

## Article

# Changes in Chemical Properties and Fungal Communities of Mineral Soil after Clear-Cutting and Reforestation of Scots Pine (*Pinus sylvestris* L.) Sites

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**Abstract:** This study aimed to assess the changes in chemical properties and fungal communities in the upper mineral soil layer in managed Scots pine (*Pinus sylvestris* L.) ecosystems. Study sites were located in the three largest *P. sylvestris* massifs in Lithuania, and six sampling sites, representing different development stages of the *P. sylvestris* forest ecosystem, were selected in each of them: mature *P. sylvestris* forest stands; clear-cuts of former *P. sylvestris* mature stand; and the *P. sylvestris* plantations aging from the 1st to 4th year. High-throughput sequencing was performed to evaluate the soil fungus community at clear-cuts, early-stage reforested sites, and mature forests in Lithuania. This study has shown that, among other chemical soil parameters, the mean concentrations of mineral nitrogen (N), total phosphorus (P), and P<sub>2</sub>O<sub>5</sub> were slightly higher in the clear-cut sites, and significantly higher in the 1st year plantations compared to the mature forests. The quality filtering after PacBio sequencing showed the presence of 60,898 high-quality fungal sequences, and 1143 fungal operational taxonomic units (OTUs). The most abundant fungal OTU in our study was *Archaeorhizomyces* sp. 5425\_1. In total, 70 mycorrhizal fungal OTUs were found in the soil samples at the studied sites. The most abundant ectomycorrhizal fungus identified was *Amanita fulva* (Schaeff.) Fr. The highest amount of ectomycorrhizal fungal OTUs was found in the clear-cut sites and in the mature forests. The concentrations of mineral N and P<sub>2</sub>O<sub>5</sub> in the upper mineral soil layer did not significantly affect fungal OTUs diversity. Conversely, a relatively strong correlation was obtained between the number of mycorrhizal fungal OTUs and the concentrations of total N and soil organic carbon (SOC), as well as between the numbers of saprotrophic fungal OTUs and the concentration of magnesium ions (Mg<sup>2+</sup>).

**Keywords:** *Pinus sylvestris*; high-throughput sequencing; upper mineral soil layer; soil chemistry; fungal communities; ectomycorrhizal fungi



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## 1. Introduction

Major parts of the world's forests are managed to achieve different goals, combining the controversial challenges of producing higher amounts of timber and simultaneously conserving biodiversity [1]. Intensive forest management often leads to drastic changes in forest ecosystems such as loss of habitats and biodiversity [2–5]. Temperate and boreal forests are increasingly affected by anthropogenic disturbances when forest biomass is harvested at various intensities. Clear-cutting is a common forest management practice to achieve maximum efficient harvesting, but it results in severe abiotic and biotic changes in the ecosystem during the reforestation stages [6,7]. Reforestation of clear-cut sites with suitable tree species is a priority for sustainable forest management, but it is also essential to maintain the stability of other live components of ecosystems during forest harvesting

and reforestation stages. The forest ecosystem components, related by common habitats, and trophic, symbiotic, and mutualistic relationships, are important for the maintenance of sustainable forest ecosystems [8–11].

Intensive harvesting of forest biomass promotes changes in chemical properties of forest soil and soil mycobiota, including mycorrhizal fungi, which are important for seedling growth [12–16]. The removal of forest biomass reduces the soil organic matter and carbon stocks. Relatively high removals of forest biomass could lead to nutrient loss, which could potentially affect soil biodiversity and reduce site productivity in the long term. Soil organic matter, providing nutrients for soil biota and plants, maintains the ability to retain soil water and air, and, therefore, it is one of the essential conditions for tree growth [17].

Mycorrhizal fungi are obligate symbionts with forest tree species, where they scavenge soil nutrients and water from the soil in exchange for photosynthate from the tree [18]. Without their fungal symbionts, the trees cannot acquire enough nutrients, and fungi have insufficient energy to carry out their life cycle without the trees. Intensive forest harvesting alters the changes in ECM fungal communities by changing the age structure and species composition of the forest trees and other plants serving as hosts.

In recent decades, numerous studies on the abundance and diversity of soil fungi and their interrelationship with plants have been conducted [19–22]. Forest soil properties, vegetation and tree species, cover, human activities, and forest management regimes affect all fungal communities. All ecophysiological groups of soil fungi, saprotrophs, symbionts, and pathogens are involved in decomposition and mineralization processes [23,24]. Ectomycorrhizal (ECM) fungi are particularly sensitive to forest harvesting [25,26] because they depend on the carbon released from tree hosts [27,28]. Intensive forest management, shortly disturbing the balance of the ecosystem, affects both the abundance and species composition of mycorrhizal fungi. Therefore, the observed changes in the structure and species composition of the mycorrhizal community may be one of the indicators caused by the impact of clear-cutting on the forest ecosystem [29].

In Lithuania, the composition of fungal communities in forest soil and the impact of forest management regimes, including clear-cutting, on soil mycobiota have not been studied extensively. Among other stands, *Pinus sylvestris* L. stands are economically valuable and cover 34.5% of the total forest area in Lithuania [30]. Due to its widespread distribution globally and regionally, *P. sylvestris* is considered a suitable species for reforestation and afforestation in the European region [31].

Recently, the differences in soil fungal communities in managed and unmanaged forests have been studied in other countries, such as Germany [32–34] and France [35]. In Poland, the comparative studies were mainly conducted on the abundance and diversity of soil mycobiota in the forest after the applications of different harvesting of woody debris and pre-planting soil preparations [36,37]. The influence of different forest litter types on tree growth and ectomycorrhizal formation of *P. sylvestris* seedlings were reported in Lithuania [38,39].

The aim of this study was to evaluate the changes of chemical properties and fungal communities in the upper mineral soil layer in the managed Scots pine (*Pinus sylvestris* L.) ecosystems, including mature forest stands, clear-cuts, and 1st, 2nd, 3rd, and 4th-year plantations. We hypothesized that changes in forest soil chemical parameters could have an impact on soil fungal communities obtained in different study sites.

This study was performed in a frame of the Lithuanian National Program (2020–2022), which was initiated based on intense debates among citizens and governmental and non-governmental organizations opposing forest management activities that may have a negative impact on biodiversity. Annually, an average of 11,968 ha of the State *P. sylvestris* forests in Lithuania are affected by forest management activities, including clear-cutting and other final felling for timber production.

Molecular methods based on PCR and ribosomal DNA sequencing have been successfully used to identify fungi at different taxonomic levels in different environmental samples and helped to elucidate the ecological conditions affecting the structure and diversity of

fungus communities [31,40–44]. To assess fungus communities in forest soil we used one of the molecular methods—high-throughput sequencing (HTS). In our study, PCR products were sequenced using a PacBio RSII platform and one SMRT cell at a SciLifeLab facility in Uppsala, Sweden. This third-generation high-throughput sequencing PacBio platform produces long reads averaging 20–25 kilobases (up to 100 kilobases). The DNA template is circularized via hairpin adaptors and recorded multiple times to offer read qualities comparable to that of traditional Sanger sequencing. The new Sequel instrument generates ~400,000 reads per SMRT cell (3.2 million reads per run), which is much fewer reads than produced by Illumina platforms, but it produces high-quality sequences of amplicons or DNA fragments of ~3 kilobases in length. This makes the platform ideal for sequencing short-to-medium length amplicons such as the full ITS region and perhaps its flanking conserved genes for precise phylogenetic placement [45]. This technology provides powerful tools to explore fungus diversity and enable identification of complex fungus communities and individual community components even at low abundances.

## 2. Materials and Methods

### 2.1. Study Sites

Study sites were in 3 different regions of Lithuania, representing the nearby locations of Alytus, Labanoras, and Varėna (Figure 1). At each location, the study sites were selected based on the forest inventory data from the Lithuanian State Forest Cadastre database. For the evaluation of the dynamic changes of chemical soil properties and fungus communities after the clear-cuttings, the study sites were selected at each region in six sampling sites representing different stages of the *P. sylvestris* forest ecosystem: (1) mature *P. sylvestris* forest stands; (2) clear-cuts of *P. sylvestris* (felled in 2019); (3) 1st year *P. sylvestris* plantations (planted in 2018); (4) 2nd year *P. sylvestris* plantations (planted in 2017); (5) 3rd year *P. sylvestris* plantations (planted in 2016); and (6) 4th year *P. sylvestris* plantations (planted in 2015). In total, 18 sampling sites were selected in May 2020 (Table 1). The study sites were selected in the clear-cuts and *P. sylvestris* plantations of different ages established after final clear-cutting of mature forest (130 years old; average tree height was 26.6–31.5 m; average tree diameter at breast height (DBH) was 37 cm). All sampling sites were described as the sites with comparable soil and climatic conditions. During the sampling period in May–September 2020, the mean air temperature was 15.8 °C, and the mean precipitation was 64 mm (data obtained from the Lithuanian Hydrometeorological Service).

**Table 1.** Characteristics of the sampling sites in the selected *Pinus sylvestris* L. stands according to the State Forest Cadastre (2021).

| Region (Code) | Sampling Site Code * | Site Description    | Geographic Coordinates          | Forest Vegetation Type ** | Stand Age (years) | Mean Tree DBH *** (cm) | Mean Tree Height (m) |
|---------------|----------------------|---------------------|---------------------------------|---------------------------|-------------------|------------------------|----------------------|
| Alytus (A)    | A1                   | Mature forest       | 54°31'29.4" N,<br>24°0'52.9" E  | vm                        | 94                | 35.9                   | 31.2                 |
|               | A2                   | Clear-cut           | 54°32'33.4" N,<br>24°8'22.1" E  | v                         | -                 | -                      | -                    |
|               | A3                   | 1st year plantation | 54°34'13.3" N,<br>23°57'59.2" E | vm                        | 3                 | -                      | -                    |
|               | A4                   | 2nd year plantation | 54°31'31.3" N,<br>24°0'50.1" E  | vm                        | 4                 | -                      | -                    |
|               | A5                   | 3rd year plantation | 54°31'26.1" N,<br>23°56'2.6" E  | vm                        | 5                 | -                      | -                    |
|               | A6                   | 4th year plantation | 54°30'39.2" N,<br>23°58'25.0" E | vm                        | 6                 | -                      | -                    |

Table 1. Cont.

| Region (Code) | Sampling Site Code * | Site Description    | Geographic Coordinates          | Forest Vegetation Type ** | Stand Age (years) | Mean Tree DBH *** (cm) | Mean Tree Height (m) |
|---------------|----------------------|---------------------|---------------------------------|---------------------------|-------------------|------------------------|----------------------|
| Varėna (V)    | V1                   | Mature forest       | 54°10′45.2″ N,<br>24°35′9.8″ E  | vm                        | 85                | 28.1                   | 24.4                 |
|               | V2                   | Clear-cut           | 54°8′32.9″ N,<br>24°34′36.1″ E  | v                         | -                 | -                      | -                    |
|               | V3                   | 1st year plantation | 54°11′51.9″ N,<br>24°35′48.7″ E | vm                        | 3                 | -                      | -                    |
|               | V4                   | 2nd year plantation | 54°9′18.4″ N,<br>24°31′48.3″ E  | vm                        | 4                 | -                      | -                    |
|               | V5                   | 3rd year plantation | 54°10′18.7″ N,<br>24°35′48.9″ E | vm                        | 5                 | -                      | -                    |
|               | V6                   | 4th year plantation | 54°6′7.5″ N,<br>24°34′4.8″ E    | vm                        | 6                 | -                      | -                    |
| Labanoras (L) | L1                   | Mature forest       | 55°10′36.7″ N,<br>25°45′28.3″ E | vm                        | 132               | 36.1                   | 31.1                 |
|               | L2                   | Clear-cut           | 55°9′10.8″ N,<br>25°47′42.8″ E  | v                         | -                 | -                      | -                    |
|               | L3                   | 1st year plantation | 55°10′35.0″ N,<br>25°45′11.5″ E | vm                        | 3                 | -                      | -                    |
|               | L4                   | 2nd year plantation | 55°8′35.9″ N,<br>25°48′37.0″ E  | vm                        | 4                 | -                      | -                    |
|               | L5                   | 3rd year plantation | 55°8′17.9″ N,<br>25°45′28.3″ E  | vm                        | 5                 | -                      | -                    |
|               | L6                   | 4th year plantation | 55°10′37.7″ N,<br>25°45′33.4″ E | vm                        | 6                 | -                      | -                    |

\* A1–A6: sampling sites in Alytus region; V1–V6: sampling sites in Varėna region; L1–L6: sampling sites in Labanoras region, also shown in Figure 1. \*\* v: *vacciniosa*, m: *myrtillosa*; \*\*\* DBH: tree diameter at breast height (measured at 1.3 meters above the ground).



**Figure 1.** Location of the three study regions in Lithuania: Alytus (A, on the map), Labanoras (L), Varėna (V). In each region, the following sampling sites were included: (1) mature *Pinus sylvestris* L. forest stands; (2) clear-cuts of *P. sylvestris* (felled in 2019); (3) 1st year *P. sylvestris* plantations (planted in 2018); (4) 2nd year *P. sylvestris* plantations (planted in 2017); (5) 3rd year *P. sylvestris* plantations (planted in 2016); (6) 4th year *P. sylvestris* plantations (planted in 2015).

For the selection of three study sites, the following criteria were used: the pure *P. sylvestris* stands growing on typical forest site type prior to clear-cutting, and the adjacent sites, reforested by *P. sylvestris*, representing the annual ecosystem stages; comparable forest soil and forest vegetation type [46]. All selected study sites were characterized by the *Pinetum vacciniosa* and *P. vaccinio-myrtilliosa* forest type; the oligotrophic mineral soil of a normal moisture regime (Nbl) [47]; and nutrient-poor sandy Arenosols with a light soil texture [48]. The mean tree diameter and mean height were available for mature forest sites (Table 1). The species composition of ground vegetation was similar in all studied sites with the dominant herbs and dwarf shrubs such as *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. and dominant mosses such as *Hyloclium splendens* (Hedw.) Schimp., *Pleurozium schreberi* (Brid.) Mitt., and *Ptilium crista-castrensis* (Hedw.) De Not.

## 2.2. Soil Sampling and Chemical Analyses

The soil sampling was performed according to the ICP-Forests methodology [49]. For the determination of basic soil properties, soil was sampled from the upper (0–20 cm) mineral soil layer, composing three samples from five subsamples per each sampling site at each study region. Soil samples were taken at 1 m from tree stems or stumps, maintaining the same distance at all study sites. In total, 54 soil samples were taken in the field. In each sampling site, the points for soil sampling were allocated systematically in two diagonals. The soil samples were individually placed into the plastic bags, labelled, and transported to the laboratory. The following chemical parameters were analysed: pH was determined in a 0.01 M CaCl<sub>2</sub> suspension (ISO 10390:2005), organic carbon (C) was determined using a dry combustion method with a total carbon analyzer Analytic Jena multi EA 4000 Germany (ISO 10694:1995) with a Heraeus apparatus (ISO 10694, dry combustion at 900 °C), and total nitrogen (N) analyzed using the Kjeldahl method (ISO 11261). In addition, mineral N was determined in 1 M KCl extraction by the spectrometric method (ISO 14256-2); mobile potassium (K<sub>2</sub>O) and phosphorus (P<sub>2</sub>O<sub>5</sub>) were determined using a solution of ammonium lactate by the Égnér-Riehm-Domingo (A-L) method. The laboratory analyses were provided by the Agrochemical Research Laboratory of the Lithuanian Research Centre for Agriculture and Forestry (LAMMC).

## 2.3. Fungal Sampling

The fungal core samples were sampled next to the soil samples as additional samples. The core samples were taken to represent each of the selected study sites, i.e., the clear-cut, the 1st–4th years plantation, and the mature forest with the corresponding age of the trees. The effects of clear-cutting on forest soil mycobiota (saprotrophic, mycorrhizal, pathogenic, and endophytic fungi) in *P. sylvestris* ecosystems were assessed. The soil sampling for fungal identification was conducted in May–September 2020 in all sampling sites (Table 1). Five sample plots were randomly selected in each sample site. For the assessment of forest soil mycobiota, soil samples were collected in four replicates at each sample plot, obtaining one composite sample per plot. For fungal identification, the soil (ca. 100 g) was sampled using a 2 cm diameter soil core from the 5–20 cm mineral soil layer. Between different samples, the soil core was thoroughly cleaned with ethanol. The soil samples were individually placed into the plastic bags, labelled, transported to the laboratory, and stored at –20 °C. In total, 90 soil samples were collected.

## 2.4. Fungal DNA Isolation, Amplification, and Sequencing

The standard CTAB (cetyltrimethylammonium bromide) method was used to isolate genomic DNA from living tissue [50]. Prior to the DNA isolation, each soil sample was freeze-dried at –60 °C for 48 h. After the freeze-drying, ca. 20–30 mg of soil was used for the DNA isolation from each sample using a NucleoSpin<sup>®</sup>Soil kit (Macherey-Nagel GmbH & Co., Duren, Germany), according to the manufacturer's recommendations. Following the DNA isolation, the DNA concentration in the individual samples was determined using a NanoDrop<sup>™</sup> One spectrophotometer (Thermo Scientific, Rodchester, NY, USA).

The amplification of the internal transcribed spacer (ITS) rRNA region was achieved using a forward primer gITS7 (5'-GTGARTCATCGARTCTTTG-3') [51] and a barcoded universal primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [52].

The polymerase chain reaction (PCR) was performed in 50 µL reactions and consisted of the following final concentrations: 1% DreamTaq Green Polymerase (5 µ/µL) (Thermo Scientific, Waltham, MA, USA); 11% 10X Buffer; 11% dNTPs (10 mM); 1% MgCl<sub>2</sub> (25 mM); 2% of each primer (200 nM); 72% sterile milli-Q water.

The amplifications were performed using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR program started with an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, and annealing at 55 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. The PCR products were assessed using gel electrophoresis on 1.5% agarose gels stained with GelRed (Biotium, Fremont, CA, USA).

The PCR products were purified using 3 M sodium acetate (pH 5.2) (Applichem GmbH, Darmstadt, Germany) and 96% ethanol mixture (1:25). After the quantification of all PCR products using a Qubit fluorometer 4.0 (Life Technologies, Stockholm, Sweden), they were pooled in an equimolar mix and sequenced using a PacBio RSII platform and one SMRT cell at a SciLifeLab facility in Uppsala, Sweden.

### 2.5. Bioinformatics

Identification of fungal species was performed by molecular method—high-throughput sequencing (HTS). The sequences were filtered and clustered using the Sequence Clustering and Analysis of Tagged Amplicons (SCATA) next-generation sequencing (NGS) pipeline, available online <http://scata.mykopat.slu.se>. The sequences were filtered for quality, removing short sequences (<20), primer dimers, and homopolymers, which were collapsed to three base pairs (bp) before clustering. The sequences were screened for primers and sample-identifying barcodes, and sequences that were missing a barcode or primer were removed. The sequences were clustered into different taxa by single linkage clustering, with a 2.0% maximum distance allowed for the sequences to enter the clusters. The fungal taxa were taxonomically classified using a Ribosomal Database Project (RDP) pipeline classifier (<https://pyro.cme.msu.edu/index.jsp>, accessed on 8 October 2022). Fungal taxa were identified using GenBank (NCBI) database and the BLASTn algorithm. The criteria used for taxonomic identification were sequence coverage > 80%; similarity to species level 98%–100%; and similarity to genus level 94%–97%. Sequences not matching these criteria were considered as unidentified and were given unique names. Representative sequences of the fungal non-singletons are available from GenBank under accession numbers ON962810–ON963944.

### 2.6. Statistical Analyses

The soil chemical parameters were analyzed for differences in concentrations between the study sites: mature forest stands, clear-cuts, and 1st–4th year *P. sylvestris* plantations. For the normality of the variables, the Shapiro–Wilk test was used, and the normality hypothesis was rejected with a 0.05 significance level. Therefore, the Kruskal–Wallis analysis of variance (ANOVA) test was used to ascertain the significant differences in variables between the sites.

For the identification of the diversity and composition of the fungal communities, the Shannon diversity (H) index was evaluated [53]. The assignment of ecological roles was based on FUNGuild [54]. The rarefaction analysis was performed using Analytical Rarefaction v.1.3, available at <http://www.uga.edu/strata/software/index.html> (accessed on 20 February 2022). Rarefaction curves showing the relationship between the cumulative number of fungal operational taxonomic units (OTUs) and the number of ITS rDNA sequences were obtained from soil samples in different study sites.

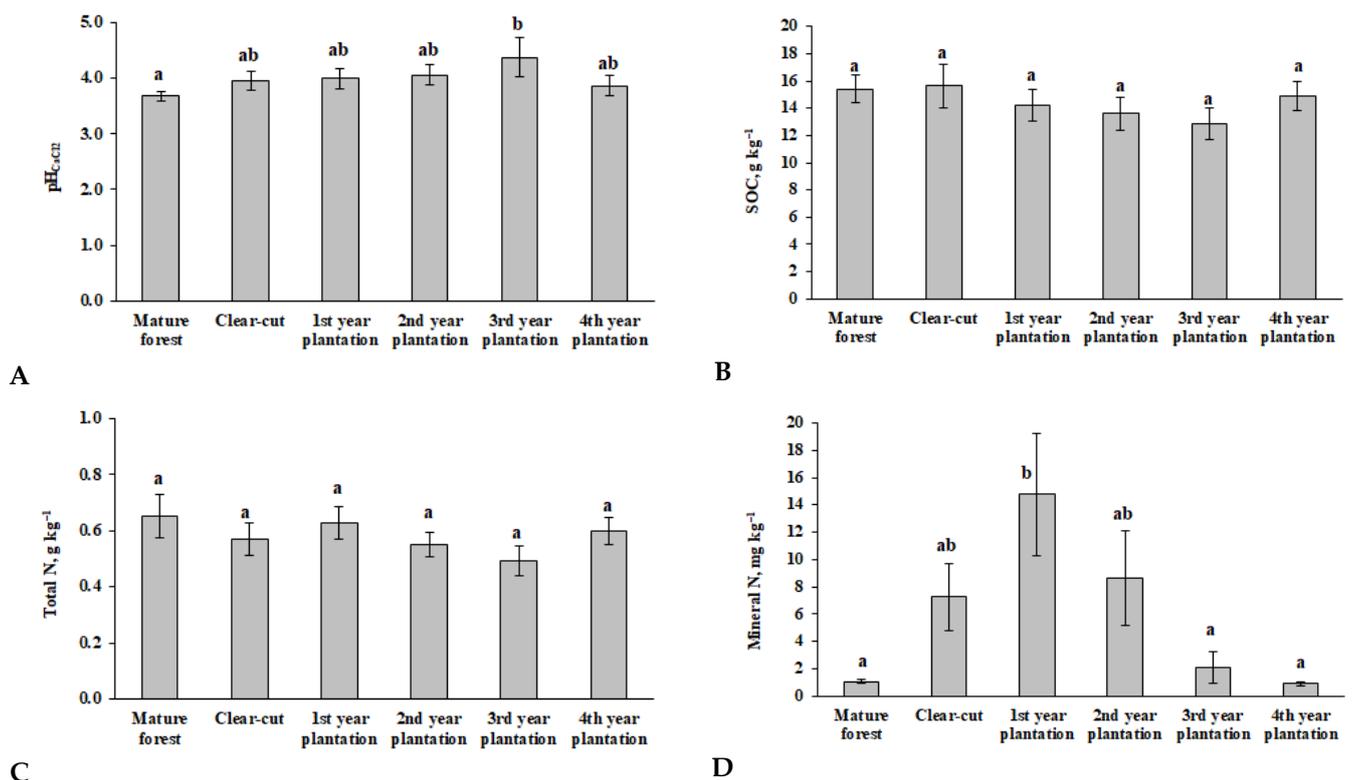
To calculate and draw a Venn diagram, we used an online program (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) (accessed on 25 August 2022). The differences in the rela-

tive abundance of fungal OTUs between study sites were tested using the Kruskal–Wallis test. Comparison of fungal composition was calculated using the Bray–Curtis similarity coefficient, which was used in the analysis of similarity to determine whether the fungal communities differed between study sites. Nonmetric multidimensional scaling (NMDS) was used to provide a visual summary of the pattern Bray–Curtis values. Relationships between soil chemical parameters and fungal variables were examined using the Pearson correlation and canonical correspondence analysis (CCA). Statistical analyses were performed using the statistical software package XLSTAT (Addinsoft, N.Y., USA) and open access software R version 4.0.5 (R Core team, Austria (<http://www.R-project.org/>) (accessed on 10 June 2021).

### 3. Results

#### 3.1. Chemical Properties of Mineral Topsoil

The mean  $\text{pH}_{\text{CaCl}_2}$  value was slightly lower in the mineral 0–20 cm topsoil layer at the clear-cut sites than in the mature *P. sylvestris* forest stands (Figure 2A). Although no clear trend was identified in the reforested sites after clear cuttings of the mature forest stands, the highest mean pH value ( $\text{pH}_{\text{CaCl}_2} 4.4 \pm 0.2$ ) was obtained in the 3rd year plantations. No significant differences were obtained for the mean concentrations of SOC and total N (Figure 2B,C), as well as K, Ca, and Mg (Table 2) across all studied sites. A statistically significantly higher mean concentration of mineral N was found in the 1st year plantations (Figure 2D).



**Figure 2.** Mean  $\text{pH}_{\text{CaCl}_2}$  (A), mean concentrations of soil organic carbon (SOC,  $\text{g kg}^{-1}$ ) (B), total nitrogen (Total N,  $\text{g kg}^{-1}$ ) (C), and mineral nitrogen (Min N,  $\text{g kg}^{-1}$ ) (D) in the 0–20 cm topsoil layer in the mature forest, clear-cut, and reforested 1st–4th year *Pinus sylvestris* L. plantations. The data from three study sites (A, L, and V) were combined. The values followed by the same letter indicate no significant difference between the sampling sites at  $p < 0.05$ .

**Table 2.** Mean EC ( $\text{mS m}^{-1}$ ), mean concentrations of total phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), chlorides ( $\text{Cl}^-$ ) ( $\text{mg kg}^{-1}$ ), and  $\text{P}_2\text{O}_5$ ,  $\text{K}_2\text{O}$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  in the 0–20 cm topsoil layer in the mature forest, clear-cut, and reforested 1st–4th year *Pinus sylvestris* L. plantations. The data from three study sites (A, L, and V) were combined. The values followed by different letters a and b within each column indicate statistically significant differences between the sampling sites at  $p < 0.05$ .

| Site Description    | EC                     | Total P                | Total K              | Total Ca            | Total Mg           |
|---------------------|------------------------|------------------------|----------------------|---------------------|--------------------|
|                     | ( $\text{mS m}^{-1}$ ) | (mg $\text{kg}^{-1}$ ) |                      |                     |                    |
| Mature forest       | $2.5 \pm 0.1$ ab       | $195.6 \pm 19.1$ a     | $146.3 \pm 8.3$ a    | $186.7 \pm 27.3$ a  | $242.4 \pm 33.8$ a |
| Clear-cut           | $2.6 \pm 0.2$ ab       | $232.3 \pm 29.4$ ab    | $137.2 \pm 7.9$ a    | $151.2 \pm 9.6$ a   | $229.0 \pm 27.8$ a |
| 1st year plantation | $3.6 \pm 0.5$ b        | $302.8 \pm 33.9$ b     | $145.4 \pm 12.7$ a   | $191.5 \pm 29.6$ a  | $228.8 \pm 40.0$ a |
| 2nd year plantation | $2.6 \pm 0.2$ ab       | $250.4 \pm 14.5$ ab    | $122.6 \pm 8.8$ a    | $179.7 \pm 20.2$ a  | $248.2 \pm 48.5$ a |
| 3rd year plantation | $2.3 \pm 0.1$ a        | $231.9 \pm 20.1$ ab    | $138.6 \pm 9.3$ a    | $206.2 \pm 19.4$ a  | $242.3 \pm 41.1$ a |
| 4th year plantation | $2.9 \pm 0.3$ ab       | $205.8 \pm 11.9$ a     | $152.0 \pm 6.1$ a    | $211.1 \pm 21.0$ a  | $263.3 \pm 24.7$ a |
|                     | $\text{Cl}^-$          | $\text{P}_2\text{O}_5$ | $\text{K}_2\text{O}$ | $\text{Ca}^{2+}$    | $\text{Mg}^{2+}$   |
|                     | (mg $\text{kg}^{-1}$ ) |                        |                      |                     |                    |
| Mature forest       | $5.8 \pm 0.2$ a        | $79.7 \pm 9.2$ a       | $34.1 \pm 2.9$ a     | $132.6 \pm 10.8$ ab | $41.8 \pm 1.5$ a   |
| Clear-cut           | $6.1 \pm 0.4$ a        | $122.6 \pm 26.2$ ab    | $35.3 \pm 4.0$ a     | $121.3 \pm 6.1$ ab  | $39.9 \pm 2.1$ a   |
| 1st year plantation | $6.7 \pm 0.3$ a        | $169.1 \pm 27.8$ b     | $36.6 \pm 4.3$ a     | $122.8 \pm 12.5$ ab | $40.8 \pm 3.1$ a   |
| 2nd year plantation | $6.7 \pm 0.4$ a        | $109.8 \pm 12.1$ ab    | $31.3 \pm 4.2$ a     | $120.4 \pm 10.8$ a  | $37.7 \pm 2.9$ a   |
| 3rd year plantation | $6.7 \pm 0.4$ a        | $101.0 \pm 11.4$ ab    | $27.2 \pm 2.4$ a     | $161.6 \pm 9.7$ b   | $48.3 \pm 5.6$ a   |
| 4th year plantation | $6.9 \pm 0.4$ a        | $107.9 \pm 16.5$ ab    | $32.1 \pm 3.8$ a     | $140.8 \pm 12.3$ ab | $50.6 \pm 6.7$ a   |

The highest electrical conductivity (EC) was found in the 1st year plantation, but in other studied sites, the EC varied within a relatively narrow range from  $2.3 \pm 0.1$   $\text{mS m}^{-1}$  to  $3.6 \pm 0.5$   $\text{mS m}^{-1}$  (Table 2). A higher concentration of total P was found in the 1st year plantation, but in the 2nd, 3rd, and 4th year plantations, the total P concentration did not differ from the P concentration in the mature forest. The concentration of  $\text{P}_2\text{O}_5$  in the mineral 0–20 cm topsoil layer was 1.5 times higher in the clear-cut, and 2.1 times higher in 1st year plantations than in the mature forest stands.

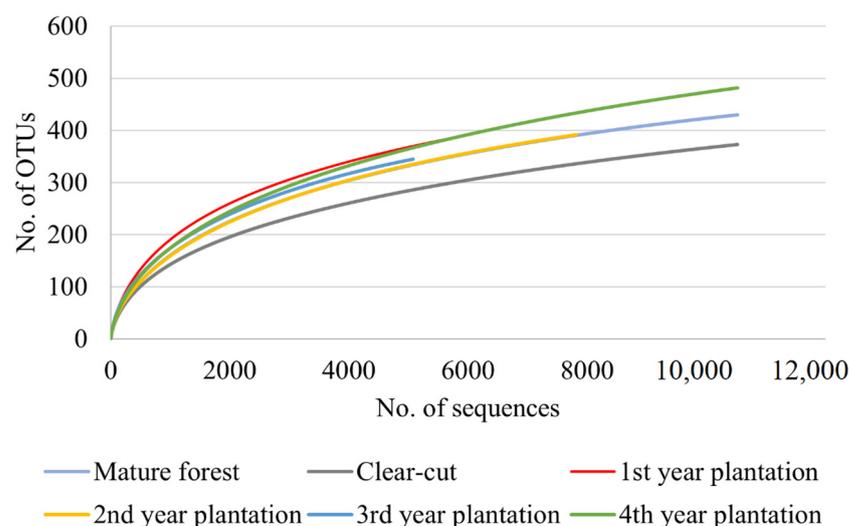
The highest concentration of  $\text{Ca}^{2+}$  was found in the 3rd year plantation. However, there were no statistically significant differences in the concentrations of  $\text{K}_2\text{O}$  and  $\text{Mg}^{2+}$  across all studied sites (Table 2). Overall, the soil nutrient concentrations in the 4th year *P. sylvestris* plantations were comparable with those obtained in the mature forest stands.

### 3.2. Fungal Community

The quality filtering after PacBio sequencing showed the presence of 60,898 high-quality fungal sequences representing 1143 fungal operational taxonomic units (OTUs) (Table 3 and Figure 3), and the non-fungal OTUs were excluded.

**Table 3.** Generated high-quality fungal sequences and detected diversity of the fungal taxa in the soil at different *Pinus sylvestris* L. sites in sampling sites. The data from the three study sites (A, L, and V) were combined.

| Site Description    | No. of Fungal Sequences | No. of Fungal Taxa | Shannon Diversity Index H |
|---------------------|-------------------------|--------------------|---------------------------|
| Mature forest       | 10,523                  | 430                | 3.86                      |
| Clear-cut           | 14,534                  | 415                | 3.76                      |
| 1st year plantation | 5846                    | 388                | 4.30                      |
| 2nd year plantation | 7818                    | 392                | 4.00                      |
| 3rd year plantation | 5078                    | 345                | 4.30                      |
| 4th year plantation | 17,099                  | 559                | 4.21                      |
| Total               | 60,898                  | 1143               |                           |



**Figure 3.** Rarefaction curves showing the relationship between the cumulative number of fungal operational taxonomic units (OTUs) and the number of internal transcribed spacer (ITS) rRNA sequences from corresponding sampling sites; the data obtained in the three study sites (A, L, and V) were combined to give a generalized trend for the mature forest stands, clear-cuts, and reforested 1st–4th year *Pinus sylvestris* L. plantations.

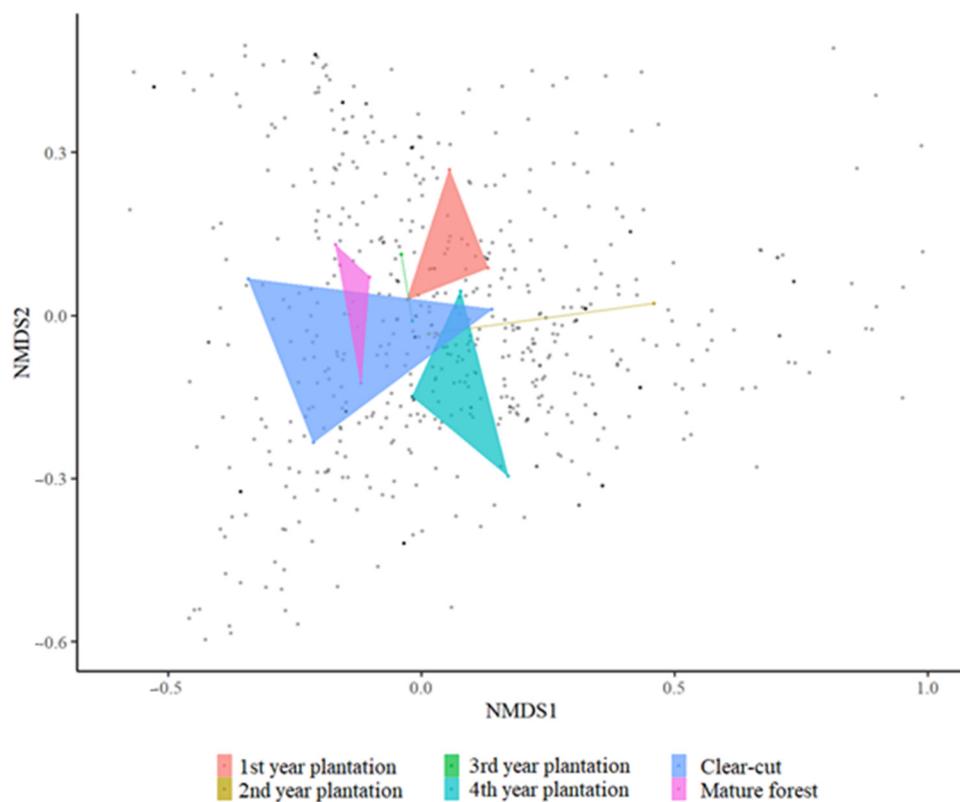
Among all the fungal OTUs, 352 OTUs (30.8%) were identified up to the species level, 161 OTUs (14.1%) up to the genus level, and 630 OTUs (55.1%) were identified only up to a higher taxonomic level. Of the 1143 fungal OTUs across all the soil samples, *Ascomycota* was the most dominant phylum, and accounted 26.4% of all the fungal OTUs, followed by *Basidiomycota* (23.3%), *Mucoromycota* (3.3%), *Chytridiomycota* (0.7%), and *Zoopagomycota* (0.1%).

The rarefaction analysis showed that the number of fungal OTUs did not reach species saturation (Figure 3), indicating that a potentially higher diversity of taxa could be detected by a deeper sequencing.

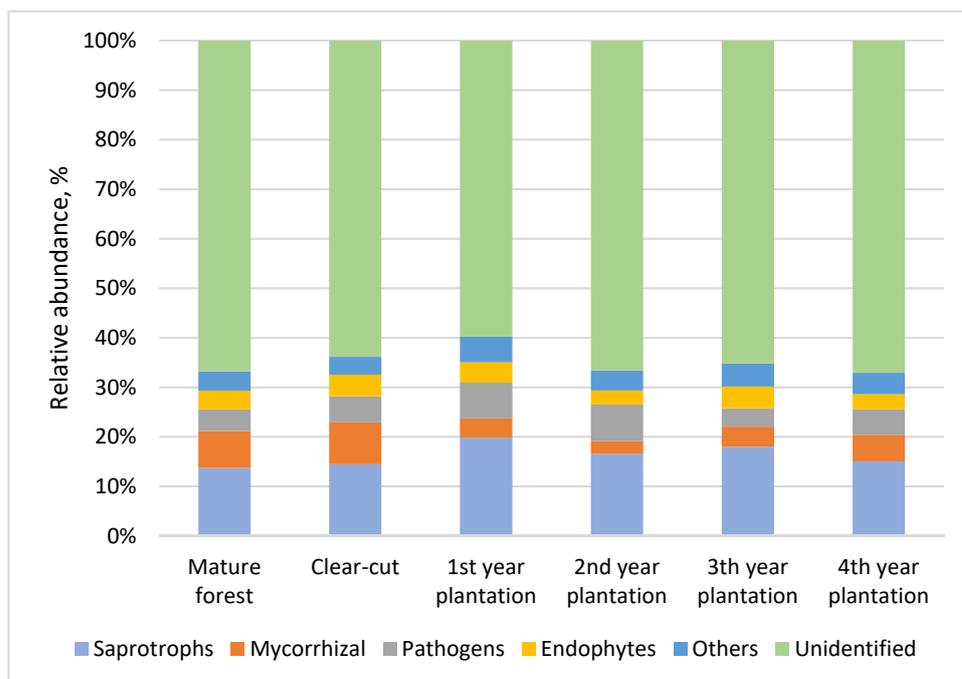
The non-metric multidimensional scaling shows that the fungal communities in the mature forest stands, clear-cuts, and the 1st–4th year *P. sylvestris* plantations did not differ significantly from each other (Figure 4).

The assignment of fungal ecological roles (trophic groups) is represented in Figure 5. The most common fungal OTUs in the soil samples were identified as of unknown ecological roles (59.8%–67.1%, representing all studied study sites), followed by saprotrophs (13.7%–19.8%), mycorrhizal fungi (2.6%–8.4%), pathogens (3.8%–7.4%), endophytes (3.0%–4.3%), and others (3.6%–5.2%).

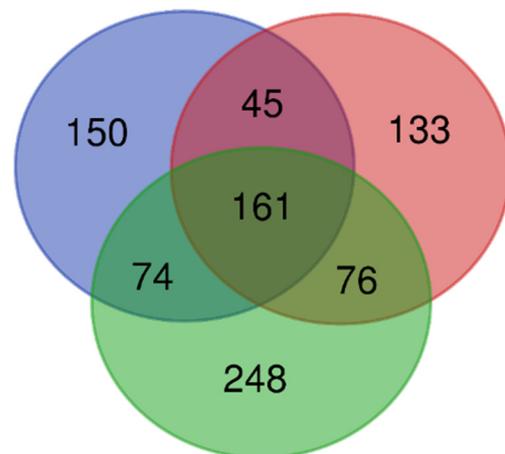
Among all obtained fungal OTUs, 91 fungal OTUs (8.0%) were detected in all studied sites. The numbers of obtained unique and common fungal OTUs in between different sites are shown in the Venn diagrams (Figures 6 and 7). The 161 common fungal OTUs were found in the mature forest stands, clear-cuts, and 4th year plantations. A similar number of unique fungal OTUs were found in the mature forest and in the clear-cut sites: 150 and 133 OTUs, respectively). In the 4th year plantations, the number of unique fungal OTUs increased significantly. The lowest number (45 OTUs) of shared fungal OTUs was found in the mature forest stands and the clear-cuts. A similar number of shared OTUs were found in the mature forest stands and the 4th year plantations, and in the clear-cuts and the 4th year plantations (74 and 76 OTUs, respectively) (Figure 6).



**Figure 4.** Nonmetric multidimensional scaling (NMDS) ordination of the fungal communities in the study sites (Bray–Curtis index). Each point represents separate fungal OTUs. The data obtained in three study sites (A, L, and V) were combined to show the overall situation for the mature forest stands, clear-cuts, and reforested 1st–4th year *Pinus sylvestris* L. plantations.

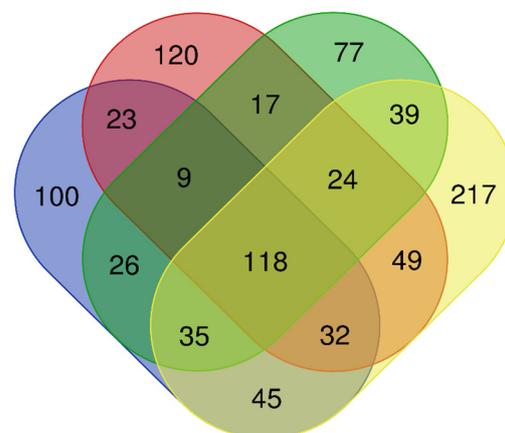


**Figure 5.** Ecological roles of the fungal OTUs in the soil sampled from different study sites. The data for the mature forest stands, clear-cuts, and reforested 1st–4th year *Pinus sylvestris* L. plantations were combined correspondingly from three study sites (A, L, and V).



**Figure 6.** Venn diagram indicating the diversity of the fungal OTUs found in the mature forests, clear-cuts, and 4th year plantations, and the number of fungal OTUs shared across studied sites; the data obtained in three study sites (A, L, and V) were combined. Different colours represent different study sites: blue indicates mature forest stands, red—clear-cuts, and green—4th year plantations.

Among all fungal OTUs found in *P. sylvestris* plantations, 100 OTUs were exclusively found in the 1st year plantations, 120 OTUs in the 2nd year plantations, 77 OTUs in the 3rd year plantations, and 217 OTUs in the 4th year plantations. The highest number of OTUs was common for the 2nd year and the 4th year plantations. The lowest number of shared fungal OTUs was between the 2nd and 3rd year plantations, i.e., 17 OTUs. In total, 118 fungal OTUs were shared between all plantations (Figure 7).



**Figure 7.** Venn diagram indicating the diversity of the fungal OTUs found in the 1st–4th year *Pinus sylvestris* L. plantations and the number of fungal OTUs shared across all plantations; the data obtained at three study sites (A, L, and V) were combined. Different colours represent different study sites: blue—1st year plantations, red—2nd year plantations, green—3rd year plantations, and orange—4th year plantations.

The 30 most common fungal OTUs, representing 62.03% of all high-quality fungal sequences in the soil sampled from the different study sites, are given in Table 4.

**Table 4.** Relative abundance and similarity of the 30 most common fungal OTUs (shown as a proportion of all the high-quality fungal sequences) in the soil samples from the *Pinus sylvestris* L. sites, representing mature forests, clear-cuts, and reforested 1st–4th year plantations; the data from three study sites (A, L, and V) were combined. The values followed by different letters within each row indicate statistically significant differences at  $p < 0.05$ .

| OTU                                  | Phylum <sup>a</sup> | References | Similarity, (%) <sup>b</sup> | Relative Abundance (%) |           |                     |                     |                     |                     |       |
|--------------------------------------|---------------------|------------|------------------------------|------------------------|-----------|---------------------|---------------------|---------------------|---------------------|-------|
|                                      |                     |            |                              | Mature Forest          | Clear-Cut | 1st Year Plantation | 2nd Year Plantation | 3rd Year Plantation | 4th Year Plantation | Total |
| <i>Archaeorhizomyces</i> sp. 5425_1  | A                   | MH248043   | 207/207 (100)                | 21.59 b                | 18.52 ab  | 5.03 ab             | 0.97 a              | 2.40 ab             | 9.01 ab             | 11.49 |
| <i>Helotiaceae</i> sp. 5425_2        | A                   | MK131613   | 240/240 (100)                | 1.84 a                 | 9.67 a    | 2.51 a              | 12.13 a             | 5.45 a              | 6.91 a              | 6.82  |
| Unidentified sp. 5425_18             | A                   | MN902668   | 258/258 (100)                | 1.22 a                 | 4.67 a    | 1.54 a              | 3.61 a              | 10.73 a             | 8.30 a              | 5.16  |
| <i>Oidiodendron chlamydosporicum</i> | A                   | MG597466   | 235/235 (100)                | 2.26 ab                | 6.31 b    | 1.66 ab             | 1.06 a              | 5.71 ab             | 7.94 b              | 4.90  |
| <i>Penicillium camemberti</i>        | A                   | MT355566   | 251/251 (100)                | 3.24 a                 | 2.51 a    | 2.10 a              | 5.95 a              | 6.64 a              | 3.54 a              | 3.67  |
| <i>Cladosporium</i> sp. 5425_16      | A                   | MT645945   | 243/243 (100)                | 4.68 a                 | 1.42 a    | 5.61 a              | 2.88 a              | 4.21 a              | 1.53 a              | 2.84  |
| <i>Amanita fulva</i>                 | B                   | MT229863   | 260/260 (100)                | 2.14 a                 | 7.08 a    | -                   | -                   | -                   | -                   | 2.06  |
| <i>Jackrogersella multififormis</i>  | A                   | MT573483   | 254/254 (100)                | 9.52 a                 | 0.05 a    | 1.54 a              | 0.06 a              | 1.93 a              | 0.05 a              | 1.99  |
| <i>Piloderma sphaerosporum</i>       | B                   | MK131527   | 292/292 (100)                | 1.45 a                 | 6.75 a    | -                   | 0.01 a              | 0.10 a              | 0.01 a              | 1.88  |
| <i>Placynthiella oligotropha</i>     | A                   | MK812378   | 248/248 (100)                | 0.02 a                 | -         | 0.10 a              | 11.61 a             | 0.10 a              | 1.08 a              | 1.81  |
| <i>Malassezia</i> sp. 5425_17        | B                   | MT594863   | 369/369 (100)                | 4.68 b                 | 0.96 ab   | 2.82 ab             | 0.84 a              | 1.26 ab             | 0.87 ab             | 1.77  |
| Unidentified sp. 5425_68             | A                   | EU292507   | 216/217 (99)                 | 0.29 ab                | -         | 0.26 ab             | 0.43 ab             | 0.10 a              | 5.22 b              | 1.60  |
| <i>Mycena zephrus</i>                | B                   | MH856339   | 304/304 (100)                | -                      | -         | 16.08               | -                   | -                   | -                   | 1.54  |
| <i>Phanerochaete</i> sp. 5425_7      | B                   | KP135164   | 293/293 (100)                | 0.45 a                 | 0.94 ab   | 6.55 b              | 1.28 ab             | 2.38 ab             | 0.56 ab             | 1.45  |
| <i>Cladophialophora minutissima</i>  | B                   | MT644883   | 313/313 (100)                | 0.11 a                 | 0.21 a    | 0.46 a              | 8.14 a              | 0.20 a              | 0.97 a              | 1.45  |

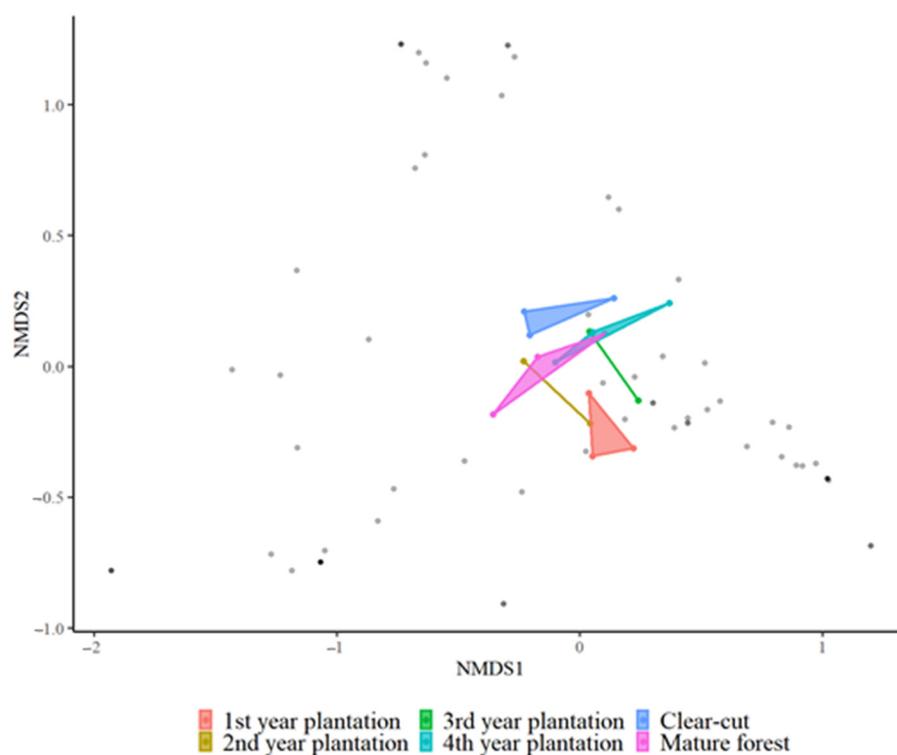
Table 4. Cont.

| OTU                                  | Phylum <sup>a</sup> | References | Similarity, (%) <sup>b</sup> | Relative Abundance (%) |           |                     |                     |                     |                     |       |
|--------------------------------------|---------------------|------------|------------------------------|------------------------|-----------|---------------------|---------------------|---------------------|---------------------|-------|
|                                      |                     |            |                              | Mature Forest          | Clear-Cut | 1st Year Plantation | 2nd Year Plantation | 3rd Year Plantation | 4th Year Plantation | Total |
| <i>Thelephora terrestris</i>         | A                   | MG597448   | 253/254 (99)                 | 0.01 ab                | 0.01 a    | 0.65 ab             | -                   | 1.50 ab             | 4.49 b              | 1.45  |
| <i>Mortierella</i> sp. 5425_53       | M                   | HQ021936   | 342/344 (99)                 | 1.59 a                 | 0.39 a    | 0.33 a              | 0.93 a              | 2.58 a              | 1.39 a              | 1.12  |
| <i>Russula decolorans</i>            | B                   | MN992510   | 359/359 (100)                | 3.79 b                 | 1.73 ab   | 0.03 a              | -                   | -                   | 0.05 ab             | 1.08  |
| <i>Unidentified</i> sp. 5425_0       | A                   | JQ312914   | 240/241 (99)                 | 0.68 a                 | 1.22 a    | 1.03 a              | 0.91 a              | 1.44 a              | 1.16 a              | 1.07  |
| <i>Russula vinosa</i>                | B                   | MK838342   | 366/367 (99)                 | -                      | -         | 0.02 a              | -                   | -                   | 3.63 a              | 1.02  |
| <i>Collophora</i> sp. 5425_14        | A                   | MW449039   | 244/244 (100)                | 1.28 a                 | 0.72 a    | 2.28 a              | 0.69 a              | 1.81 a              | 0.45 a              | 0.98  |
| <i>Xylaria hypoxylon</i>             | A                   | MK577428   | 252/252 (100)                | 4.41 b                 | 0.03 ab   | 0.51 ab             | 0.04 a              | 0.85 ab             | 0.04 ab             | 0.90  |
| <i>Alternaria infectoria</i>         | A                   | MT635276   | 253/253 (100)                | 0.29 a                 | 0.24 a    | 1.80 a              | 0.52 a              | 2.09 a              | 1.35 a              | 0.90  |
| <i>Mortierella</i> sp. 5425_47       | M                   | MT601876   | 293/293 (100)                | 1.37 b                 | 0.50 ab   | 0.19 a              | 0.23 ab             | 2.97 ab             | 0.34 ab             | 0.75  |
| <i>Cladophialophora</i> sp. 5425_111 | A                   | AB986417   | 268/271 (99)                 | -                      | -         | -                   | -                   | -                   | 2.54                | 0.71  |
| <i>Mortierella macrocystis</i>       | M                   | MH859487   | 330/330 (100)                | 0.45 ab                | 1.14 b    | 1.25 ab             | 0.15 a              | 1.34 ab             | 0.30 ab             | 0.68  |
| <i>Glonium pusillum</i>              | A                   | MT635300   | 249/249 (100)                | 0.48 a                 | 0.43 a    | 0.94 a              | 1.91 a              | 0.51 a              | 0.32 a              | 0.65  |
| <i>Unidentified</i> sp. 5425_67      | A                   | HQ433092   | 300/300 (100)                | 0.13 ab                | 1.86 b    | 0.02 a              | 0.18 ab             | 0.02 a              | 0.51 ab             | 0.64  |
| <i>Solicoccozyma terricola</i>       | B                   | MH487580   | 329/329 (100)                | 0.22 a                 | 0.52 a    | 0.21 a              | 1.04 a              | 0.20 a              | 1.06 a              | 0.63  |
| <i>Lecanoromycetes</i> sp. 5425_115  | A                   | MT027951   | 234/234 (100)                | 0.07 a                 | 0.09 a    | 0.31 a              | 0.01 a              | 0.96 a              | 1.66 a              | 0.61  |
| Total of OTUs                        |                     |            |                              | 68.25                  | 67.96     | 55.82               | 55.59               | 57.48               | 65.26               | 63.63 |

<sup>a</sup> Ascomycota (A), Basidiomycota (B), Mucoromycota (M). <sup>b</sup> Sequence similarity column shows base pairs between the query sequence and the reference at GenBank, and the percentage of sequence similarity.

The most abundant fungi in the studied sites were *Archaeorhizomyces* sp. 5425\_1 (11.5% of all high-quality sequences), *Helotiaceae* sp. 5425\_2 (6.8%), Unidentified sp. 5425\_18 (5.2%), *Oidiodendron chlamydosporicum* Morrall (4.9%), *Penicillium camemberti* Thom (3.7%), and *Cladosporium* sp. 5425\_16 (2.8%) (Table 4). Among the obtained most common fungal OTUs, five species represented ectomycorrhizal (ECM) fungi, which included *Amanita fulva* (Schaeff.) Fr. (2.1% of all high-quality sequences), *Piloderma sphaerosporum* (Peck) Julich (1.9%), *Thelephora terrestris* Ehrh. (1.5%), *Russula decolorans* Fr. (1.1%), *Russula vinosa* Lindblad (1.0%), and only one OTU represented the plant pathogen *Collophora* sp. 5425\_14 (1.0%). The dominant endophytes in the studied fungal community were *Mortierella* sp. 5425\_53 and *Mortierella macrocystis* W.Gams.

The non-metric multidimensional scaling of the mycorrhizal fungal communities showed a partial separation of the samples obtained from the different sites. The mycorrhizal fungal communities did not differ significantly among the mature forest stands and the 4th year plantations ( $p > 0.05$ ). A fungal community between 1st year plantations and the clear-cut site differed significantly ( $p < 0.05$ ) (Figure 8).



**Figure 8.** Nonmetric multidimensional scaling (NMDS) ordination of the mycorrhizal fungi abundance in study sites (Bray–Curtis index). Each point represents a separate mycorrhizal fungal OTU. The data obtained in three study sites (A, L, and V) were combined to show the overall situation for the mature forest stands, clear-cuts, and reforested 1st–4th year *Pinus sylvestris* L. plantations.

In total, 70 mycorrhizal fungal OTUs were found in the soil samples in the studied sites. The relative abundances of the thirty most common ectomycorrhizal (ECM) fungi are given in Table 5.

The most abundant ECM fungi were *Amanita fulva* (2.1% of all the high-quality sequences), *Piloderma sphaerosporum* (1.9%), *Thelephora terrestris* (1.5%), *Russula decolorans* (1.1%), *Russula vinosa* (1.0%), and *Pseudotomentella* sp. 5425\_255 (0.6%) (Table 5).

**Table 5.** Relative abundance and similarity of the 30 most common ectomycorrhizal (ECM) fungal OTUs in the soil samples from the *Pinus sylvestris* L. sites, representing mature forest stands, clear-cuts, and reforested 1st–4th year plantations; the data from three study sites (A, L, and V) were combined. The values followed by different letters within each row indicate statistically significant differences at  $p < 0.05$ .

| OTU  | Phylum <sup>a</sup> | References | Similarity, (%) <sup>b</sup> | Relative Abundance (%) |           |                     |                     |                     |                     |       |
|--|---------------------|------------|------------------------------|------------------------|-----------|---------------------|---------------------|---------------------|---------------------|-------|
|  |                     |            |                              | Mature Forest          | Clear-Cut | 1st Year Plantation | 2nd Year Plantation | 3rd Year Plantation | 4th Year Plantation | Total |
| <i>Amanita fulva</i>                       | B                   | MT229863   | 260/260 (100)                | 2.14 a                 | 7.08 a    | -                   | -                   | -                   | -                   | 2.06  |
| <i>Piloderma sphaerosporum</i>             | B                   | MK131527   | 292/292 (100)                | 1.45 a                 | 6.75 a    | -                   | 0.01 a              | 0.10 a              | 0.01 a              | 1.88  |
| <i>Thelephora terrestris</i>               | B                   | MT644883   | 313/313 (100)                | 0.01 ab                | 0.01 a    | 0.65 ab             | -                   | 1.50 ab             | 4.49 b              | 1.45  |
| <i>Russula decolorans</i>                  | B                   | MN992510   | 359/359 (100)                | 3.79 b                 | 1.73 ab   | 0.03 b              | -                   | -                   | 0.05 ab             | 1.08  |
| <i>Russula vinoso</i>                      | B                   | MK838342   | 366/367 (99)                 | -                      | -         | 0.02 a              | -                   | -                   | 3.63 a              | 1.02  |
| <i>Pseudotomentella</i> sp.<br>5425_119    | B                   | HM146848   | 345/345 (100)                | -                      | 2.46      | -                   | -                   | -                   | -                   | 0.59  |
| <i>Elaphomyces</i> cf.<br><i>muricatus</i> | A                   | KR029741   | 287/288 (99)                 | -                      | 2.20      | -                   | -                   | -                   | -                   | 0.53  |
| <i>Amphinema</i> sp.<br>5425_155           | B                   | MK838190   | 288/288 (100)                | 1.43 a                 | 0.01 a    | -                   | 0.04 a              | -                   | -                   | 0.25  |
| <i>Laccaria proxima</i>                    | B                   | MN663149   | 301/301 (100)                | -                      | -         | 0.43 a              | -                   | 1.75 a              | 0.08 a              | 0.21  |
| <i>Tricholoma portentosum</i>              | B                   | LC375763   | 304/304 (100)                | 0.1a                   | 0.65 a    | -                   | 0.01 a              | 0.02 a              | -                   | 0.19  |
| <i>Amanita pantherina</i>                  | B                   | MZ410633   | 310/310 (100)                | 0.13 a                 | -         | -                   | -                   | -                   | 0.59 a              | 0.19  |
| <i>Russula vesca</i>                       | B                   | MZ410735   | 320/321 (99)                 | 0.76 a                 | 0.13 a    | -                   | 0.04 a              | -                   | -                   | 0.17  |
| <i>Russula albonigra</i>                   | B                   | DQ422029   | 321/321 (100)                | 0.95                   | -         | -                   | -                   | -                   | -                   | 0.16  |
| <i>Russula adusta</i>                      | B                   | MG687346   | 321/321 (100)                | -                      | 0.65 a    | 0.07 a              | -                   | -                   | -                   | 0.16  |
| <i>Piloderma bicolor</i>                   | B                   | MH809947   | 283/283 (100)                | -                      | 0.65      | -                   | -                   | -                   | -                   | 0.15  |
| <i>Xerocomus badius</i>                    | B                   | MN947388   | 284/284 (100)                | 0.04 a                 | -         | 0.03 a              | -                   | -                   | 0.46 a              | 0.14  |
| <i>Russula paludosa</i>                    | B                   | LC192779   | 358/358 (100)                | -                      | 0.44 a    | 0.02 a              | -                   | -                   | -                   | 0.11  |
| <i>Pseudotomentella</i> sp.<br>5425_255    | B                   | MK290703   | 316/316 (100)                | -                      | 0.36      | -                   | -                   | -                   | -                   | 0.09  |
| <i>Tomentella stiposa</i>                  | B                   | MK838269   | 313/314 (99)                 | -                      | 0.30      | -                   | -                   | -                   | -                   | 0.07  |

Table 5. Cont.

| OTU                                      | Phylum <sup>a</sup> | References | Similarity, (%) <sup>b</sup> | Relative Abundance (%) |           |                     |                     |                     |                     |       |
|--|---------------------|------------|------------------------------|------------------------|-----------|---------------------|---------------------|---------------------|---------------------|-------|
|  |                     |            |                              | Mature Forest          | Clear-Cut | 1st Year Plantation | 2nd Year Plantation | 3rd Year Plantation | 4th Year Plantation | Total |
| <i>Tricholoma virgatum</i>               | B                   | DQ389735   | 302/302 (100)                | -                      | 0.30      | -                   | -                   | -                   | -                   | 0.07  |
| <i>Amanita porphyria</i>                 | B                   | MT345267   | 296/296 (100)                | 0.01 a                 | 0.21 a    | 0.02 a              | -                   | -                   | -                   | 0.05  |
| <i>Cortinarius</i> sp. 5425_463          | B                   | MG597372   | 303/305 (99)                 | 0.28                   | -         | -                   | -                   | -                   | -                   | 0.05  |
| <i>Wilcoxina</i> sp. 5425_54             | A                   | MW238026   | 257/257 (100)                | -                      | 0.01 a    | -                   | -                   | -                   | 0.11 a              | 0.03  |
| <i>Pseudotomentella griseopergamacea</i> | B                   | MG597410   | 321/321 (100)                | -                      | -         | -                   | -                   | -                   | 0.11                | 0.03  |
| <i>Russula aeruginea</i>                 | B                   | MG680182   | 301/301 (100)                | 0.13 a                 | 0.01 a    | -                   | 0.04 a              | -                   | -                   | 0.03  |
| <i>Albatrellus</i> sp. 5425_551          | B                   | HQ204631   | 330/338 (98)                 | -                      | 0.12      | -                   | -                   | -                   | -                   | 0.03  |
| <i>Inocybe</i> sp. 5425_176              | B                   | MK838270   | 303/303 (100)                | -                      | -         | -                   | -                   | -                   | 0.10                | 0.03  |
| <i>Piloderma olivaceum</i>               | B                   | MH864982   | 281/281 (100)                | 0.14 a                 | 0.01 a    | -                   | -                   | -                   | -                   | 0.03  |
| <i>Inocybe lutescens</i>                 | B                   | MW845894   | 295/295 (100)                | 0.13 a                 | 0.01 a    | -                   | -                   | -                   | 0.01a               | 0.03  |
| <i>Tylospora fibrillosa</i>              | B                   | MF926576   | 287/287 (100)                | -                      | 0.10      | -                   | -                   | -                   | -                   | 0.02  |
| Total of OTUs                            |                     |            |                              | 11.57                  | 24.17     | 1.27                | 0.14                | 3.37                | 9.63                | 10.89 |

<sup>a</sup> Ascomycota (A), Basidiomycota (B). <sup>b</sup> Sequence similarity column shows base pairs between the query sequence and the reference at GenBank, and the percentage of sequence similarity.

Among all 70 mycorrhizal fungal OTUs, 9 OTUs were exclusively found in the soil of the mature forest stands, and 13 OTUs were found in the clear-cut sites. However, only three mycorrhizal fungal OTUs—*Amanita fulva*, *Piloderma olivaceum* (Parmasto) Hjortstam, and *Elaphomyces granulatus* Fr.—were identified as the common OTUs in the soil at the mature forest stands and the clear-cut sites. Among all mycorrhizal fungal OTUs, two OTUs were exclusively identified in the soil of the 1st and 3rd year plantations. No unique mycorrhizal fungal OTUs were identified in the 2nd year plantations. In total, 11 OTUs were exclusively found in the soil at the 4th year plantations. Across all 70 mycorrhizal fungal OTUs found in the soil, *Basidiomycota* was the most dominant phylum, accounting for 88.6%. The highest number of ectomycorrhizal fungal OTUs (30 OTUs) were found in the clear-cut sites, and 27 ECM fungal OTUs were found in the mature forest stands. The number of ECM fungal OTUs significantly decreased in 1st, 2nd, and 3rd year plantations (10, 7, and 8 OTUs, respectively), and their number significantly increased up to 24 OTUs in the 4th year *P. sylvestris* plantations.

### 3.3. Relations between Chemical Parameters and Fungal Community in the Mineral Topsoil

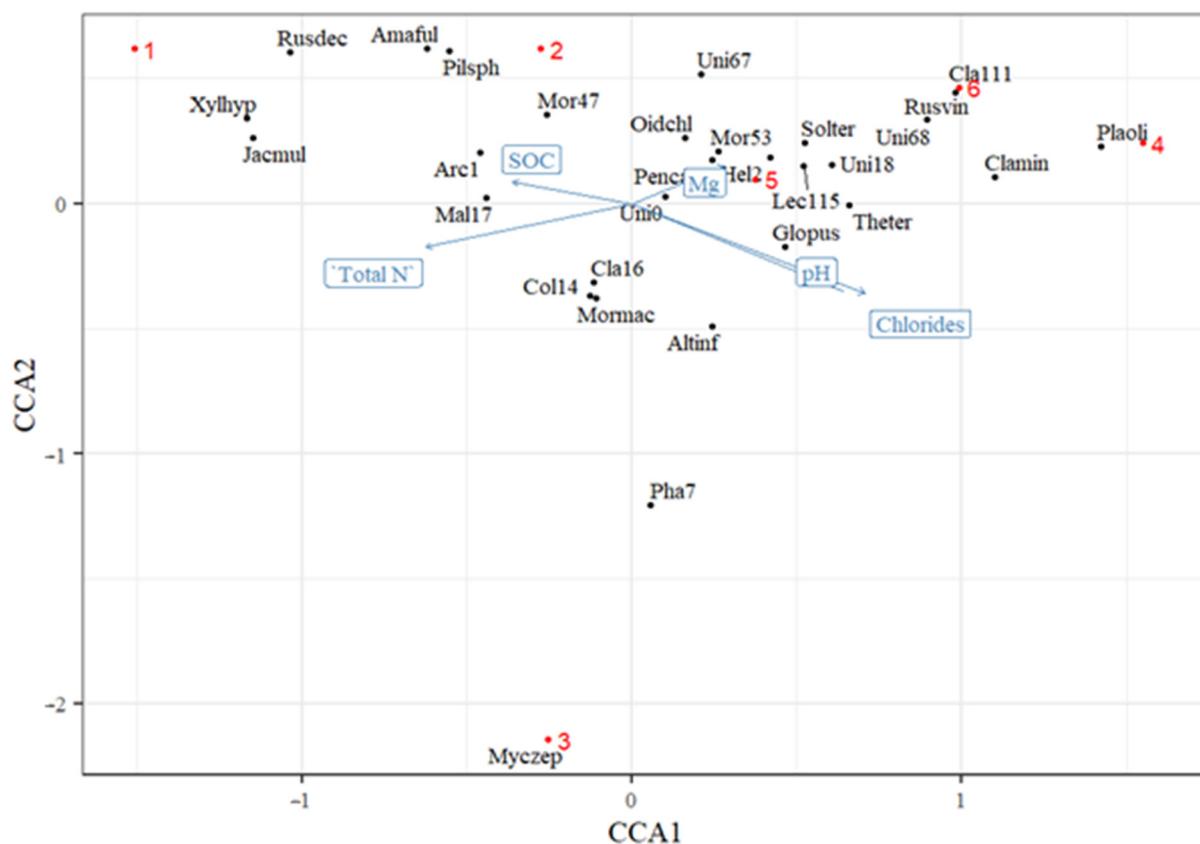
Relatively strong positive significant correlations were found between the number of mycorrhizal fungal OTUs and the concentrations of total N and SOC, as well as between the number of saprotrophic fungal OTUs and the  $Mg^{2+}$  concentrations (Table 6).

**Table 6.** Pearson’s correlation between chemical soil parameters ( $pH_{CaCl_2}$ , SOC, total N, P, K, Ca, and Mg, and also mobile  $Ca^{2+}$ ,  $Mg^{2+}$ , and  $Cl^{-}$ ) and the parameters of soil fungi in the studied sites.

| Variables                 | $pH_{CaCl_2}$ | g kg <sup>-1</sup> |         |        |        |        | mg kg <sup>-1</sup> |                  |                  |                 |
|---------------------------|---------------|--------------------|---------|--------|--------|--------|---------------------|------------------|------------------|-----------------|
|                           |               | SOC                | N       | P      | K      | Ca     | Mg                  | Ca <sup>2+</sup> | Mg <sup>2+</sup> | Cl <sup>-</sup> |
| No. of fungal sequences   | 0.050         | 0.041              | 0.114   | −0.342 | 0.262  | 0.099  | 0.327               | −0.079           | −0.153           | 0.493           |
| No. of fungal OTUs        | −0.212        | 0.321              | 0.336   | 0.011  | 0.324  | 0.299  | 0.303               | 0.152            | 0.110            | 0.483           |
| No. of mycorrhizal fungi  | −0.413        | 0.518 *            | 0.498 * | −0.177 | 0.477  | 0.225  | 0.479               | 0.212            | 0.066            | 0.021           |
| No. of pathogenic fungi   | −0.028        | 0.064              | 0.087   | 0.263  | −0.085 | 0.078  | −0.120              | −0.110           | −0.181           | 0.389           |
| No. of saprotrophic fungi | −0.173        | 0.271              | 0.350   | 0.262  | 0.406  | 0.493  | 0.298               | 0.477            | 0.505 *          | 0.206           |
| No. of endophytes         | −0.207        | 0.436              | 0.380   | 0.141  | 0.435  | 0.142  | 0.384               | 0.068            | 0.158            | 0.245           |
| Shannon_H                 | −0.274        | 0.183              | 0.003   | 0.363  | −0.090 | −0.099 | −0.200              | 0.192            | 0.383            | −0.015          |

\* Statistically significant correlations at  $p < 0.05$ .

The relations between the relative abundance of the 30 most common fungal OTUs and chemical soil parameters ( $pH_{CaCl_2}$ , SOC, total N,  $Mg^{2+}$ , and  $Cl^{-}$ ) in the study sites were evaluated using canonical correspondence analysis (CCA) (Figure 9). The relative abundance of fungi *Archaeorhizomyces* sp. 5425\_1 and *Malassezia* sp. 5425\_17 significantly correlated with the SOC concentration. Relative abundance of fungus *Glonium pusillum* H.Zoog correlated with the soil pH value. The relative abundance of fungi *Penicillium camemberti*, *Helotiaceaea* sp. 5425\_2, and *Mortierella* sp. 5425\_53 significantly correlated with the  $Mg^{2+}$  concentration (Figure 9).



**Figure 9.** Canonical correspondence analysis (CCA) of the selected chemical variables ( $\text{pH}_{\text{CaCl}_2}$ , SOC, total N,  $\text{Mg}^{2+}$ , and  $\text{Cl}^-$ ) and the most common 30 fungal OTUs. Red numbers represent study sites: 1 is the mature forest stands; 2—clear-cuts; 3—the 1st year plantations; 4—the 2nd year plantations; 5—the 3rd year plantations; 6—the 4th year plantations. The first and second axes explain 32% and 30% of the variance. Abbreviations of fungal species are composed of the three-letter abbreviation of the species scientific name. If the fungal taxa were identified only to the genus level, abbreviations are composed of the three-letter abbreviation of the genus name and the last number of the identification code (full names of fungal out are given in Table 4).

#### 4. Discussion

The current study showed significantly higher concentrations of mineral N,  $\text{P}_2\text{O}_5$ , and  $\text{K}_2\text{O}$  in the mineral 0–20 cm soil layer of the 1st and 2nd year *P. sylvestris* plantations, established on clear-cut sites. The obtained values were from 7% for  $\text{K}_2\text{O}$ , to 2.1 times higher than in the adjacent mature forest stands. In the period between 1 and 3 years after clear-cutting of mature pine forest, changes in mineral N concentrations have been observed in the previous studies [55–57]. Several studies have shown that nitrate leaching was observed to be particularly intense in the clear-cut sites, and higher concentrations of phosphates, potassium, and SOC were often obtained [56,58–60]. In the mineral soils of clear-cut sites, more intensive nitrification processes are recorded due to the changes in the site temperature and humidity, which causes the decomposition of the forest floor, the mineralization and decrease of organic matter content, as well changes in the mechanisms of plant competition for nutrients [57,61–64]. The abovementioned processes intensify the leaching of N compounds, especially nitrates, into the deeper soil layers. Therefore, a rapid increase in the concentrations of mineral N forms ( $\text{NO}_3\text{-N}$ ;  $\text{NO}_2\text{-N}$ , and  $\text{NH}_4\text{-N}$ ) may impair the recovery and development of the ground vegetation [65]. However, the growth of young *P. sylvestris* at the early stages of stand development is unlikely to be significantly affected. However, other studies indicated the opposite results, showing

that the concentrations of mobile  $P_2O_5$  and  $K_2O$  in the 0–10 cm layer of mineral soils of clear-cuts were lower than in the stand [65].

The SOC concentrations depend on the balance between carbon input with plant litter and carbon loss due to the mineralization of soil organic matter [66,67]. Due to the more intensive mineralization of soil organic matter, clear-cutting has been found to enhance SOC fluxes, including the leaching of total and labile SOC [68–72]. Although in the clear-cut sites more intensive mineralization and an increase in SOC in the upper mineral soil layer could be expected, the current study did not reveal such a trend.

One of the most important indicators is the C: N ratio, which directly determines the decomposition and transformation of soil organic matter [73]. This study analyzed the C: N ratio for determining soil quality and microbiological activity, but no clear differences were found between the C: N values in the mature forest, clear-cut, and reforested sites. As reported by Siebers et al. [74], more significant changes in the C: N ratios in the upper mineral soil layer after clear-cutting of the mature forest are often recorded after 24 months, indicating the relatively slow decomposition of forest litter and felling residues, followed by the gradual migration of elements into deeper soil layers.

Changes in chemical soil parameters certainly affect the diversity and abundance of soil mycobiota. The removal of the main source of plant biomass C and the concomitant physical and chemical changes in the soil leads to a change in the composition of fungal communities towards species that can more easily spread, establish, or survive under new conditions [25,29,75]. For example, ectomycorrhizal (ECM) fungi may induce N limitation of free-living microbial decomposers, and this competition between ECM fungi and free-living decomposers for N can slow soil C cycling and increase soil C storage [76,77].

In the current study, the higher concentrations of measured chemical soil parameters, such as mineral N,  $P_2O_5$ , and  $K_2O$  in the upper mineral 0–20 cm soil layer in the plantations did not cause significant changes in fungal operational taxonomic units (OTUs) diversity. A relatively strong correlation was found between the number of mycorrhizal fungal OTUs and the concentrations of total N and SOC, as well as between the number of saprotrophic fungal OTUs and the  $Mg^{2+}$  concentrations. Some relations were evaluated between separate fungal OTUs and chemical soil parameters. The relative abundance of fungi *Archaeorhizomyces* sp. 5425\_1 and *Malassezia* sp. 5425\_17 significantly correlated with the SOC concentration. The relative abundance of fungus *Glonium pusillum* correlated with the soil pH value. The relative abundances of fungi *Penicillium camemberti*, *Helotiaceae* sp. 5425\_2, and *Mortierella* sp. 5425\_53 significantly correlated with the  $Mg^{2+}$  concentration.

In the current study, a total of 1143 fungal OTUs were found. Although the number of species found is not small, the main dominant species are the same as in the studies conducted by other authors. The most abundant fungal OTU in our study was *Archaeorhizomyces* sp. 5425\_1, which represented 11.5% of the total fungal OTUs. *Archaeorhizomyces*, a widespread fungal class with a dominant presence in many soils, contain cryptic filamentous species forming plant–root associations whose role in terrestrial ecosystems remains unclear [78–80]. The second most abundant fungus *Helotiaceae* sp. 5425\_18 represented 6.8% of the total fungal OTUs. The distribution of species of the *Helotiaceae* family is usually widespread, and therefore it is difficult to assign this fungal OTU species to any trophic group. *Oidiodendron chlamydosporicum* was also identified as one of the most common fungal OTU in our study. This fungal OTU was found in all study sites—the mature forest stands, clear-cuts, and 1st–4th year *P. sylvestris* plantations. In accordance with previous studies [81,82], the *Oidiodendron* sp. was more abundant in undisturbed soil.

In the current study, a similar number of unique fungal OTUs were detected in the mature forest stands and in the clear-cuts. This tendency was comparable for both analyses—soil fungal OTUs and mycorrhizal fungal OTUs. In the 1st–3rd year *P. sylvestris* plantations, the number of unique fungal OTUs decreased. In the 4th year plantations, the number of unique fungal OTUs significantly increased. Based on the results of our study, the number of fungal OTUs was able to recover four years after clear-cutting, but the composition of fungal communities remained different.

Mycorrhizal fungi are a very important component in assessing the reforestation process after clear-cutting [83–88]. Therefore, in our study, more attention was paid to mycorrhizal fungi and changes in relative abundance in different study sites. One of the functions of mycorrhizal fungi, especially in the early stages of forest ecosystem development, is protection against soil-borne pathogens and nematode infections in pine forests [89–91]. Furthermore, when colonized with mycorrhizal fungi, tree seedlings are known to tolerate different environmental stresses, such as drought or high temperatures, more efficiently [31,92–95]. In the temperate and boreal forests, most woody plant species are obligate partners with ectomycorrhizal fungi, which provide nutrients (for example, N) and water to host trees in exchange for photosynthetically fixed carbon (C) [75,88,90,96]. Among other forest tree species, *P. sylvestris* has been recognized as an obligate mycotroph, known as a host of 200–300 ectomycorrhizal fungal species [97–101]. In our study, a total of 70 mycorrhizal fungal OTUs were found in the soil sampled from the *P. sylvestris* study sites. The highest number of ECM fungal OTUs was found in the clear-cut sites, and the lowest number was found in the 2nd and 3rd year *P. sylvestris* plantations. The most abundant ECM fungi in our study were: *Amanita fulva*, *Piloderma sphaerosporum*, and *Thelephora terrestris*. *Amanita fulva* was found only in the mature forest stands and in the clear-cuts, while it was not detected in the 1st–4th year plantations. *T. terrestris* is considered a pioneer, multi-host mycobiont, common in nurseries, as well as young and old forests [82,102]. Among all ECM fungal OTUs, only three—*Elaphomyces* cf. *muricatus* Fr, *Wilcoxina* sp. 5425\_54, and *Elaphomyces granulatus*—represented the phylum Ascomycota. According to other studies, *Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf was the most abundant and most frequently occurring species in the forest nurseries and clear-cuts [31,103]. *Wilcoxina* species form ectendomycorrhiza and are the most common colonizers of young conifers.

According to previous studies, clear-cutting has negative short-term effects on ectomycorrhizal fungal biomass and diversity [25,104,105]. However, other findings indicated that clear-cutting has profound long-lasting effects on the abundance and composition of ectomycorrhizal fungal communities [25,103,106]. Clear-cutting may cause a complete disappearance of energy supply to mycorrhizal fungi, while thinning only reduces energy input [25,27]. However, mycorrhizal fungi may remain unchanged due to clear cuttings if the fungi are associated with the retained trees or with the tree roots, extending into the felled area from the forest edges [107–109]. According to the study by Sterkenburg [75], several ectomycorrhizal fungal species were still detected in the soil three years after clear cutting with no retained trees, although at very low abundance. This could be related to the high number of naturally grown *P. sylvestris* seedlings prior to the forest felling [75,110]. In contrast, in our study, there were no common ECM fungal species detected in the soil in the 1st–4th year plantations. Only one ECM fungus, *Thelephora terrestris*, was found in all study sites, except for the 2nd year *P. sylvestris* plantations.

An important aspect of our study was that the evaluated effects reflected a period of four years after clear-cutting, and the obtained data were compared to those obtained in a mature *P. sylvestris* forest typical for the hemiboreal forest zone. This study contributes to existing knowledge on changes in soil chemistry and mycobiota during the early stages of forest regeneration, both nationally and regionally.

## 5. Conclusions

The study aim was to determine the fungal communities in relation to soil chemical parameters in the upper mineral soil layer of Scots pine (*Pinus sylvestris* L.) ecosystems, including mature forest stands, clear-cuts, and 1st, 2nd, 3rd, and 4th year plantations. A higher electrical conductivity (EC), higher concentrations of mineral nitrogen (N), total phosphorus (P), and P<sub>2</sub>O<sub>5</sub> were obtained in the soil of the 1st year plantations than the mature forest. The highest soil pH values and Ca<sup>2+</sup> concentrations were found in the 3rd year plantations. The soil chemical parameters obtained in the 4th year plantations were comparable with those obtained in the mature forest stands.

A total of 1143 fungal operational taxonomic units (OTUs) were identified in *P. sylvestris* forest soils. The most dominant phyla were *Ascomycota*, *Basidiomycota*, *Mucoromycota*, *Chytridiomycota*, and *Zoopagomycota*. The highest Shannon diversity index was obtained in the 1st and 3rd year plantations, while the lowest was found in the clear-cuts. The fungal communities identified in all studied sites of *P. sylvestris* plantations did not differ significantly. The number of common and unique fungal OTUs varied among the study sites, but a higher number of unique fungal OTUs was more typical for the 4th year plantations than other sites. The most abundant fungi were *Archaeorhizomyces* sp. 5425\_1, *Helotiaceae* sp. 5425\_2, Unidentified sp. 5425\_18, and *Oidiodendron chlamydosporicum*. The most abundant ectomycorrhizal (ECM) fungal OTUs were found in the clear-cut and mature forest stand sites. The most abundant ECM fungi were *Amanita fulva*, *Piloderma sphaerosporum*, *Thelephora terrestris*, *Russula decolorans*, and *Russula vinosa*.

The soil of *P. sylvestris* sites was inhabited by different fungal communities during the first four years after clear-cutting. The assumption that changes in forest soil chemical parameters can affect soil fungal communities in different study sites was partially confirmed, although the identified changes in soil parameters did not reveal significant effects on fungal abundance and diversity in all cases.

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