

Article

Ectomycorrhizal Diversity and Exploration Types in *Salix caprea*

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Abstract: The tree species *Salix caprea* shows high adaptability to different habitat conditions and is economically valuable as a woody crop for biomass production. Moreover, *S. caprea* is dependent on mycorrhizal fungi, which are crucial for its growth and adaptability in different environments. Hence, this study explores the ectomycorrhizal diversity of *S. caprea* by utilizing the taxonomy (morphotyping and a molecular approach using the ITS and LSU regions) and trait diversity (exploration types) at two test sites in Germany and Poland. In total, 19 ectomycorrhizal (EM) morphotypes of *S. caprea* were characterized. Seven taxa were identified at the species level (*Hebeloma populinum*, *Cortinarius atrocoeruleus*, *Inocybe hirtella*, *Laccaria* cf. *ochropurpurea*, *Tuber maculatum*, *Cenococcum geophilum*, and *Phialophora finlandia*) and twelve at the genus level (*Tomentella* spp. 1–8, *Hebeloma* sp. 1, *Inocybe* sp. 1, and *Tuber* spp. 1–2). The EM colonization ranged from 14 to 28% of the fine root tips. At both test sites, the largest portion of the total EM colonization consisted of Thelephoraceae. The exploration types were classified as medium-distance smooth (*Tomentella* spp. 1–8 and *L. ochropurpurea*) and medium-distance fringe (*C. atrocoeruleus*), while the other taxa were short-distance exploration types, highlighting their potential functional role in the adaptation and growth of *S. caprea*.

Keywords: *Salix caprea*; willow; mycorrhiza; ectomycorrhizal; morphotypes; pioneer



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1. Introduction

Ectomycorrhizal (EM) fungi are symbionts associated with the roots of most tree species in boreal and temperate forest ecosystems [1]. Most of these tree species are dependent on EM fungi for their growth and survival [2]. Researchers have shown that EM fungi can positively influence the performance of the tree host by improving soil fertility and seed germination rates, enhancing soil nutrient mobilization (e.g., nitrogen, N) and water uptake, and modulating plant traits to alleviate biotic and abiotic stresses. In exchange, EM fungi receive photosynthetically fixed carbon (C) [3–7]. Mycorrhizal fungi help extend the area of nutrient and water acquisition through the extraradical hyphal network, and they can also aid in the distribution of hydraulic lift water to neighboring plants by forming common mycorrhizal networks, especially during drought [8]. Additionally, many EM fungal partners can efficiently increase carbon sequestration and contribute to soil nutrient mobilization by producing enzymes involved in the hydrolysis of nitrogen and phosphorus compounds from the soil organic matter, and they contribute to the weathering of minerals, e.g., through the release of organic acids [9]. Ectomycorrhizal fungi can alleviate abiotic (e.g., heavy metals) stress through metal exclusion (forming a barrier around plant roots), production of chelating compounds that bind heavy metals (reducing the toxicity), or through metal accumulation in fungal tissue; they can also improve soil structure by forming soil aggregates, reducing the mobility and bioavailability of heavy metals in soil, thereby preventing the metals from reaching the plant roots [10,11]. The EM fungi can also protect plants from biotic stress (e.g., pathogens) through the production of pathogenesis-related biomolecules or they can produce antagonist substances and increase plant fitness resulting from an enhanced nutrient supply [12].

A high diversity of EM fungi is frequently reported in forests; however, the vegetation type and abiotic soil characteristics can either decrease or increase the frequency and diversity of EM formation [13]. Successful EM colonization is problematic at some primary successional forest sites, especially at sites that are degraded or disturbed due to volcanic activity, dunes, mining, clear-cut logging, wildfires, glacier retreat, soil erosion, and the scarcity of ectomycorrhizal vegetation [2].

Willows (*Salix* species) are well known as pioneer vegetation in disturbed and polluted environments comprising fast-growing shrubs and deciduous trees with high aerial biomass and deep roots [14,15]. They can colonize nutrient-limited sites, such as roadside ditches, abandoned agricultural fields, railroads, old mine tailings, and gravel pits, as well as burned fields or flooded areas where disturbance results in an open community with less competition [14,15]. These species exhibit specific physiological adaptations and are particularly resilient in the face of climate change [16]. *Salix* spp. are economically valuable as woody crops for biomass production [17]. They are also extensively used for the phytoremediation of trace-element-contaminated environments and to minimize the leaching of pesticides from agricultural soils [18]. *Salix caprea* (goat willow or pussy willow) is a shrubby tree which is popularly known for its low nutrient requirements and its high tolerance of heavy metals [19]. In general, *Salix* species are naturally associated with both arbuscular mycorrhizal (AM) and EM fungi. Ectomycorrhizas can colonize more than 80% of the willow's fine roots, while AM fungi are mainly limited to <5% of the fine roots [20–23]. Previous studies on the EM fungi of *Salix* spp. have reported the dominance of Thelephoraceae in different environments, such as contaminated sites [24], *S. alba* in riparian edge forests [25], and *S. viminalis* in short rotation coppices [26] and saline soils [27].

Moreover, it is important to understand not only the shifts in EM diversity but also the functional differences in EM taxa based on the development and differentiation of their external mycelium (exploration types) [28]. To understand the functional traits of EM taxa and ecosystem functioning, the classification of EM fungi according to the different exploration types (e.g., short-distance, long-distance, and mat formation), the presence or absence of rhizomorphs, and hyphae type can be relevant [29,30]. The variation in EM exploration types, which differ among EM taxa and differing environments, allows EM fungi to acquire nutrients under unfavorable conditions [31]. In this context, the starting point of field investigation is EM fungal characterization at the species level. This goal can be achieved by combining detailed light microscope-based morphology and anatomical description (morphotyping) [28] with molecular approaches (Sanger sequencing).

Therefore, the present study aims to investigate the abundance and diversity of EM fungi on the fine roots of the dominant shrub species *S. caprea* (1) and the functional traits (exploration types) of the fungal partners associated with *S. caprea* species growing at two sites (2); the study also investigates the relationships of the host tree species and soil chemistry with EM composition (3). The recognition of well-adapted EM strains of *S. caprea* may be an important step before the selection of an EM inoculum for the remediation of poor soils.

2. Materials and Methods

2.1. Test Sites and Sampling

Samples of the fine roots of *S. caprea* and soil were collected from two test sites: Neuhaus (site 1: 54.277556, 12.298263; 11 m above sea level, northern Germany) and Bydgoszcz (site 2: 53.099306, 18.294809; 36 m above sea level, northern Poland). Site 1 is located close to the dunes of the Baltic Sea (maritime climate) and the neighboring dune vegetation (*Ammophila arenaria*); site 2 (temperate climate) is situated near agricultural land (without mechanical or chemical human intervention) with surrounding vegetation, including grasses, e.g., perennial ryegrass (*Lolium perenne*), couch grass (*Elymus repens*), and dicotyledonous plants, e.g., goosefoot (*Chenopodium album*), knotgrass (*Polygonum aviculare*), and common nettle (*Urtica dioica*).

The vegetation at the two test sites was dominated by 20–30-year-old pioneer individuals of *S. caprea*. The soil and root samples of *S. caprea* were taken (20 cm × 20 cm × 20 cm soil cubes) with a sharp knife, c. 20 cm from the stem base of the willow plants. The roots of willows grow highly irregularly with partially concentrated fine root bunches [32]. Bunches of this type were selected for sampling. Five replicates (each from under a different tree; the trees grew 5–6 m apart) were taken randomly from each test site during three sampling seasons in fall 2002, spring 2003, and fall 2003, for a total of fifteen samples per site. The samples were collected in plastic bags and immediately transported at 4 °C to the laboratory for analysis.

2.2. Soil Analysis

The determination of soil pH was performed electrometrically using a glass electrode in 0.01 M CaCl₂ with a soil solution ratio of 1:2.5. The C and N contents of the soils were determined using Foss Heraeus CNS—Vario EL (Elementar Analysentechnik GmbH, Langenselbold, Germany). Total phosphorus (Pt) was determined according to the method of Dick and Tabatabai [33]. The P concentration was analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES, JY 238, HORIBA Jobin Yvon, Lille, France, wavelength 214 nm). The soil texture was determined using Köhn's method [34].

2.3. Determination of Ectomycorrhizas Colonization

The roots from each sample were carefully separated and washed with distilled water for examination. The root samples of *S. caprea* at the two study sites (site 1 and site 2) were taken during three seasons: fall_2002, spring_2003, and fall_2003. A total of 15 samples (two sites × three sampling seasons × five replications) from each of the two sites during one sampling season were examined. As the roots of *S. caprea* grow highly irregularly under natural conditions, the number of root tips varied considerably among the samples taken from different locations and at different times of the year and ranged from 1400 to 5200 root tips (vital and non-vital, mycorrhizal, and non-mycorrhizal). The living roots were identified based on a turgid appearance and the possession of white cortical cells. The EM frequencies associated with each site and each sampling season were calculated (numbers of EM root tips × 100%/total numbers of root tips).

2.4. Morphological and Anatomical Description of the Ectomycorrhizas

The EM types (morphological–anatomical types) were distinguished by the macroscopic characteristics of their fungal mantles, such as color, surface appearance, and the presence of emanating hyphae and hyphal strands, as well as microscopic features such as mantle type [35,36]. The EM types were also classified into exploration types [37]. Based on microscopic identification, two-to-five root tips per EM type were frozen in Eppendorf tubes and stored at –20 °C for molecular analyses.

2.5. Molecular Analysis of the EM Fungi

Analyses of the DNA sequences of the D1/D2 region of the nuclear-encoded large subunit RNA gene (LSU) and the ITS region were used to identify the EM-forming fungi. The DNA was isolated from frozen mycorrhizal samples using the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After extraction, fungal DNA was amplified using the universal primers ITS1 or ITS1F and ITS4 or ITS4B [37,38], and some fungal DNA samples were amplified using the primer LSU with the primer pair NL1–NL4 [39]. The PCR reaction was performed according to Haug's method [40]. The PCR products obtained were purified using the QIAquick protocol (Qiagen, Hilden, Germany). For cycle sequencing, the ABI PRISM Dye-Terminator Cycle Sequencing Ready Reaction Kit was used (Applied Biosystems, Foster City, CA, USA), followed by electrophoresis on an automated sequencer (ABI 373A Stretch, Applied Biosystems, Foster City, CA, USA). The sequencing was carried out according to the manufac-

turer's protocol, but the cycle sequencing reaction volumes were reduced by half. Both forward and reverse DNA strands were sequenced.

The sequences were edited using Sequencher TW Version 4.1 (Gene Codes, Ann Arbor, MI, USA). The NCBI BLAST searches with the LSU and ITS sequences were performed on the GenBank [41] and/or UNITE database [42]. For species identification, the ITS region was chosen since the interspecific variability of this region was shown to be fairly high compared to its intraspecific variation [43]. For the identification of mycorrhizal tips formed by *Inocybe*, we used LSU sequences, since most fungi belonging to this species have less than 3% intraspecific variation in the ITS region of the nuclear ribosomal DNA. Only sequences of EM fungi showing 98% identity over the whole length of the sequence with a known fungus were assumed to be fungal partners of the mycorrhiza. If the identity was 99% or 100%, it was certain that this taxon was involved in the mycorrhiza. The DNA sequences determined for this study were submitted to GenBank with the accession numbers. The evolutionary history was inferred by using the maximum likelihood method and the Jukes–Cantor model. Initial tree(s) for the heuristic search were obtained automatically by applying the neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then by selecting the topology using the superior log likelihood value. Evolutionary analyses were conducted in MEGA X [44].

2.6. Statistical Analysis

Using the Statistica software ver.13.3 (StatSoft, Kraków, Poland), one-way analysis of variance (ANOVA), followed by the Newman–Keuls post hoc test with a significance level of $p < 0.05$, was carried out to demonstrate the differences between the two test sites during the three sampling seasons in terms of the degree of EM colonization in the fine root tips of *S. caprea*.

3. Results

The chemical and physical properties of the soil (<2 mm) at the two sites are presented in Table 1. The total P concentration of the soil was below the standards observed in the agricultural soils or short rotation coppices (4.5–6.8 mg/100 g; [45]), which is why these sites were classified as nutrient-deficient. The soil pH in which *S. caprea* was growing was 6.5 at both test sites.

Table 1. Chemical and physical properties of soils (0–10 cm depth) at the two test sites: site 1 (Neuhaus, northern Germany) and site 2 (Bydgoszcz, northern Poland). Abbreviations: C_{org}—organic carbon, N_t—Total nitrogen, P_t—Total phosphorous.

	pH (CaCl ₂)	C _{org} (g kg ⁻¹)	N _t (g kg ⁻¹)	P _t (g kg ⁻¹)	Clay (g kg ⁻¹)	Silt (g kg ⁻¹)	Sand (g kg ⁻¹)
Site 1 (NEU)	6.5	54.6	4.3	1.3	20	40	940
Site 2 (BYD)	6.5	58.5	5.1	0.5	60	90	850

The degree of EM colonization in *S. caprea* was significantly higher at site 1 during fall (in both the fall sampling seasons) compared to site 2 (Figure 1). The ectomycorrhizal fungal colonization was generally poor in *S. caprea* during spring at both test sites. The number of different EM morphotypes per site ranged from nine at site 2 to 10 at site 1 (Table 2).

Table 2. The sampling seasons: fall 2002 (1), spring 2003 (2), fall 2003 (3) and description of ectomycorrhizal morphotypes of *S. caprea* at two test sites: site 1 (NEU) and site 2 (BYD). The nomenclature corresponds to that of Agerer (1991) [35].

Taxa	Sampling Seasons			Morphotype Description							
	(1)	(2)	(3)	Color	Shape of Mycorrhiza	Surface of Mantle	Cystidia	Emanating Hyphae	Rhizomorph	Exploration Types	Structure of Mantle
<i>Tomentella</i> sp. 1 (NEU14)			+	Dark brown/black	Unramified, straight	Grainy	Bristle-like, type A	Infrequent, septate with clamps	n.o.	medium-distance smooth	Pseudoparenchymatous, type M
<i>Tomentella</i> sp. 2 (BYD9)			+	Gold brown	Unramified, straight	Rough	Bristle-like, type A	Infrequent, septate with clamps	n.o.	short distance	Outer layers: pseudoparenchymatous, type L; inner layers: plectenchymatous, type A–B
<i>Tomentella</i> sp. 3 (NEU1)	+			Dark brown	Unramified or ramified, straight	Grainy	n.o.	n.o.	n.o.	short distance	Pseudoparenchymatous, type P
<i>Tomentella</i> sp. 4 (NEU11)		+		Dark brown	Ramified, straight	Woolly with soil particles	n.o.	Branched, septate with clamps	n.o.	short distance	Pseudoparenchymatous, type Q
<i>Tomentella</i> sp. 5 (BYD2)	+			Dark brown	Unramified or ramified, straight	Woolly with soil particles	n.o.	Branched, septate with clamps	n.o.	short distance	Pseudoparenchymatous, type Q
<i>Tomentella</i> sp. 6 (BYD3)	+			Dark brown/black	Unramified, bent	Grainy	n.o.	n.o.	n.o.	short distance	Plectenchymatous, type A
<i>Tomentella</i> sp. 7 (BYD5)		+		Dark brown	Ramified, bent	Rough	n.o.	Branched, rarely septate with clamps	n.o.	short distance	Plectenchymatous, type A
<i>Tomentella</i> sp. 8 (BYD6)		+		Black	Unramified, straight	Grainy	n.o.	Branched, septate with clamps	n.o.	short distance	Plectenchymatous, type A
<i>Hebeloma populinum</i> (NEU13)			+	White-beige with shining mantle	Unramified, straight	Cottony/woolly	n.o.	Branched, septate with clamps	Infrequent, undifferentiated	short distance	Plectenchymatous, type B
<i>Hebeloma</i> sp. (NEU4)	+			White-beige with shining mantle	Unramified, straight	Cottony	n.o.	Branched, septate with clamps	n.o.	-	Plectenchymatous, type A
<i>Cortinarius atrocoeruleus</i> (NEU7)	+			Gold orange	Unramified, bent	Woolly	n.o.	Branched, septate with clamps	Infrequent, smooth	medium-distance fringe	Plectenchymatous, type A

Table 2. Cont.

Taxa	Sampling Seasons			Morphotype Description							
	(1)	(2)	(3)	Color	Shape of Mycorrhiza	Surface of Mantle	Cystidia	Emanating Hyphae	Rhizomorph	Exploration Types	Structure of Mantle
<i>Inocybe</i> sp. 1 (NEU10)		+	+	White-beige	Unramified, slightly bent	Smooth	n.o.	n.o.	n.o.	short distance	Plectenchymatous, type A
<i>Inocybe hirtella</i> (BYD1)	+			White-beige	Monopodial-pinnate	Smooth	n.o.	n.o.	n.o.	short distance	Plectenchymatous, type A–E
<i>Laccaria</i> cf. <i>ochropurpurea</i> (NEU3)	+			White	Unramified, straight	Smooth	n.o.	n.o.	n.o.	medium-distance smooth	Plectenchymatous, type A
<i>Tuber</i> sp. 1 (NEU5)	+			Gold-brown	Unramified, slightly bent	Smooth	Awl-shape, type A	n.o.	n.o.	short distance	Pseudoparenchymatous, type H–M
<i>Tuber maculatum</i> (BYD4)	+			Gold-brown	Unramified, beaded	Smooth	Awl-shape, type A	n.o.	n.o.	short distance	Pseudoparenchymatous, type H–M
<i>Tuber</i> sp. 3 (BYD8a)			+	Gold-brown	Unramified, beaded	Smooth	Awl-shape, type A	n.o.	n.o.	short distance	Pseudoparenchymatous, type H–M
<i>Cenococcum geophilum</i> (NEU9)		+	+	Black	Unramified, straight	Grainy	n.o.	Unbranched, not septate	n.o.	short distance	Plectenchymatous, type G
<i>Phialophora finlandia</i> (NEU2)	+			Dark brown	Unramified, slightly bent	Grainy	Missing	Branched, septate with clamps	n.o.	short distance	Pseudoparenchymatous, type Q

n.o.—not observed; Present (+).

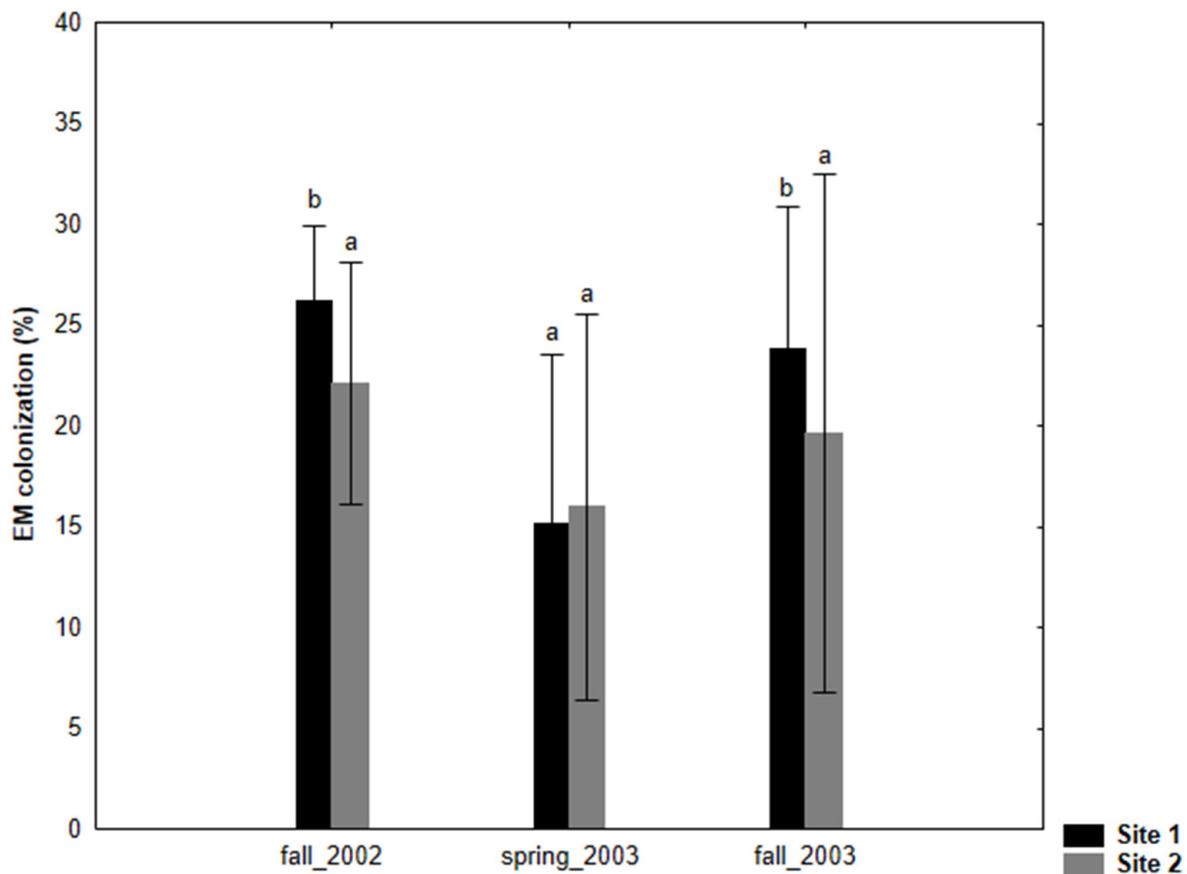


Figure 1. Ectomycorrhizal (EM) fine root colonization of *Salix caprea* (% colonized fine root tips, mean value \pm SD) at 0–10 cm soil depth grown at two test sites (site 1: NEU and site 2: BYD) during three sampling seasons (fall 2002, spring 2003, fall 2003). The small letters denote significant differences between sampling sites in each year.

Identification of Ectomycorrhizal Morphotypes

A total of 1400 to 5200 root tips (vital and non-vital, mycorrhizal and non-mycorrhizal) of *S. caprea* from the two sites, site 1 and site 2, taken during three sampling seasons, namely, fall_2002, spring_2003, and fall_2003, were analyzed. Nineteen different morphotypes of *S. caprea* were identified (Figure 2). These morphotypes mainly comprised the phyla Basidiomycota (14 morphotypes) and Ascomycota (five morphotypes) (Figures 3 and 4). The Basidiomycota group was dominated by the families *Thelephoraceae* (eight taxa), *Hymenogastraceae* (two taxa), and *Inocybaceae* (two taxa), followed by *Cortinariaceae* and *Hydnangiaceae*, while Ascomycota was represented by the families *Tuberaceae* (three taxa), *Gloniaceae*, and *Herpotrichiellaceae*.

Seven taxa were identified at the species level: *Hebeloma populinum*, *Cortinarius atrocoeruleus*, *Inocybe hirtella*, *Laccaria* cf. *ochropurpurea*, *Tuber maculatum*, *Cenococcum geophilum*, and *Phialophora Finlandia* (Figures 3 and 4). Twelve were identified at the genus level: *Tomentella* sp. 1–8, *Hebeloma* sp. 1, *Inocybe* sp. 1, and *Tuber* sp. 1–2 (Table 3 and Figure 3). The EM roots of *Inocybe* sp. 1 and *C. geophilum* were sampled at site 1 during two sampling seasons: spring_2003 and fall_2003. The other sixteen morphotypes were found only once. The dominant morphotypes of *S. caprea* were formed by *Thelephoraceae* (Table 3 and Figure 3). They were found at both sampling sites and across all sampling seasons (with the exception of site 1 in spring_2003) (Figure 3).

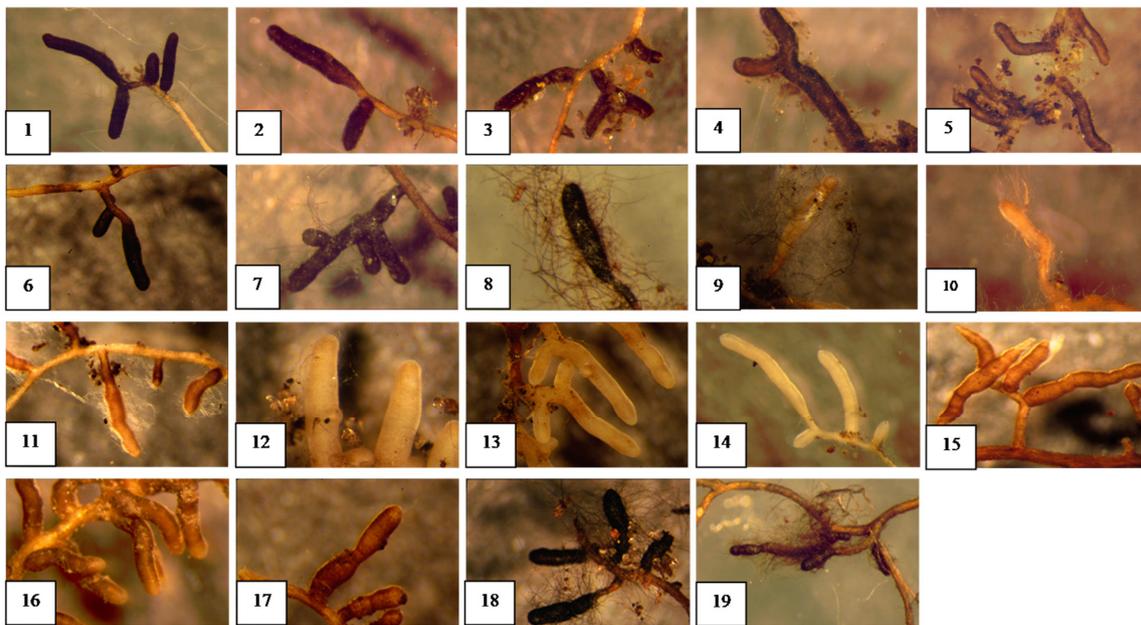


Figure 2. Ectomycorrhizal (EM) morphotypes on *Salix caprea* at the two test sites, site 1 (NEU) and site 2 (BYD). Ectomycorrhizal morphotypes: (1) *Tomentella* sp. 1 (NEU14), (2) *Tomentella* sp. 2 (BYD9), (3) *Tomentella* sp. 3 (NEU1), (4) *Tomentella* sp. 4 (NEU11), (5) *Tomentella* sp. 5 (BYD2), (6) *Tomentella* sp. 6 (BYD3), (7) *Tomentella* sp. 7 (BYD5), (8) *Tomentella* sp. 8 (BYD6), (9) *Hebeloma populinum* (NEU13), (10) *Hebeloma* sp. (NEU4), (11) *Cortinarius atrocoeruleus* (NEU7), (12) *Inocybe* sp. 1 (NEU10), (13) *Inocybe hirtella* (BYD1), (14) *Laccaria* cf. *ochropurpurea* (NEU3), (15) *Tuber* sp. 1 (NEU5), (16) *Tuber maculatum* (BYD4), (17) *Tuber* sp. 3 (BYD8a), (18) *Cenococcum geophilum* (NEU9), (19) *Phialophora finlandia* (NEU2).

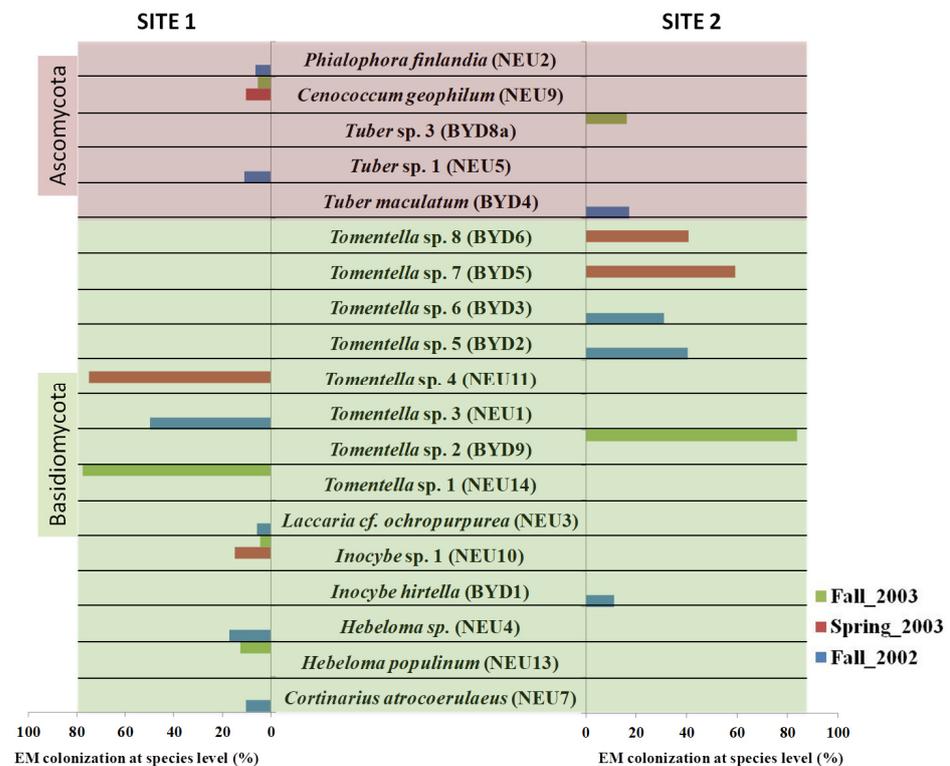


Figure 3. The colonization percentage of the identified EM morphotypes at the species level at the two test sites: site 1 (Neuhaus) and site 2 (Bydgoszcz) during three sampling seasons (fall 2002, spring 2003, fall 2003).

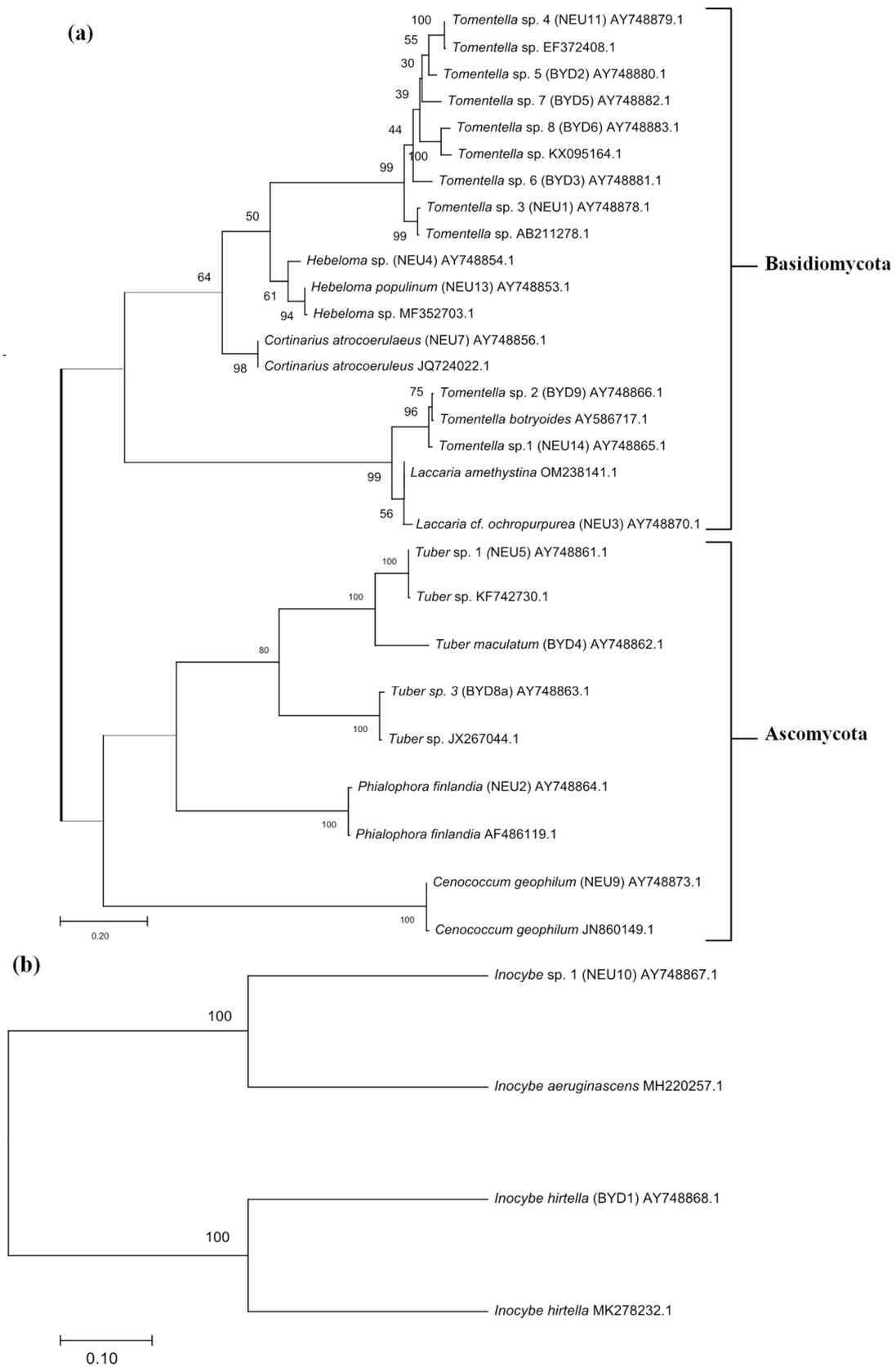


Figure 4. Phylogenetic tree depicting the different ectomycorrhizal taxa identified from two phyla Ascomycota and Basidiomycota based on ITS (a) and LSU (b) regions of the nuclear ribosomal DNA. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

Table 3. Molecular identification and closest BLAST sequence match of ectomycorrhizas sampled from *S. caprea* from two sites, site 1 (NEU) and site 2 (BYD), during three sampling seasons (fall 2002, spring 2003, and fall 2003).

Taxa	T bp	Acc nr	BLAST and/or UNITE Search Results	H bp	%
<i>Tomentella</i> sp. 1 (NEU14)	650 *	AY748865	<i>Tomentella botryoides</i> [AY586717] †	640/649	99
			<i>Tomentella sublilacina</i> [UDB002972] ††	635/651	97
<i>Tomentella</i> sp. 2 (BYD9)	652 *	AY748866	<i>Tomentella botryoides</i> [AY586717] †	644/651	99
			<i>Tomentella sublilacina</i> [UDB002972] ††	637/653	97
<i>Tomentella</i> sp. 3 (NEU1)	727 *	AY748878	<i>Tomentella</i> sp. [AB211278] †	643/649	99
			Ectomycorrhiza of <i>S. reinii</i> [AB096871] †	645/654	99
<i>Tomentella</i> sp. 4 (NEU11)	705 *	AY748879	<i>Tomentella</i> sp. [EF372408] †	617/621	99
			<i>Tomentella</i> sp. [UDB003321] ††	616/647	95
<i>Tomentella</i> sp. 5 (BYD2)	679 *	AY748880	<i>Tomentella</i> sp. [EF644156] †	618/630	99
			<i>Tomentella coerulea</i> [UDB003307] ††	611/645	94
<i>Tomentella</i> sp.6 (BYD3)	670 *	AY748881	<i>Tomentella</i> sp. [EU668199] †	641/673	96
			<i>Tomentella stuposa</i> [UDB002429] ††	602/652	92
<i>Tomentella</i> sp. 7 (BYD5)	697 *	AY748882	<i>Tomentella</i> sp. [HM146872] †	667/685	98
			<i>Tomentella coerulea</i> [UDB003329] ††	628/645	97
<i>Tomentella</i> sp. 8 (BYD6)	710 *	AY748883	Uncultured Thelephoraceae ThelPop [GU990355] †	682/684	99
			<i>Tomentella cinerascens</i> [TCU83483] †	647/694	94
			<i>Tomentella subtestacea</i> [UDB000034] ††	610/671	90
<i>Hebeloma populinum</i> (NEU13)	670 *	AY748853	<i>Hebeloma populinum</i> [EF644107] †	659/663	99
<i>Hebeloma</i> sp.(NEU4)	776 *	AY748854	<i>Hebeloma mesophaeum</i> [FJ845404] †	746/767	98
<i>Cortinarius atrocoeruleus</i> (NEU7)	514 *	AY748856	<i>Cortinarius atrocoeruleus</i> [AY083178] †	512/515	99
			<i>Cortinarius atrocoeruleus</i> [UDB001011] ††	512/515	99
<i>Inocybe</i> sp. 1 (NEU10)	599 **	AY748867	<i>Inocybe godeyi</i> [FN550897] †	578/600	97
			<i>Inocybe queletii</i> [EU307813] †	581/604	97
<i>Inocybe hirtella</i> (BYD1)	624 **	AY748868	<i>Inocybe hirtella</i> [AM882932] †	623/624	99
			<i>Inocybe</i> cf. <i>hirtella</i> [EU307826] †	611/626	98
<i>Laccaria</i> cf. <i>ochropurpurea</i> (NEU3)	637 *	AY748870	<i>Laccaria ochropurpurea</i> [AF261494] †	622/630	99
<i>Tuber</i> sp. 1 (NEU5)	678 *	AY748861	<i>Tuber</i> sp. [GQ267493] †	673/677	99
<i>Tuber maculatum</i> (BYD4)	677 *	AY748862	<i>Tuber maculatum</i> [AJ969627] †	665/667	99
			<i>Tuber maculatum</i> [UDB000121] ††	665/667	99
<i>Tuber</i> sp. 3 (BYD8a)	759 *	AY748863	<i>Tuber rufum</i> f. <i>nitidum</i> [FM205606] †	465/506	92
<i>Cenococcum geophilum</i> (NEU9)	565 *	AY748873	<i>Cenococcum geophilum</i> [AY394919] †	554/565	99
<i>Phialophora finlandia</i> (NEU2)	745 *	AY748864	<i>Phialophora finlandia</i> [AF486119] †	736/749	99

Taxa = the assigned species or genus names according to sequence homology with sequences from GenBank † or UNITE ††. T bp = length of the sequence. * ITS sequence. ** LSU sequence. Acc nr—GenBank accession number. H bp = the number of base pairs over which the homology is calculated. % = the degree of homology, presented as the percent base pair identity between the match and the sequence of the mycorrhizal fungus. BLAST (National Centre for Biotechnology Information, Bethesda, MD, USA): threshold level > 97%; UNITE: threshold level 1.5%.

Based on the morphotyping of the EM roots, we observed the black, dark brown, or brown color of the hyphal mantle, which was characteristic of all *Thelephoraceae* morphotypes (Figure 2). Cystidia were observed in three morphotypes of *Tomentella* sp., and rhizomorphs were found only in one. All these morphotypes were identified at the genus level (*Tomentella* sp. 1–8) (Table 2). As most *Inocybe* species have less than 3% intraspecific variation in the ITS region of the nuclear ribosomal DNA [46], we used the LSU sequences of these fungal partners for identification (Table 3 and Figure 4). All *Hebeloma* species have very similar morphology (Table 3). Their characteristics include a white beige shining mantle and cottony emanating hyphae. The *Cortinariaceae* had a plectenchymatous mantle.

The white and smooth EM fungi of *Laccaria* cf. *ochropurpurea* were found at site 1 (Table 2 and Figure 2).

Only five morphotypes were identified among the ascomycetes. The EM fungi of *Tuber* sp. 1 were found at site 1, while *T. maculatum* and *Tuber* sp. 2 were found at site 2 (Table 2). All the EM roots of *Tuber* sp. were gold-brown with cystidia (Table 2 and Figure 2). *Cenococcum geophilum* (two sampling seasons: spring_2003 and fall_2003) and *P. finlandia* (fall_2002) were found only at site 1 (Table 2).

The exploration types of the identified EM fungi were classified as medium-distance smooth (*Tomentella* sp. 1–8 and *Laccaria* cf. *ochropurpurea*) and medium-distance fringe (*Cortinarius atrocoeruleus*), while the other taxa were short-distance exploration types with no rhizomorphs (Table 2).

4. Discussion

Combining morphotyping with molecular approaches is a well-accepted method for the characterization of different ectomycorrhizas and the analysis of their community structure [47]. Following this strategy, we highlighted the importance of combining these two methods for the selection and identification of the ectomycorrhizal diversity of *Salix caprea*, a pioneer tree at two sites.

Species of *Betula*, *Salix*, *Populus*, *Alnus*, and *Sorbus aucuparia* L. represent deciduous pioneer tree species in Europe. Pioneer tree species are very common in early successional stages and, due to their rapid regeneration and high seed production, they mitigate the negative consequences associated with disturbed areas, such as soil erosion and the loss of nutrients [48]. It is known that EM fungi may also aid in the establishment and facilitation of early colonizing tree species at disturbed sites. A study by Nara et al. [49] reported that the first plant to colonize the volcanic desert on Mount Fuji, Japan, was an alpine dwarf willow *Salix reinii*, which is strictly ectomycorrhizal. Similar research at the same site showed that *Salix* seedlings developed easily only when beside the established *Salix* shrubs, and no seedling growth was observed in areas without established *Salix* [50]. Further investigations proved that the growth of successive seedlings was dependent on the EM association of the neighboring EM shrubs, which significantly improved as the early established *Salix* shrubs facilitated the subsequent establishment of conspecific seedlings by providing EM fungal symbionts [51].

In the present study, a total of 19 different EM morphotypes were characterized in *S. caprea* from both test sites using an EM morphology examination and ITS/LSU sequencing (Figures 3 and 4). The molecular analysis of EM roots indicated that the EM fungal communities of *S. caprea* from the sites contained a diverse and broad array of fungi, including basidiomycetes and ascomycetes. Well-known fungal associates of the members of the *Salicaceae* (*Salix* and *Populus*) are, for example, *Lactarius controversus*, *Russula atrorubens*, *Russula persicina*, and *Tricholoma cingulatum* [24,52]. Many species of *Cortinarius* [53,54] and *Hebeloma* [55] also preferentially associate with members of the *Salicaceae* family. It is striking that only EM fungi formed by Thelephorales (*Tomentella* spp.) and Agaricales (*Inocybe* spp.) were observed at both the investigated sites. In addition, *Tomentella* spp., *Inocybe* spp., and *Cenococcum geophilum* were found during both spring and fall sampling seasons. These three EM species can be classified as early colonizers of *S. caprea*, since they occur in their host in the early stages of growth. The other morphotypes (*Hebeloma* sp. 1, *Hebeloma populinum*, *Cortinarius atrocoeruleus*, *Laccaria* cf. *ochropurpurea*, *Tuber* sp. 1–2, *Tuber maculatum*, and *Phialophora finlandia*) occurred only during the fall sampling season, mostly at site 1, though *Tuber* species were also found at site 2. There are two explanations for this phenomenon: either the host tree determines the type of EM recruitment, or the spatial heterogeneity may be very high and, consequently, with each sampling, only a small portion of the fungi is cached. Thus, we speculate that further sampling may result in more species at the investigated sites. A report on the underground EM fungal diversity of the pioneer tree *S. reinii* shows that species of *Thelephoraceae*, *Cortinarius*, and *Hebeloma* were recruited in the later stages of *S. reinii* growth and that a limited assimilate supply

from the plant host inhibited the colonization of some fungi (such as *Inocybe lacerna* and *Scleroderma bovista*) and supported the colonization of non-conspicuous sporocarp species such as *Sebacina*, *Thelephoraceae*, and *C. geophilum* [49].

The diversity of EM assemblages also manifests in the functional diversity that is relevant to tree host health and ecosystem functioning [56]. The potential functions of the identified EM fungal isolates are presented in Table 4. Furthermore, the exploration type may also partly explain the dynamics of EM fungi in their host. In this study, *Tomentella* sp. 1–8 and *L. ochropurpurea*, which were classified as being of the medium-distance smooth exploration type, had internally undifferentiated rhizomorphs, and the ectomycorrhizal mantles had few or no radiating hyphae close to the root and a smooth appearance. On the other hand, the medium-distance fringe types, i.e., *C. atrocoeruleus*, had rhizomorphs with a hairy surface, and they formed fans of emanating hyphae with extended contacts with the soil. The others were identified as short-distance exploration types characterized by the production of abundant, short, non-aggregated hyphae in the vicinity of the root tip; they also produced no rhizomorphs [28]. In the present study, most of the identified EM fungal species were short-distance exploration types, which are known to be present in the fluctuating nutrient pools that are characteristic of disturbed soils [57]. Previously, as Beccarra et al. (2009) [58] and Nara et al. (2003) [49] have suggested, species of *Inocybe* and *Tomentella* were well-known as colonizers of EM plants in disturbed or primary habitats.

The most common morphotypes in this study were formed by *Tomentella* sp., which is a known ‘short-distance explorer’ and is found in forests as well as in disturbed soils [10,28,59]. Most tomentelloid species have a worldwide distribution, with the highest species richness being found in temperate coniferous and broad-leaved forests [60]. The genus *Thelephora* displays characteristics similar to those of tomentelloid fungi and comprises about 40 species [61]. The ecological characteristics of tomentelloid fungi remain largely unexplored [61,62]. The color of *Tomentella* sp. morphotypes in this study was always dark (from completely black through to dark brown and brown). Their characteristic dark color results from the incorporation of melanin, a natural dark pigment and a common fungal wall component. Melanized cell walls are not common among EM fungi, except for tomentelloid species [62] and a few other EM fungal taxa. Fungal melanin may act as a barrier between fungal cells and their surrounding environment and protect them against physical, chemical, and biological stress [63]. In addition, the melanized hyphae of the outer mantle layer and the extramatrical mycelium may function as protective barriers between the external environment and the active fungus–plant symbiosis inside the root.

Inocybe sp. is a widely distributed species (mainly generalist fungi) [64]. Ectomycorrhizal fungal species of *I. lacera* were previously found during the early primary succession of *S. reinii* [50]. In our study, the morphotypes of *Inocybe* sp. on *S. caprea* were characteristic of both sites and occurred across all sampling seasons. *Inocybe* spp. are abundant in contaminated sites and dry and disturbed habitats and are tolerant to drought conditions [10,11,65]. Another species, *Cenococcum geophilum*, was observed in both spring and fall of 2003, but only at site 1. This species is cosmopolitan and is well recognized for its extremely wide host and habitat range (e.g., it is found in post-fire soils and colonizes exotic trees) [66,67]. According to Park [56], the early stage of the symbiosis is characterized by white and brown EM fungi, while the black EM roots characterize the most mature stage of EM development. This was confirmed in the present study.

Truffles of the genus *Tuber* are ascomycetous fungi which form EM with the roots of trees such as *Quercus*, *Populus*, and *Salix* [24,52,68], as well as shrubs such as *Cistus* and *Corylus* [69,70]. In the present research, *Tuber* sp. was isolated from both sites, but was more common at site 2 during fall. *Tuber* spp. are known to adapt and tolerate extreme conditions [11,24,52].

The genus *Cortinarius* has a widespread distribution in pioneer trees [53,54]. Many studies have provided reports on different species of *Cortinarius* found on *Salix* spp.: *C. decipiens* and *C. tenebricus* [71], on *S. barratiana*—*Cortinarius* spp. [54], on *S. herbacea*—*C. favrei* [72], on *S. repens*—*Cortinarius* spp. [73], and on *S. reinii*—*C. alboviolaceus* [49]. Similarly,

this genus was very common in the present study, but none of the above-mentioned fungal species was identified. The fungi with gold-orange EM roots from site 1 were identified as *C. atrocoeruleus*. *C. atrocoeruleus* has been previously reported to play a role in assisting plant nutrition [10,74].

Two morphotypes of *Hebeloma* sp., identified as *H. populinum* and *Hebeloma* sp. 1, were found at site 1. *Hebeloma* species have a broad host range with little ambiguity as the occurrence of these species is naturally associated with various gymnosperms and dicots in the field [75]. *Hebeloma populinum*, which was found in this study, is also frequently reported as a specialist fungal partner of *Populus* spp. [10,11].

Laccaria sp. has been reported to form EM associations with numerous tree species, including *Pinaceae*, *Dipterocarpaceae*, *Fagaceae*, *Myrtaceae*, *Tiliaceae*, and *Salicaceae* [76]. An association with *S. caprea* and *L. ochropurpurea* was observed at site 1. Hryniewicz and colleagues isolated *L. ochropurpurea* from *S. caprea* obtained from an ore mining site [24]. The presence of *L. ochropurpurea* increased pathogen resistance in chestnut plantations (*Castanea dentata*) [77].

A dark brown morphotype with abundant emanating hyphae, a pseudo-parenchymatous mantle, and fan-like rhizomorphs was found at site 1: it was formed by *Phialophora finlandia*. Fungus *P. finlandia* is commonly referred to as *Mycelium radices-atrovirens* (MRA) or dark septate endophyte (DSE) [78] and forms ectomycorrhiza and/or ectendomycorrhiza with hardwoods and conifers, respectively [79,80]. *Phialophora finlandia* has been reported to produce EM morphotypes ranging from light amber, brown, or bicolored to black [79,80], corresponding to the observed morphotype in the present study. This strain can play a role in plant growth promotion and survival in disturbed soils [81] and in the nursery of *Picea abies* [82].

Table 4. Potential role of the identified ectomycorrhizal strains reported in the research literature. Abbrev., not defined (ND).

Identified Ectomycorrhiza	Isolated from Pioneer Tree sps.	Generalist or Specialist Fungi	Type of Environment	Potential Function of EM	NCBI Accession No.	Citation
<i>Tomentella</i> sp.	<i>Populus</i> sp.	Generalist	Forest/dry habitat/heavy metal-polluted area	Tolerance to drought or increased concentrations Of heavy metals in the soil	JQ898563	[10]
	<i>Salix alba</i>	Generalist	Saline meadow near a soda factory	Adaptation to saline conditions	KP745608	[59]
<i>Hebeloma populinum</i>	<i>Populus</i> sp.	Specialist	Forest/dry habitat	Tolerance to drought	JQ898545	[10]
	<i>Populus tremula</i>	Specialist	Former lead/zinc smelter	Fungal immobilization of heavy metals	EF644130	[11]
<i>Hebeloma</i> sp.	<i>Populus</i> sp.	Generalist	Heavy metal-polluted area	Tolerance to increased concentrations Of heavy metals in the soil	JQ898546	[10]
	<i>Salix caprea</i>	Generalist	Former ore mining area, Heavy metal-polluted soil	Tolerance against toxic metal concentrations in the soil	AY748855	[24]
<i>Cortinarius atrocoeruleus</i>	<i>Populus</i> sp.	Generalist	Forest	Plant nutrition	JQ898541	[10]
	<i>Quercus</i> sp.	Generalist	Mature mixed forest in the southern Appalachian Mountains	Supplies carbohydrates to seedlings	AY656961	[74]
<i>Inocybe</i> sp.	<i>Populus</i> sp.	ND	Forest/dry habitat/heavy metal-polluted area	Tolerance to drought or increased concentrations of heavy metals in the soil	JQ898552	[10]
	<i>Populus tremula</i>	Generalist	Former lead/zinc smelter	Fungal immobilization of heavy metals	EF644135	[11]
<i>Inocybe hirtella</i>	<i>Populus</i> sp.	Nd	Forest	Plant nutrition	JQ898549	[10]
	<i>Quercus ilex</i>	Nd	Mediterranean forest	Adaptation to climate change	ND	[65]
<i>Laccaria ochropurpurea</i>	<i>Salix caprea</i>	Nd	Former ore mining area, heavy metal-polluted soil	Tolerance against toxic metal concentrations in the soil	AY748870	[24]
	<i>Castanea dentata</i>	Nd	Chestnut plantation	Resistance to pathogens	MK077757	[77]

Table 4. Cont.

Identified Ectomycorrhiza	Isolated from Pioneer Tree sps.	Generalist or Specialist Fungi	Type of Environment	Potential Function of EM	NCBI Accession No.	Citation
<i>Tuber</i> sp.	<i>Salix caprea</i>	Nd	Former ore mining area, heavy metal-polluted soil	Tolerance against toxic metal concentrations in the soil	AY748870	[24]
	<i>Populus tremula</i>	Generalist	Former lead/zinc smelter	Fungal immobilization of heavy metals	EF644167	[11]
<i>Tuber maculatum</i>	<i>Populus alba</i>	Nd	Marsh in nature reserve	Adaptation to ecological conditions	HG937633	[52]
	<i>Populus nigra</i> , <i>Pinus nigra</i> , <i>Tilia x vulgaris</i>	Generalist	Man-made park, Mediterranean area	Nutritional exchange or competition for the main nutritional resources in fungal communities	ND	[83]
<i>Cenococcum geophilum</i>	<i>Carya ovata</i>	Generalist	Old experimental plantation of exotic trees, temperate mixed forest	Acclimatization of exotic trees outside of their native Range	ND	[67]
	<i>Quercus</i> sp.	Generalist	Oak savanna/ forest, post-fire site	Survival in the extreme environment (after burning)	ND	[66]
<i>Phialophora finlandia</i>	<i>Picea abies</i>	Generalist	Forest nursery	Improvement of the growth and survival of outplanted tree seedlings	DQ508807	[81]
	<i>Pinus sylvestris</i>	Generalist	Forest, post-agricultural land (slightly polluted soil) and buffer zone of the copper smelter (heavy metal-polluted soil)	Adaptation to different soil conditions	ND	[83]

5. Conclusions

In the present study, only the EM symbioses formed by Thelephorales (*Tomentella* spp.) and Agaricales (*Inocybe* spp.) were observed at both investigated sites. They can be classified as early colonizers of *S. caprea*, since they are well known to occur in their host during the early stages of growth. The identified EM taxa were mainly short-distance exploration types, and these taxa typically occurred in the fluctuating nutrient pools that are characteristic of disturbed soils. The EM fungi described in this study were previously reported by other researchers in disturbed environments, e.g., dry and polluted soils, and they have potential functions in the adaptation and growth of *S. caprea*.

Author Contributions: K.H. participated in all analyses and wrote the first version of the manuscript. BF participated in the preparation of the manuscript. K.H., B.U.F. and J.S. analyzed the results and the statistical output. K.H. and C.B. performed sampling at the locations, selected plant genotypes for the experiment, and provided input for the manuscript. C.B. conducted soil analyses. K.H. designed and managed the field and lab experiments. C.B. was the respondent for the study conceptualization, supervision, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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