhizal root-tips expressed as a percentage, (iv) and frequency of occurrence of species/taxa of mycorrhizal fungi defined as the number of soil cores colonised by the given mycorrhizal species/taxon. To assess mycorrhizal species/taxon diversity, the Dominance (D), Shannon-Wiener (*H'*), Simpson (1-D), and Evenness (*e^H/S*) diversity indices were determined. To compare the ECM communities of different age classes, the Jaccard similarity index was calculated for each pair of age classes. True taxon richness was estimated with *Chao 2*, *Jackknife 1* and *Jackknife 2* measures calculated using 1,000 randomised bootstrap runs without sample replacement. These coefficients were calculated both for the whole data set as well as for individual age classes. To assess the difference among analysed age classes ANOSIM (using the Bruy-Cutis dissimilarity measure) was performed which was then followed up with SIMPER analysis to detect which species contribute the most to differences amongst the analysed age classes.

Table 1.

Molecular identification, relative abundance on root tips [%] and frequency (in soil cores, n=48) of mycorrhizal fungal taxa on the roots of silver birch stands

Fungal taxa	GenBank accession numbers	Best match sequence	Sequence similarity [%]	Relative abundance	Frequency
Basidiomycota					
Amanita muscaria (L.) Lam.	OR458466-OR458468	AB080980	100.0	5.6	6
Amanita spissa (Fr.) P. Kumm.	OR458469	EF493271	100.0	1.6	1
Boletus edulis Bull.	OR458470-OR458474	KC184481	100.0	7.6	5
Cortinarius valgus Fr.	OR458475	KF961225	100.0	0.4	1
Inocybe moravica Hruby	OR458513-OR458540	OP164099	100.0	34.6	31
Inocybe lacera (Fr.) P. Kumm.	OR458510-OR458512	OP164102	100.0	7.2	3
Lactarius glyciosmus (Fr.) Fr.	OR458541-OR458542	MZ955936	100.0	8.8	5
Lactarius rufus (Scop.) Fr.	OR458543-OR458544	KF241543	100.0	1.7	2
Lactarius like				3.3	1
Paxillus involutus (Batsch) Fr.	OR458545-OR458551	HQ604826	100.0	3.9	20
Thelephora terrestris Ehrh.	OR458553 OR462248	JQ711902	100.0	0.4	2
Tomentella sublilacina (Ellis & Holw.) Wakef.	OR458556-OR458561	JQ888214	100.0	2.9	5
Tomentella stuposa (Link)	OR458554-OR458555	MK602778	98.5	0.2	2
Tomentella like				2.1	2
Tricholoma fulvum (DC.) Stalpers Bigeard & H. Guill.	OR458562	FJ845446	100.0	>0.1	1
Ascomycota					
Cenococcum geophilum Fr.				4.1	15
Hyaloscypha finlandica (C.J.K. Wang & H.E. Wilcox) Vohník, Fehrer & Réblová	OR458476-OR458495	HQ406816 MT321760 OQ418585	100.0 99.7 99.7	10.9	11
Hyaloscypha spinulosa (Beverw.) K. Yamag., Chuaseehar. & Nakagiri	OR458508	LC332950	100.0	0.7	1
Hyaloscypha sp.	OR458496-OR458507	MG230329 MK529861 MT321760	100.0 99.7 96.4	3.2	3
Phialocephala fortini C.J.K. Wang & H.E. Wilcox 1986	OR458552	MT294419	100.0	0.6	1
Unidentified 1				>0.1	1
Unidentified 2				0.1	2
Unidentified 3				>0.1	1

As distributions of all analysed parameters significantly diverged from the norm, the Kruskal--Wallis test was used to check for differences amongst the analysed age classes, and if one was found, a Mann-Whitney paired comparison followed.

All calculations and data processing were carried out in PAST 4.1 software (Hammer *et al.*, 2001). A significance level of 0.05 was assumed for all the analyses.

Results

Soil properties. Soil reactions in all stands at both analysed depths were acidic and ranged from 4.3 to 4.7 (in H_2O) and 3.8 to 4.0 (in KCl). The content of C, N, and P_2O_5 was very low, while the content of base cations was high. The C/N ratio varied greatly between the analysed stands and soil levels ranged from 7.6 to 21.1. The lowest values were recorded in the upper topsoil layer in age classes II and III (Table 2).

Root characteristics. Root length and mass, as well as the number of tips in the soil core, increased with stand age. For all these traits, significant differences were observed among age classes (H=28.86, p<0.001; H=33.6, p<0.001; H=16.23, p<0.001; respectively).

In the case of root length, significantly shorter roots were found in the first age class (41.5 ±7.2 cm). Roots in samples from the remaining three age classes did not differ in length significantly (Fig. 2). The youngest trees were significantly characterised by the lowest root mass (0.078 ±0.035 g), while the highest significant weight was obtained from root samples taken from the oldest stand (1.682 ±0.243 g) (Fig. 3). In turn, the number of tips in the soil core had the significantly lowest value in the youngest birches, while in the older stands we observed similar values for that feature (Table 3).

Mycorrhizal root colonisation. On average, VM root tips constituted 58.9 ±4%, NM 10.5 ±2% and NV ones 30.6 ±4% of all tips. The structure of root tips showed remarkable differences that can be observed with aging stands (Fig. 4). Mean fractions of VM tips were very variable (from 43.5 ±7% in the IV age class up to 70.5 ±3% in the II age class), however, observed differences among age classes turned out to not be significant (H=7.72, p=0.052). On the other hand, for NM and NV tips we found a significant effect of stand age (H=15.47, p=0.001 and H=17.2, p=0.001, respectively). In the case of non-mycorrhizal tips, significantly lower values were observed in age classes III and IV (3.4 ±1%, 4.4 ±1%, respectively) and higher ones in age

Table 2.

Selected parameters of topsoil layers (0-5 cm and 5-15 cm) in the chronosequence of silver birch stands (age classes: I-IV)

Feature	I		II		III		IV	
	0-5	5-15 c	0-5 c	5-15 c	0-5 c	5-15 c	0-5 c	5-15 c
	[cm]							
pH in H_2O	4.46	4.56	4.29	4.55	4.74	4.32	4.54	4.70
pH w KCl	3.84	3.91	3.88	3.89	3.96	3.99	3.78	4.03
Total N [g kg^{-1}]	0.106	0.109	0.197	0.079	0.201	0.071	0.162	0.097
Total C [g kg^{-1}]	1.67	1.68	1.50	1.45	1.66	1.50	1.97	1.67
C/N	15.75	15.41	7.61	18.4	8.26	21.13	12.16	17.22
P_2O_5 [mg kg^{-1}]	9.7	10.9	13.9	15.4	9.2	8.1	19.8	9.9
Ca^{2+} [mg kg^{-1}]	312	296	291	352	311	309	390	304
K^+ [mg kg^{-1}]	475	445	437	429	397	402	398	486
Mg^{2+} [mg kg^{-1}]	204	196	202	211	214	229	232	241
Na^+ [mg kg^{-1}]	57.8	53.5	61.0	57.0	46.2	50.8	44.0	61.0

class II (21.4 ±4%). The fraction of non-vital mycorrhizal root tips was the highest in age class IV (52.6 ±4%) and the lowest in age class II (8.1 ±3%).

From 48 soil samples, 93,523 mycorrhizal root tips were examined. ITS PCR was successful for all 117 samples selected for analysis (PCR product present), while only 102 among 117

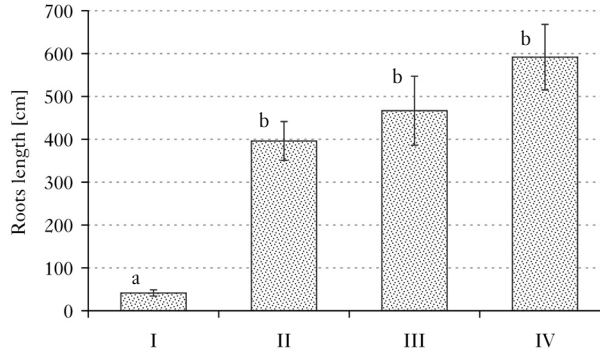

Fig. 2.

Mean (bar) and standard error (whiskers) of root length [cm] for naturally regenerated silver birch stands in different age classes (I-IV)

different letters indicate homogenous groups determined with the Kruskal-Wallis test (P≤0.05)

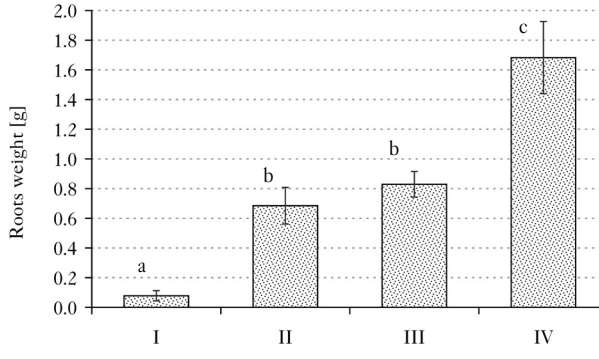

Fig. 3.

Mean (bar) and standard error (whiskers) of root weight [g] for naturally regenerated silver birch stands in different age classes (I-IV)

different letters indicate homogenous groups determined with the Kruskal-Wallis test (P≤0.05)

Table 3.

Diversity parameters of mycorrhizal fungal communities in a chronosequence of silver birch stands. Different letters indicate a significant difference according to the Kruskal-Wallis test

Diversity parameters	Age class				P
	I	II	III	IV	
No of taxa per stand	3	12	11	13	nd
Mean no of taxa per soil core	1.4 a	2.8 b	2.9 b	3.0 b	<0.0001
Root tips per soil core	191 a	2760 b	2747 b	1961 b	<0.0001
Dominance (D)	0.686 a	0.636 a	0.719 a	0.801 a	0.368
Shannon-Wiener (*H'*)	0.326 a	0.616 a	0.464 a	0.353 a	0.169
Simpson (1-D)	0.230 a	0.364 a	0.281 a	0.199 a	0.232
Evenness (e^H/S)	0.897 a	0.702 b	0.571 b	0.546 b	0.001

nd – not determined

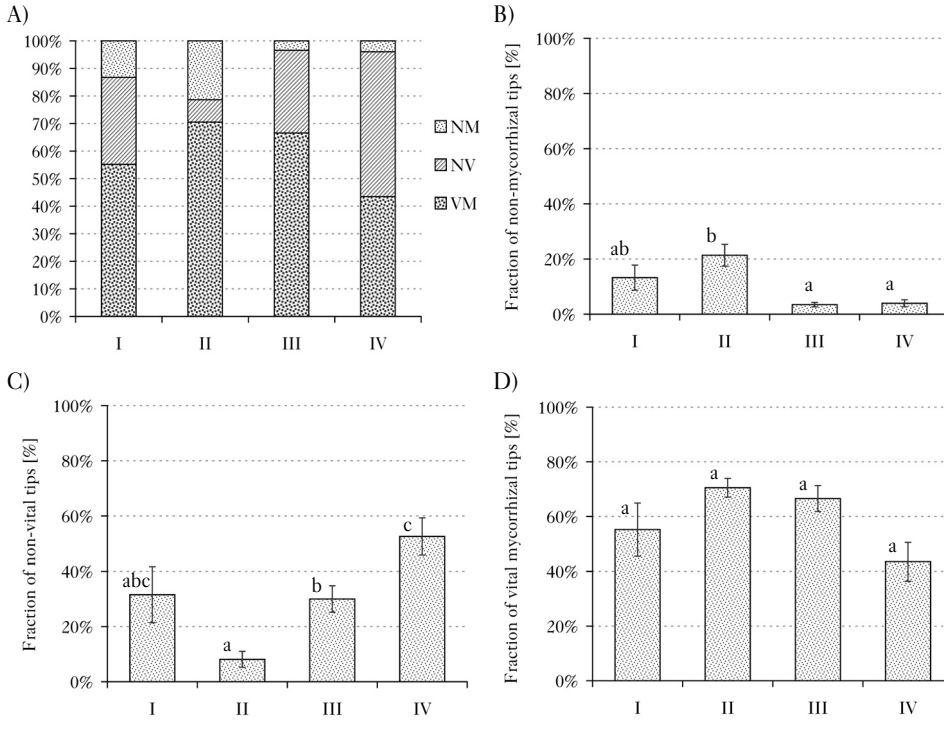

Fig. 4.

Structure (%) of mycorrhizal root tips (VM – vital, NM – non-mycorrhizal, NV – non-vital) (A) and mean (bar) and standard error (whiskers) of the fraction of a given root tips (%) (B-D) for naturally regenerated silver birch stands in different age classes (I-IV)

within each class of root-tips, different letters indicate homogenous groups determined with the Kruskal-Wallis test (P≤0.05)

PCR products were subjected to sequencing (87.2% rate of sequence identification). The remaining DNA extracts yielded mixed PCR products, thereby, excluding them from direct sequencing. ITS fungal rDNA identified 20 fungal taxa of which 17 were assigned to species and 3 to genus. Of the 20 fungal taxa, 15 (81%) belonged to Basidiomycetes and 5 (19%) to Ascomycetes (Table 1).

Composition of the mycorrhizal assemblage. *Inocybe moravica* Hruby (relative abundance of 34.6%, frequency in 31 soil cores) was the most frequent and abundant fungus. Another species that was present in all age classes was *Paxillus involutus* (Batsch) Fr. Most taxa (11 identified and 3 unidentified) occurred only in a single age class (Tables 1, 4).

There were only 3 ECM fungal species in the youngest stand. A higher number of fungal taxa was observed in three older age classes (12, 11, and 13 respectively). The mean number of ECM taxa per soil core ranged from 1.4 for the first age class to 3.0 for the fourth and was significantly lower in the youngest birch ($H=19.7$, $p<0.001$). Also, the Evenness index indicated a significant difference in the first age class from the older stands ($H=17.5$, $p=0.001$). Furthermore, the other species richness and diversity indicators (Dominance, Shannon-Wiener, Simpson) showed no significant differences between analysed age classes (Table 3).

The *Chao 2*, *Jackknife 1* and *Jackknife 2* richness estimators for the whole dataset equalled 24.43 ±9.07, 23.49 ±6.14, and 25.35 ±8.4, respectively. In the case of the youngest stand, these values were low (2.89 ±0.32, 3.15 ±0.58, and 3.15 ±1.06). For the older stands, they were substan-

tially higher and fairly equal and amounted to 11.49 ±2.48, 12.63 ±2.02 and 13.14 ±3.40 (II age class), 10.66 ±3.12, 11.49 ±2.32 and 12.10 ±3.72 (III age class), and 12.70 ±4.24, 13.29 ±2.77 and 14.39 ±4.49 (IV age class).

The number of taxa shared between age classes ranged from 2 to 7 (Table 5). Only *I. moravica* and *P. involutus* were commonly found in all the groups which resulted from low species richness noted in the youngest stand. For pairs formed by the older age classes, *I. moravica*, *P. involutus*, *Hyaloscypha finlandica* (C.J.K. Wang & H.E. Wilcox) Vohník, Fehrer & Réblová, *Cenococcum geophilum* and *Tomentella sublilacina* (Ellis & Holw.) Wakef. were shared by all three groups. In general, Jaccard similarity indices were low, however, particularly low values were observed for pairs formed with the first age class (Table 5).

Table 4.

Ectomycorrhizal fungal communities in a chronosequence of silver birch stands (RA – relative abundance on root tips [%], F – total frequency in soil cores (n=12 per age class)

Fungal taxa	Age class							
	I		II		III		IV	
	RA	F	RA	F	RA	F	RA	F
Inocybe moravica	70.2	9	28.5	8	47.3	8	21.9	6
Thelephora terrestris	15.7	2						
Paxillus involutus	14.1	6	8.1	4	0.1	2	2.2	8
Lactarius glyciosmus			15.8	3	8.7	2		
Amanita muscaria			15.6	6				
Hyaloscypha finlandica			12.7	2	15.3	6	3.3	3
Hyaloscypha sp.			8.8	3				
Inocybe lacera			8.2	2	11.7	1		
Cenococcum geophilum			1.4	1	2.2	8	11.1	6
Tomentella stuposa			0.7	2				
Tomentella sublilacina			>0.1	1	7.9	3	>0.1	1
Tomentella like			>0.1	1			8.4	1
Boletus edulis					2.8	1	25.6	4
Hyaloscypha spinulosa					2.0	1		
Phialocephala fortini					1.6	1		
Lactarius like							12.8	1
Lactarius rufus							6.7	2
Amanita spissa							6.1	1
Cortinarius vulgaris							1.7	1
Tricholoma fulvum							>0.1	1
Unidentified 1			0.1	1				
Unidentified 2					0.4	2		
Unidentified 3							0.1	1

Table 5.

The number of shared ECM taxa (upper triangle matrix) and Jaccard similarity index (lower triangle matrix) between age classes of silver birch stands

Age class	I	II	III	IV
I		2	2	2
II	0.154		7	6
III	0.167	0.438		6
IV	0.143	0.316	0.333	

Results of ANOSIM suggest an even distribution of the ranked dissimilarities among analysed groups (R=-0.034, p=0.888) which confirms the results above of the rather indistinct differences in ECM assemblages.

Moreover, SIMPER analysis reveals that the greatest influence on the dissimilarities among investigated age classes can be attributed to *P. involutus*, *Amanita muscaria* (L.) Lam. and *I. moravica* (17.14, 17.07, and 14,18%, respectively) (Table 6).

Discussion

The level of colonization of silver birch roots with ECM fungi was rather low. The mean fraction of VM root tips was the highest in age class II (70.5%) and the lowest in age class IV (43.5%). On the contrary, Twieg *et al*. (2007) analysed paper birch growing in forest habitats and found that 97% of roots were mycorrhizal. A high number of NV root tips was also noted reaching 52.6% in age class IV. These results indicate that on post-agricultural land, according to this research, conditions for the formation and functioning of mycorrhizae were not favourable. It can be assumed that the reason for this was the limiting availability of inoculum (Kałucka and Jagodziński, 2016) and the presence of soil conditions differing from those typical for forest soils (Keizer and Arnolds, 1994; Visser, 1995). It is suggested that the availability of inoculum was limited in stands of younger age classes, especially in age class II (20.4% NM root tips). In the older age classes, it was lower and amounted to only 3.2% and 4.4%, in the III and IV age classes respectively which is similar to the research by Twieg *et al*. (2007).

Table 6.
Effect of individual ECM fungi on dissimilarity among analysed age classes according to SIMPER analysis

Taxon	Average dissimilarity index	Contribution [%]	Cumulative contribution [%]
Paxillus involutus	1.204	17.14	17.14
Amanita muscaria	1.198	17.07	34.21
Inocybe moravica	0.996	14.18	48.40
Hyaloscypha sp.	0.611	8.71	57.10
Lactarius glyciosmus	0.602	8.57	65.67
Tomentella stuposa	0.406	5.78	71.45
Hyaloscypha finlandica	0.401	5.71	77.16
Thelephora terrestris	0.400	5.70	82.86
Inocybe lacera	0.396	5.64	88.51
Unidentified 1	0.205	2.93	91.43
Tomentella sp.	0.205	2.93	94.36
Cenococcum geophilum	0.200	2.85	97.21
Tomentella sublilacina	0.196	2.79	100.00
Lactarius rufus	0.000	0.00	100.00
Amanita spissa	0.000	0.00	100.00
Unidentified 2	0.000	0.00	100.00
Tricholoma fulvum	0.000	0.00	100.00
Cortinarius vulgaris	0.000	0.00	100.00
Boletus edulis	0.000	0.00	100.00
Phialocephala fortini	0.000	0.00	100.00
Lactarius sp	0.000	0.00	100.00
Unidentified 3	0.000	0.00	100.00
Hyaloscypha spinulosa	0.000	0.00	100.00

The soil in stands of all age classes differed in its properties from forest soils (Aleksandrowicz-Trzcińska *et al.*, 2018a, b). Only the pH was similar to that of forest soils. The content of N, C, and P was very low (about 5-10, 13 and 7 times, respectively), while the content of cations was very high (about Ca – 5, Mg – 20, K – 17 and Na – 35 times) in relation to that of forest soils. The observed range of values of analysed soil characteristics, especially C/N, indicates a high heterogeneity of soil properties which may affect both the duration of ECM root function and the composition of ECM fungal communities (Keizer and Arnolds, 1994; Visser, 1995). Hence, it is quite probable that differences were observed in the fraction of dead mycorrhizae in individual stands as well as in the composition of ECM fungal communities regardless of their age. In contrast, Twieg *et al.* (2009) claimed that ECM community structure was more strongly influenced by stand age than soil nutrients. It should be noted that their study was conducted in typical, undisturbed forest habitats.

In this study, 23 species of ECM fungi in 4 birch stands of various ages (up to 16 years) were found. This is 10 species fewer than reported by Rosenvald *et al.* (2012), who also studied young silver birch stands. However, these stands were older (up to 32 years old) and grew in forest habitats which are generally richer in various forms of ECM fungal inoculum compared to post-agricultural soil (Boerner *et al.*, 1996). On the other hand, if we compare the number of species in the stands of particular age classes (6 years – 9 species, 14 years – 11 species and 32 years – 18 species), the number of species in our study turns out to be slightly higher or comparable.

The results confirmed the hypothesis that fungal diversity increases with stand age only partially. The increase in the number of ECM fungal species was observed only until the stand reached age class II. The three older stands were characterized by a similar number of species (11-13). Similar variation was found in the magnitude of biometric parameters of the roots including their length and weight as well as the number of root tips in the sample. These results show that the changes that occurred in young birch stands under the conditions of this experiment, *i.e.* between 7 and 16 years of age, were not large compared to those that occurred up to 7 years of age. Similar results were obtained by Rosenvald *et al.* (2012). The fastest changes in ECM root morphology of silver birch occurred at a very young age (under 6 years old). Most likely the rate of change was influenced by the fact that the stand had already reached canopy closure at age class II. Many studies indicate that an increase in ECM fungal species richness occurs until the crowns have closed (Last *et al.*, 1987; Twieg *et al.*, 2007). Under the conditions of this experiment, canopy closure occurred early due to the rapid growth of birch and the high density of natural regeneration. The results are consistent with those obtained by Jagodziński and Kałucka (2008, 2010, 2011) for *Pinus sylvestris* L. stands restored in different habitats including former farmland. They indicated that the biomass of active fine roots usually reaches a maximum in young stands (between 8-20 years old) which occurs during the canopy closure stage. The growth of fine roots is positively correlated with the growth of ECM mycelium, and the production of ECM mycelium reaches its maximum value during crown closure. The leaf area index is also highest during this period which is associated with the intensity of photosynthetic processes and carbon allocation to ECM roots (Twieg *et al.*, 2007; Kałucka and Jagodziński, 2013). This pattern of ECM fungal succession, related to tree development, both of the assimilatory apparatus and roots, has been described in studies of other tree species, both coniferous and deciduous (Keizer and Arnolds, 1994; Kranabetter *et al.*, 2005; Twieg *et al.*, 2007).

The number of taxa per soil sample and Evenness indices showed that the analysed ECM fungal community on birch roots in the first age class is significantly different (and poorer) from the communities in the older stands which differ one from another much less. Dominance, Shannon-

-Wiener and Simpson indices showed similarity of biological diversity among fungal communities of all stands, while they were not similar in terms of species composition as evidenced by the low Jaccard similarity index. The greatest influence of dissimilarities among investigated age classes can be attributed to *P. involutus*, *A. muscaria* and *I. moravica*. Changes in the composition of communities appear randomly, and it is hypothesized that they are related primarily to the availability of inoculum of particular fungal species and soil conditions. These factors are noted as the ones influencing ECM fungal communities by Gao *et al.* (2014).

Inocybe moravica was the dominant species in the birch stands studied, regardless of age. The species was first described as early as 1930 by Hruby but was often confused with *I. lacera* (Fr.) P. Kumm. and, thus, seemingly 'forgotten'. Both species are very similar in macroscopic as well as microscopic aspects and may occur at the same locations (Bandini *et al*., 2022), most often in acidic soil. They form mycorrhizae with many coniferous and deciduous tree species and are sometimes dominant in harsh environments (Cullings and Makhija, 2001; Nara, 2006).

Paxillus involutus was found in all age classes. It is known to be a wide-ranging host generalist, although it is not specifically associated with young trees (Wallander and Söderström, 1999). Similarly, it was found in two-year-old seedlings of silver birch in a forest nursery as in this study (Rudawska *et al*., 2019). Its presence in all age classes may have also resulted from the immediate proximity of the study area to a pine stand. Both pine and birch are frequent partners of this species (Wallander and Söderström, 1999).

Thelephora terrestris Ehrh. is a pioneer, multi-host mycobiont present in the nursery, young and old forests as well as in a wide variety of soils. It can be the dominant species in young stands established on former agricultural land (Hilszczańska, 2002, 2005). It is considered to be poorly competitive with other ECM fungi (Colpaert, 1999). This fact may explain the occurrence of *T. terrestris* only in age class I in our study.

Ascomycete fungi of the genus *Hyaloscypha* were found which were originally included in the *Hymenoscyphus ericae* (D.J. Read) Korf & Kernan aggregate (VrÍlstad *et al*., 2000). Members of this taxon can form ectomycorrhizal, ericoid and mycothalli associations and also grow endophytically on plant roots and hypogeous ectomycorrhizal fruitbodies (Hambleton and Sigler, 2005; Fehrer *et al*., 2019). The most abundant and frequent fungi in this study was *H. finlandica*. It is a common ectomycorrhizal fungus found on nursery-grown conifers (Trocha *et al*. 2006) as well as in Scots pine stands of different ages (Rudawska *et al*., 2018) and in mixed coniferous forest (Rosling *et al*., 2003; Tedersoo *et al*., 2003).

Typically, low-specialized fungi (non-host specific, multiple-host fungi, generalists) colonize most roots and are the dominant group, while individual fine roots are colonized by a smaller number of fungal species specialized for a given host species (host-specific fungi, specialists) (Horton and Brunus, 1998; Kałucka and Jagodzinski, 2013). The species found in this study belong exclusively to host generalists. Since the estimated number of species is higher than those found in this study, it is likely that the rarely occurring and high dispersion host-specific fungi were not found. On the other hand, it is also possible that they were not present in the studied stands at all. The main reason for this may be the lack of inoculum and favourable conditions for the establishment of symbiosis as the unfavourable conditions are known to promote generalist species while discriminating against specialized ones (Rudawska *et al*., 2018).

In the studied stands, the ECM fungal species found belonged both to early-stage *e.g.*, *Inocybe*, *Thelephora*, multi-stage *e.g.*, *Cenococcum* and late-stage fungi *e.g.*, *Amanita*, *Boletus*, *Lactarius*, *Paxillus* (Newton, 1992). This confirms that the rates of succession in birch stands are much faster than, for example, oak stands (Keizer and Arnolds, 1994). Sporocarps of the first late-stage fungi appear

as early as four years after planting and are dominant on the roots of 10-year-old and older trees (Last *et al.*, 1987). Breakdown into these simple categories was useful but insufficient to describe fungal species succession patterns (Twieg *et al.*, 2007).

This research is a case study. Although the results obtained partly confirm previous ones, they cannot be treated as universal for birch stands restored on former agricultural land.

Conclusions

Our findings indicate that the four young, different-aged stands of silver birch regenerated naturally on post-agricultural land were disturbed ecosystems. The properties of soils in all stands differ significantly from those characteristic of forest soils. The low level of ECM colonization of birch roots and the high proportion of non-vital root tips indicate unfavourable conditions for the formation and functioning of mycorrhizal relationships.

An increase in the number of ECM fungal species in young stands of silver birch was observed only up to the canopy closure stage (up to the age of 7 years). ECM fungal communities in all stands were characterized by similar biological diversity but various species composition. It appears that the presence and structure of ectomycorrhizal fungal communities in the analysed silver birch stands on former agricultural lands were more influenced by the properties of the soil and its heterogeneity as well as by the availability of the given fungi species inocula as compared to the age of the stand.

Authors' contributions

Conceptualisation – M.A.-T., MZ; methodology – M.A.-T., S.B., K.B., K.S., MZ; collection of data – M.A.-T., K.B., S.B., M.Z.; genetic analysis – K.S.; statistical analysis – S.B.; data analysis – M.A.-T., S.B., K.B., K.S., M.Z.; writing-original draft preparation – M.A.-T. with contributions from – K.B., K.S., S.B. All authors contributed to the manuscript revision, review and approval of the submitted version.

Conflicts of interest

The authors declare no conflicts of interest.

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STRESZCZENIE

Sukcesja grzybów mykoryzowych w naturalnie odnowionych drzewostanach brzozy brodawkowatej *Betula pendula* Roth na gruncie porolnym

Brzoza brodawkowata jako gatunek pionierski często odnawia się naturalnie na gruntach porolnych. Jest ona gatunkiem ektomykoryzowym tworzącym związki z grzybami workowymi i podstawkowymi. Sukcesja grzybów ektomykoryzowych brzozy była badana w latach 80. w Szkocji, głównie na podstawie obecności owocników. Z dotychczasowych badań sukcesji dotyczącej zbiorowisk grzybów ektomykoryzowych różnych gatunków drzew wynika, że liczba symbiontów grzybowych wzrasta wraz z wiekiem drzew do momentu zwarcia koron. Na skład ilościowy i jakościowy zbiorowiska ma wpływ bardzo wiele różnych czynników, takich jak warunki glebowe i klimatyczne, dostępność składników mineralnych i inokulum oraz konkurencja międzygatunkowa.

Ze względu na fakt, że sukcesja grzybów ektomykoryzowych zależy również od czynników lokalnych (środowiskowych, w tym glebowych), celem pracy była analiza zbiorowisk grzybów mykoryzowych w 4 młodych, różnowiekowych drzewostanach brzozy brodawkowatej odnowionych samosiewnie na gruncie porolnym. Założono, że wraz z wiekiem drzew będzie wzrastała liczba gatunków grzybów kolonizujących korzenie oraz będzie podlegać zmianom skład gatunkowy zbiorowisk grzybów ektomykoryzowych.

Obiektem badań były 4 drzewostany brzozy brodawkowatej rosnące w miejscowości Dobieszyn, odnowione samosiewnie na gruncie porolnym (ryc. 1), w wieku: 1-2 lata (I klasa wieku), 2-7 lat (II klasa wieku), 6-11 lat (III klasa wieku) i 11-16 lat (IV klasa wieku). Z każdego drzewostanu pobrano próby glebowe do analizy mykoryz i właściwości gleb. W próbach oznaczono długość i masę korzeni, liczbę wierzchołków korzeni oraz poziom ich zmykoryzowania. Na podstawie badań molekularnych określono skład gatunkowy zbiorowisk grzybów ektomykoryzowych badanych drzewostanów brzozowych.

Właściwości gleby odbiegały od charakterystycznych dla gleb leśnych, z wyjątkiem odczynu. Zawartość C, N i P_2O_5 była bardzo niska, a kationów wysoka. Stosunek C:N był bardzo zróżnicowany między drzewostanami oraz poziomami gleby i wynosił od 7,6 do 21,1 (tab. 2).

Długość korzeni w próbie, ich masa i liczba korzeni krótkich wzrastały wraz z wiekiem drzewostanu. Wartości cech biometrycznych korzeni były istotnie mniejsze w próbach pobranych w najmłodszym drzewostanie w porównaniu z 3 starszymi, dla których różnice w wielkości tych parametrów były mniejsze, często nieistotne statystycznie (ryc. 2 i 3; tab. 3).

Wysoki udział niemykoryzowych wierzchołków korzeni w klasach wieku I (10,8%) i II (20,4%) może być wynikiem ograniczonej dostępności inokulum. Z kolei wysoki udział wierzchołków nieżywotnych w IV klasie wieku (do 55,7%) wskazuje, że warunki glebowe nie sprzyjały funkcjonowaniu mykoryzy (ryc. 4).

We wszystkich drzewostanach stwierdzono 23 taksony grzybów ektomykoryzowych (3 w I klasie wieku, 12 – w II, 11 – w III i 13 w IV klasie). *Inocybe moravica* występował we wszystkich klasach wieku i charakteryzował się najwyższym udziałem – 34,6% oraz frekwencją – 31 (na 48 prób). Drugim gatunkiem stwierdzonym we wszystkich klasach wieku był *Paxillus involutus*. Większość taksonów (14) wystąpiła tylko w jednej klasie (tab. 1 i 4).

W warunkach doświadczenia wzrost liczby taksonów grzybów mykoryzowych miał miejsce między 1 a 7 rokiem życia drzewostanu. Wskaźniki dominancji, Shannona-Wienera i Simpsona

pokazały, że zbiorowiska grzybów w drzewostanach wszystkich klas wieku charakteryzowały się zbliżoną różnorodnością biologiczną (tab. 3), natomiast nie były podobne pod względem składu gatunkowego, co potwierdza niski indeks podobieństwa Jaccarda (tab. 5). Największy wpływ na brak podobieństwa między zbiorowiskami grzybów ektomykoryzowych w poszczególnych klasach wieku miały *P. involutus* (17,14%), *Amanita muscaria* (17,07%) i *I. moravica* (14,18%) (tab. 6). Zmiany w składzie zbiorowisk wydają się przypadkowe i związane raczej z dostępnością inokulum poszczególnych taksonów grzybów oraz warunkami glebowymi. W znacznie mniejszym stopniu były to zmiany charakterystyczne dla sukcesji grzybów ektomykoryzowych, jaka ma miejsce w drzewostanach na siedliskach leśnych. Wydaje się, że na uzyskany wynik mniejszy wpływ miał wiek drzewostanu, co może być efektem stosunkowo niedużych różnic w wieku poszczególnych drzewostanów, a większy właściwości gleby (znacznie różniące się od właściwości gleb leśnych) i dostępność inokulum.