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Mycobiome of Post-Agricultural Soils 20 Years after Application of Organic Substrates and Planting of Pine Seedlings

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Abstract: A 20-year study of a pine stand on post-agricultural land showed that woody debris in the form of organic matter can be successfully used to restore symbiotic mycorrhizal communities, as is the case with forest soils. Woody substrates restored organic matter in soils altered by long agricultural use and had a positive effect on the composition of mycobiota antagonistic to pathogens, especially to *Heterobasidion annosum*, the causal agent of the dangerous disease root and stump rot of many forest tree species, including stands of *Pinus sylvestris* (L.). In a study that started in 2001 in the forest district of Czarne Człuchowskie (northern Poland), the following organic materials were used: wood residues (W), sawdust (S), bark compost (B), and compost applied to the root zone during planting (G). The organic materials were spread in the form of mulch over the entire area during planting. After twenty years, it was found that the substrates used provided suitable growth conditions for mycobiome useful for pines. The addition of organic matter did not change the alpha biodiversity of the soil, but in the long term led to significant changes in the composition of mycobiota (beta biodiversity). The changes in the soil after the addition of organic material naturally accelerated the formation of the forest habitat. A number of fungi evolved that degraded added lignin and cellulose while being antagonists of *H. annosum* and other pine pathogens. In particular, the well-known hyperpathogens of the genus *Trichoderma* played an important role by promoting resistance of the soil environment to pathogens. Soil enrichment by bark compost and wood residues increased the relative abundance of *Trichoderma* more than fourfold.Mycorrhizal fungi became dominant in soil enriched with organic matter. After enriching the soil with bark compost, the relative abundance of *Amphinema* and *Inocybe* increased to 5%. The relative abundance of *Russula* in soil enriched with wood residues and sawdust increased to 9% and 5%, respectively. Mycorrhizal fungi, e.g., of the genus *Ąmanita*, *Rusula*, which formed root mycorrhizae, not only increased the root receiving area many times over, but also protected the roots (mechanically and chemically from pathogens). Altogether, the observed positive changes increase the chances that the first generation of pines will survive on the ground.

Keywords: afforestation; root and butt rot; *Heterobasidion annosum*; organic matter; fungi diversity, mycorrhizal fungi; *Pinus sylvestris*

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1. Introduction

The circular economy recycles the waste produced, thus protecting the environment. Forestry can also join this trend by reforesting former agricultural land [1]. From an ecological point of view, these soils have been impoverished by many years of agricultural use. The first generation of Scots pine (*Pinus sylvestris* L.) on formerly agricultural land is doomed to die already at the age of the young trees (about 40 years) due to increased activity of insect pests and fungal pathogens (short-term forecast 2002). Of the latter, the root and butt rot pathogen of pines *Heterobasidion annosum* (Fr.) Bref. is the most dangerous in post-agricultural soils, where it has no natural counterparts (fungi and bacteria) and may cause severe losses on a massive scale [2]. Therefore, the restoration of a stable forest on post-agricultural land depends primarily on soil conditions [3]. Post-agricultural soils do not yet function as forest soils, and measures to accelerate processes for their improvement, especially their biological properties, are important [4]. Post-agricultural soils lack sufficient humus, and it is the organic horizon that has a decisive influence on most soil properties, especially on their retention, microbiological properties, and fertility. Organic matter improves moisture conditions, increases biodiversity, and provides a constant supply of nutrients from decomposing organic matter to soil mineral horizons and subsequently to growing trees [5]. Soil revitalization issues on post-agricultural land have been addressed in many research papers, with the prevailing view being that they are necessary to improve the fertility of poor post-agricultural soils and to initiate the development of forest ecosystems that are more resistant to disease threats. Converting land use from agriculture to forestry is a difficult and time-consuming process. Post-agricultural soils under long-term management are highly modified and are fundamentally different from forest soils [6,7].

Some of the wood wastes generated in technological processes, such as sawdust and residues from timber harvesting, have been used to accelerate the transformation of post-agricultural soils into forest soils [8], as well as for organic fertilization of land formerly used for agriculture [9]. Sawdust has also been used in nursery substrates as a carrier of antagonistic fungi (e.g., *Trichoderma* sp.) to increase the biological activity of the soil against pathogens of roots [10].

This study aimed to evaluate the changes in the soil 20 years after the application of various organic substances in a pine stand established on former arable land in the Czarne Człuchowskie forestry. One of the objectives was to verify whether the changes in mycobiota biodiversity are in the direction of the formation of mycorrhizal fungal communities characteristic of forest ecosystems. We also wanted to use a powerful tool, namely, next-generation sequencing (NGS), to find out to what extent the addition of various organic substances has a long-term effect on the composition of the soil mycobiome.

2. Materials and Methods

Test plots of 0.3 ha of Scots pine in the Czarne Człuchowskie forest district (subdistrict Brzezie, fresh forest site) were used for soil revitalization in 2001 (after autumn preparation by full plowing). The following substrates were applied separately on each experimental plot: bark compost (B), wood residues (W), gravel from bark compost (G), and sawdust (S). No treatments were applied to the control plot (C). In 2021, after 20 years, an identification of fungal DNA from soil was performed (using NGS).

2.1. Mulching of the Soil

The restoration of the soil's organic horizon and the changes in its physicochemical properties were observed in designated experimental plots (Figure 1).

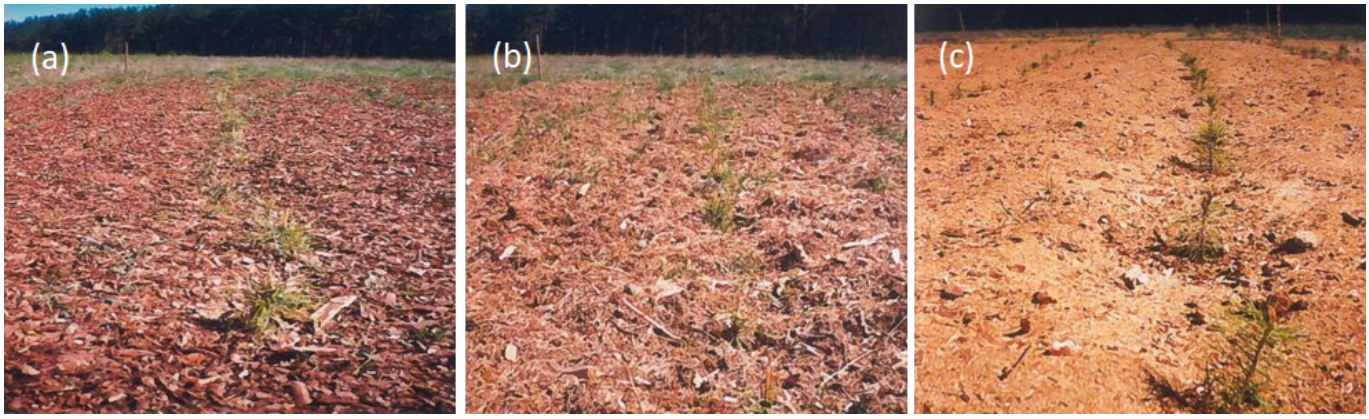

Figure 1. Soil mulching in Czarne Człuchowskie forest district: (**a**) pine bark compost—B, (**b**) logging wood residues—W, (**c**) pine sawdust—S.

2.2. DNA Extraction

For genetic analyses, 12 cylinders of 100 cm^3 of soil were taken, with pooled samples from the Czarne Człuchowskie forest district. They were taken after removing the organic layer of the soil from a depth of about 5 cm. Three replicates of each were prepared, for a total of 36 samples. The soil-containing samples were thoroughly mixed before collection of approximately 1 g.

For each sample, DNA was extracted from the soil using a NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany). Briefly, 0.5–1.0 g of soil was added to 2 mL Eppendorf tubes containing ceramic beads (1.4 mm), suspended in 500 µL of SL1 extraction buffer, and vortexed at 10,000 rpm for 10 min. The further steps were performed following the manufacturer's instructions. DNA was eluted in a final volume of 100 µL and stored at –20 °C before further analysis.

The DNA concentrations and purity of all samples were measured with a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of extracted DNA ranged from 11 ng/µL up to ~100 ng/µL, and the 260/280 ratio was in the range of 1.86–2.07. The DNA concentration of all samples was normalized to 10 ng/µL for amplicon PCR.

2.3. Libraries Preparation

Multiplexed amplicon libraries were constructed according to the two-step PCR protocol described by Rettel et al. [11]. This method consists of dual PCR amplification. The first PCR uses amplicon-specific primers, including an Illumina adapter overhang (amplicon PCR), and the second, cycle-limited PCR is used for the incorporation of Illumina index adapters for multiplexing (index PCR). The ITS2 primers (Table 1) contain a Nextera-Illumina-Adapter overhang (underlined) and the marker-specific sequence.

Table 1. Sequence of the forward primer ITS3-Mix2 [12] and reverse primers ITS4-cwmix1 and ITS4-cwmix2 [13] for amplification of the fungal ITS2 region. Nextera-Illumina-Adapter overhang sequence is underlined.

Primer Name	Primer Sequence
ITS3-Mix2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACCAWCGATGAAGAACGCAG
ITS4-cwmix1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATCCTCCGCTTAYTGATATGC
ITS4-cwmix2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATCCTCCGCTTATTRATATGC

Briefly, amplicon PCR was conducted as described below. Primers were diluted to a final concentration of 20 pmol/µL, and the reverse primers (ITS4-cwmix1 and ITS4-cwmix2) were mixed at an equimolar concentration to improve taxonomic coverage of the fungal kingdom. The PCR cocktail of 20 µL reaction volume comprised 10 µL KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), 1 µL of forward primer, and 1 µL of reverse primer mix at 20 pmol/µL, 2 µL of nuclease-free water (Qiagen, Hilden, Germany), and 2 µL of template DNA (20 ng). PCR reactions were carried out with the following program on a Veriti 96-Well Thermal Cycler (ThermoFisher Scientific, Waltham, MA, USA): initial denaturation for 3 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 57 °C, 1.5 min at 70 °C, and a final elongation cycle for 5 min at 72 °C. The first PCR product was purified with CleanNGS (CleanNA, Waddinxveen, The Netherlands). Following purification, 2 µL of the first PCR product was PCR amplified for final library construction containing the index using the NEBNext Multiplex Oligos for Illumina 96 Index Primers (New England Biolabs, UK). The cycle condition for the second PCR was the following: initial denaturation for 3 min at 95 °C followed by 8 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 sat 72 °C, and a final elongation cycle for 5 min at 72 °C. The second PCR product was purified the same way as the first PCR product.

The resulting PCR products were pooled, and the final purified product was then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification Kits for Illumina Sequencing platforms, Roche, Basel, Switzerland). The paired-end (2 × 300 bp with V3 chemistry) sequencing was performed using the MiSeq platform (Illumina, San Diego, CA, USA). Sequences were submitted in the NCBI Sequence Read Archive (SRA) under the study accession number PRJNA905069.

2.4. Processing and Analysis of Sequencing Data

Reads quality was checked at FastQC [14] and filtered using the Trimmomatic (version 0.38) [15] to exclude low-quality reads (Q < 20, sequences with any ambiguous (N) bases, more than six homopolymers). The chimera sequences identified by Mothur 1.31.2 [16] were discarded.

To analyze community composition and assign taxonomic affiliations to the amplicon sequences, we used the software pipeline CCMetagen v1.2.3—(ConClave-based Metagenomics) [17] that utilizes the ConClave sorting scheme [18]. Taxonomic assignment was carried out utilizing the entire NCBI nucleotide collection. The criteria used for taxonomic assignment in CCMetagen were as follows: species-level similarity threshold of 98.41%, genus-level of 96.31%, family-level of 88.51%, order-level of 81.21%, class-level of 80.91%, and phylum-level of 50% [19]. To account for differences in sequencing depth, rarefaction was performed in Mothur implemented in Galaxy v22.05 [16]. Fungal OTUs shared among experiment variants were analyzed using the Venn Diagram software [20].

2.5. Analysis of Fungal Biodiversity

Alpha diversity of the fungal communities was estimated by the Shannon diversity index. Evaluation of beta diversity was calculated using Bray–Curtis dissimilarity index. For the calculations, we used the Scikit-bio 0.5.8 [21] software package. The assignment of ecological roles was based on FUNGuild [22].

3. Results

Effects of Mulching on the Soil Mycobiome

Obtained sequence reads were filtered and low-quality reads were discarded. High-quality reads were checked for chimera, and the chimera was also removed. Obtained high-quality free-of-chimera reads were assigned to OTU. OTUs with reads number below 100 were discarded from further analysis. The curated datasets comprised 1,697,460 reads The per-sample values ranged between 138,800 and 917,700.

Amplicons were assigned to 186 OTUs (Supplementary Table OTU). Approximately half of them were identified to genus or species level. There were 84 genera and 45 species

found. Soil fungal communities were strongly dominated by the diverse Ascomycota and Basidiomycota (125 and 51 of total OTUs, respectively). Mucorromycota was represented by 11 OTUs.

Ascomycota was represented by 11 classes. The most numerous were Leotiomycetes (34 OTUs), Sordariomycetes (28 OTUs), Eurotiomycetes (22 OTUs), Dothideomycetes (19 OTUs), and Pezizomycetes (10 OTUs). Basidiomycota were represented by six classes, and almost all of them belonged to Agaricomycetes (42 OTUs). Similarly, Basidiomycota Mucorromycota were represented by four classes: Glomeromycetes, Mortierellomycetes, Mucoromycetes, and Umbelopsidomycetes, each of them containing a single OTU.

The most taxonomically rich phylum was also the most abundant in analyzed soil. Relative abundance of reads number belonging to Ascomycota ranged from 33.9% to 54.6%, Basidiomycota from 15.3 % to 37.3%, and Mucorromycota from 2.2% to 5.6%. Other phyla (Chytridiomycota, Cryptomycota, and Zoopogomycota) encountered below 0.01% reads. Besides known fungi phyla, a lot of reads belonging to unclassified fungi (22.4%–44.4%) were detected. Relative abundance of fungi at phylum, class, order, and family level is presented in Figures 2–4, respectively.

Within the Ascomycota, two classes are the most numerous: Leotiomycetes and Eurothiomycetes. Leotiomycetes make up about half (C—48%; B—50%; G—48%; S-57; W—61%), and Eurothiomycetes about one quarter (C—25%; B—32%; G—25%; S—26%; W—21%) of Ascomycota OTUs, respectively. In Basidiomycota, Agaricomycetes dominate;they account for over 87% of all Basidiomycota OTUs reads (C—87%; B—96%; G—87%; S—87%; W—98%). Simillarly, in Mucoromycota, Mortierellales dominate(C—69%; B—85%; G—69%; S—79%; W—78%) (Figure 3).

In Agaricomycetes, there are the most abundant orders: Agaricales and Russulales. In control, bark compost, and bark compost gravel, relative abundance of Agaricales is 72%, 63%, and 79%, respectively. In sawdust and wood residues, there are 36% and 21% Agaricales, respectively. The opposite relationship occurs in Russulales. While in the control they are <1%, in wood-residues-enriched soil, their relative abundance is about 54%, making them the most abundant order of Agaricomycetes.

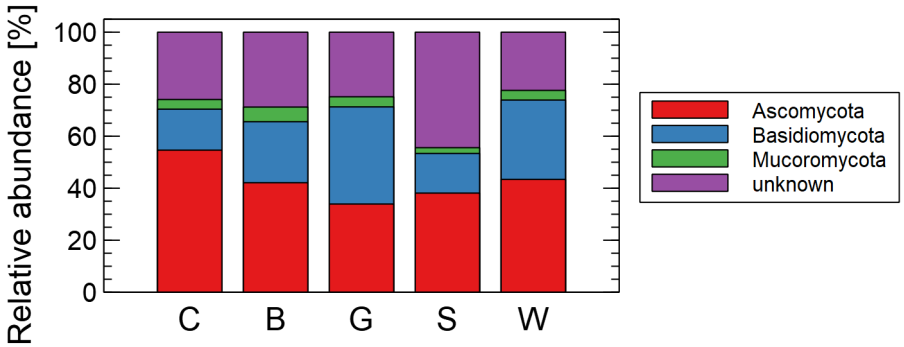

Figure 2. Relative abundance of detected fungi phylum versus various studied soil conditions: C—control, B—bark compost, G—bark compost gravel, S—sawdust, W—wood residues.

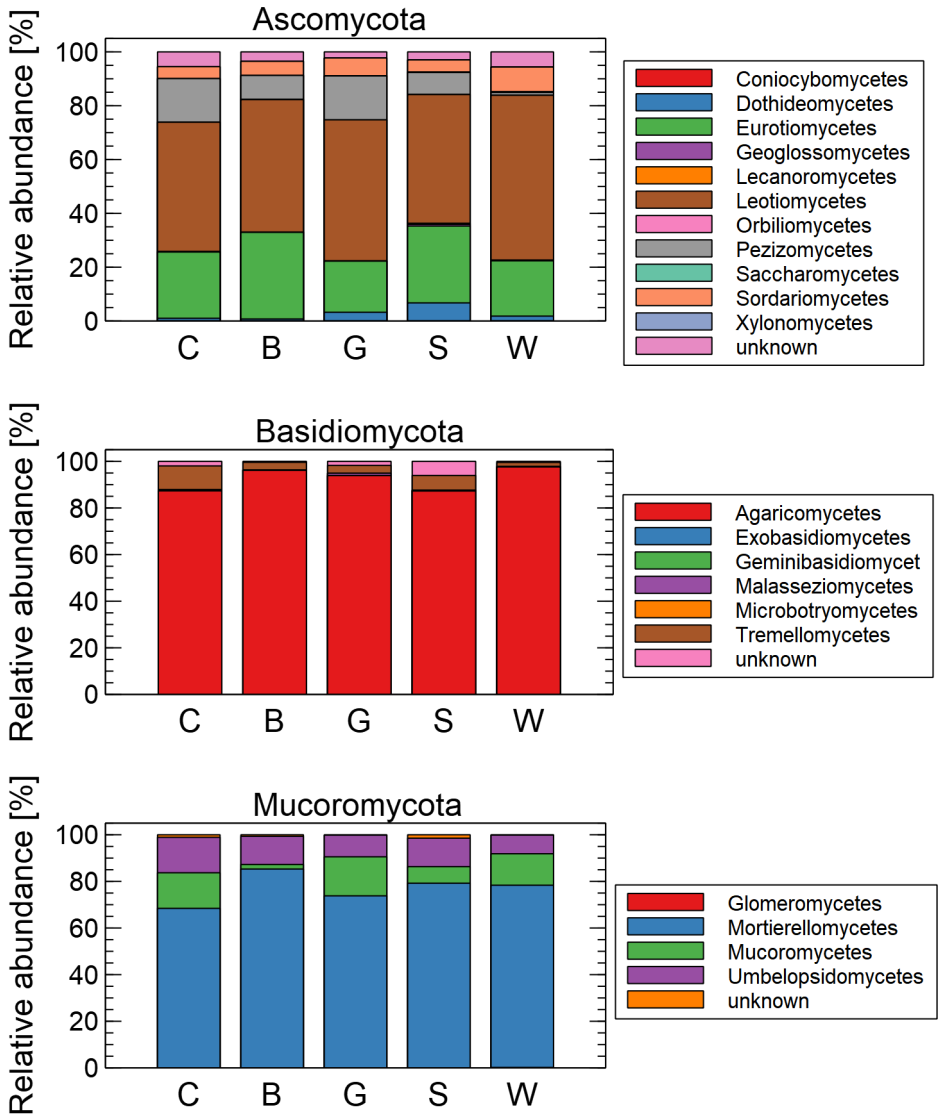

Figure 3. Relative abundance of detected fungi class versus various studied soil conditions: C—control, B—bark compost, G—bark compost gravel, S—sawdust, W—wood residues. The fungal phylum for which percentage is calculated is marked above subfigures.

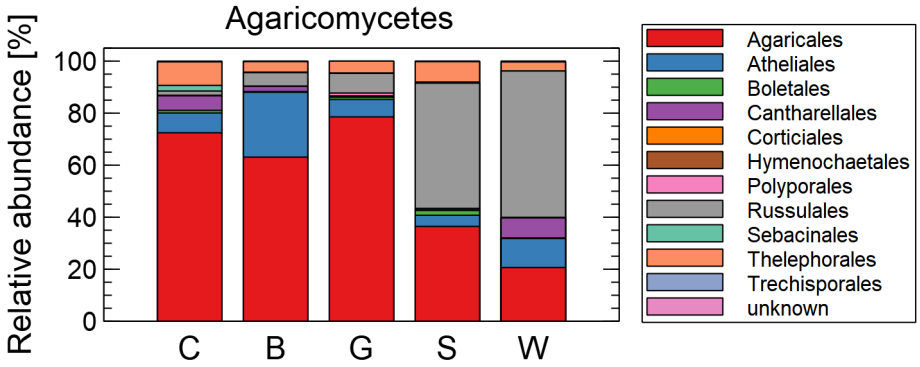

Figure 4. Relative abundance of detected fungi order of selected class Agaricomycetes of phylum Basidomycota versus various studied soil conditions: C—control, B—bark compost, G—bark compost gravel, S—sawdust, W—wood residues.

The performed analysis indicates that OTUs belonging to ten–twelve genera make up from 33% to 39% of all reads shown in Table 2.

Table 2. Relative abundance of the most abundant fungi genera (>1% in at least one experimental condition).

Genus	Fungi Relative Abundance (%)				
	Control	Bark Compost	Wood Residues	Sawdust	Bark Compost Gravel
Amphinema	0.00	5.01	0.00	0.00	0.00
Apiotrichum	0.00	0.10	0.08	0.00	1.02
Cenangium	0.10	0.09	0.40	0.10	2.01
Cenococcum	0.00	0.00	0.00	2.01	0.00
Collarina	0.06	0.00	1.01	0.00	0.00
Cortinarius	3.01	2.03	0.01	0.08	0.00
Exophiala	0.70	0.30	1.02	0.70	0.30
Geomyces	1.01	0.30	0.80	0.09	2.01
Hyaloscypha	1.10	4.00	0.21	1.03	0.20
Hydnum	0.30	0.20	2.03	0.00	0.00
Hygrophorus	0.00	0.10	0.90	0.00	1.04
Inocybe	0.90	5.02	2.00	0.00	7.01
Meliniomyces	6.10	0.40	1.01	8.02	2.00
Mortierella	2.03	2.00	0.80	1.01	0.52
Oidiodendron	6.00	5.00	7.00	5.03	2.03
Penicillium	5.02	10.01	6.03	7.01	4.04
Russula	0.10	0.10	9.04	5.04	1.00
Sagenomella	6.04	2.00	0.70	2.00	0.81
Solicoccozyma	1.03	0.60	0.30	0.51	0.40
Tricholoma	2.00	1.02	1.02	1.02	4.02
Wilcoxina	6.01	0.10	0.04	0.11	2.00

The addition of organic matter did not change the alpha biodiversity of the soil. The Shannon diversity index H (Figure 5) ranged from 3.41 to 4.53 and was not statistically significantly different between studied soil conditions. Instead of changes in biodiversity, enrichment of the soil with organic substrates led to long-term significant changes in mycobiota composition. The changes occur at qualitative and quantitative levels. Changes at qualitative level are presented in Figure 6a, and at quantitative level, in Figure 6b.

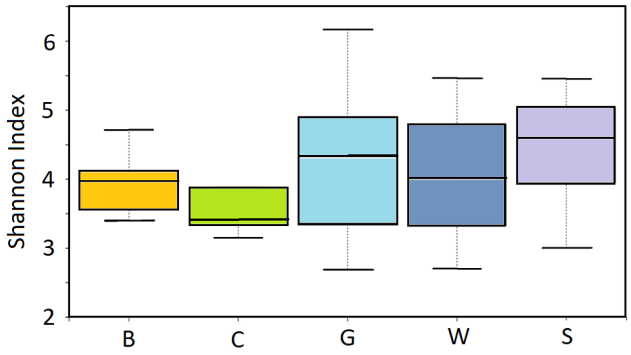

Figure 5. Alpha diversity of detected fungi based on Shannon index versus various studied soil conditions: B—bark compost, C—control, G—bark compost gravel, W—wood residues, S—sawdust.

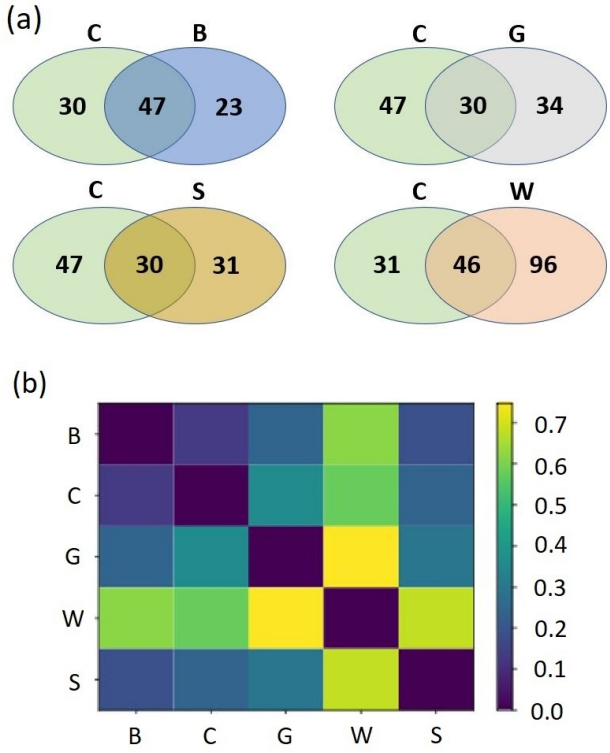

Figure 6. (**a**) Venn diagram showing the number of common OTUs between the soil conditions studied. (**b**) Bray–Curtis dissimilarity index. Comparison of species detected in studied soil conditions: C—control, B—bark compost, G—bark compost gravel, S—sawdust, W—wood residues.

In control soil, 77 OTUs were detected, 35 of which were assigned to genus and 22 to species level. The addition of sawdust, which enriched the soil with organic material consisting mainly of cellulose and lignin, slightly decreased diversity (61 OTUs, 30 genera, 10 species, respectively), but mainly led to mycobiome reconstruction. Moreover, in soil enriched by sawdust, 31 OTUs not detected in control soil appeared, among them *Aspergillus terreus*, *Yarrowia lipolytica*, *Podospora* sp., *Pseudaegerita* sp., *Pseudocamaropycnis* sp., and *Rickenella* sp. Importantly, a relative abundance of *Cenococcum geophilum* (the only species of the *Cenococcum* genus; *Gloniaceae*) increased twofold to approximately 2% of total fungal reads.

Enrichment of soil with sawdust leads not only to appearance of new species but also to the disappearance of some of the species present in the control plot (*Absidia glauca*, *Chaetomium crispatum*, *Cortinarius diasemospermus*, *Cortinarius parvannulatus*, *Entoloma sanvitalense*, *Metapochonia suchlasporia*, *Oidiodendron chlamydosporicum*, *Penicillium raphiae*, *Phialocephala cf. fortinii*, *Phialocephala fortinii*, *Pseudogymnoascus pannorum*, *Sagenomella striatispora*, *Trichoderma polysporum*, *Umbelopsis isabellina*, *Wilcoxina rehmii*). Especially strong changes in relative abundance were detected for *Cortinarius parvannulatus* (control—2%, not detected in sawdust) and *Wilcoxina* sp. (6.0% and 0.1%, respectively) (Table 2).

Enrichment of soil with bark compost also did not significantly change biodiversity (70 OTUs, 37 genera, 19 species), but, similar to sawdust,it significantly changed mycobiota composition. In bark-compost-enriched soil (but not in control soil plots), we detected *Amphinema* sp. (5% of total reads), two species of *Oidiodendron* (*O. pilicola* and *O. tenuissimum*), *Acephala macrosclerotiorum*, *Apiotrichum* sp., *Chalara* sp., *Desmazierella acicola*, *Hebeloma* sp., *Hydnotrya* sp., *Hygrophorus* sp., *Lophodermium* sp., *Metarhizium anisopliae*, *Mycena* sp., *Penicillium thomii*, *Phoma* sp., *Rhizoscyphus* sp., *Trichoderma* sp., and *Tricholoma* sp.; altogether 23 OTUs. Very abundant were *Penicillium* sp. (10% of total reads) which, together with eight other genera (*Amphinema* 5%, *Cortinarius parvannulatus* 1%, *Hyaloscypha* 4%, *Inocybe* 5%, *Mortierella* 2%, *Oidiodendron* 5%, *Sagenomella* 2%, *Tricholoma* 1%), made up 35% of total reads (Table 2).

The appearance of 23 new OTUs was associated with the disappearance of numerous OTUs present in control soil (*Absidia cylindrospora*, *Absidia glauca*, *Capronia* sp., *Collarina* sp., *Cortinarius diasemospermus*, *Entoloma sanvitalense*, *Fusarium* sp., *Helvella* sp., *Humicola* sp., *Metapochonia suchlasporia*, *Neonectria* sp., *Pseudogymnoascus pannorum*, *Rhizoscyphus cf. ericae*, *Trichoderma polysporum*, *Varicosporium* sp., *Wilcoxina* sp. and uncultured *Leptosphaeriaceae*, *Malassezia*, *Venturiaceae*, *Wilcoxina*).

Bark compost gravel had a similar effect on mycoflora to bark compost, but additional species were detected (*Cadophora* sp., *Suillus* sp., *Sordaria* sp., *Cenococcum geophilum* and *Lophodermium conigenum*. *Acephala macrosclerotiorum*, *Amphinema* sp., *Chalara* sp., *Hebeloma* sp., *Hydnotrya* sp., *Metarhizium anisopliae*, *Mycena* sp., *Oidiodendron pilicola*, *Oidiodendron tenuissimum*, *Phoma* sp., *Rhizoscyphus* sp. and *Trichoderma asperellum*).

The biggest changes in soil mycobiota were found in soil enriched by wood residues. A total of 23 genera (*Absidia* sp., *Articulospora* sp., *Botryosphaeria* sp., *Chaetothyriales* sp., *Cordyceps* sp., *Didymella* sp., *Diplodia* sp., *Erysiphe* sp., *Lactarius* sp., *Lasiodiplodia* sp., *Lecanicillium* sp., *Neofusicoccum* sp., *Paecilomyces* sp., *Paraphaeosphaeria* sp., *Peniophora* sp., *Phyllosticta* sp., *Pithomyces* sp., *Pochonia* sp., *Preussia* sp., *Pseudotomentella* sp., *Rhizopogon* sp., *Starmerella* sp., *Tylospora* sp.) and some species (*Fusarium merismoides*, *Penicillium canescens*, *P. ochrochloron*, *P. soppii*, *P. spinulosum*) were detected only in this experiment variant. The appearance of so many fungi genera was associated with the disappearance of a small number of genera (*Cortinarius* sp., *Entoloma* sp., *Helvella* sp., *Humicola* sp., *Ilyonectria* sp., *Rhizoscyphus* sp. *Wilcoxina* sp.) and *Oidiodendron chlamydosporicum* species present in control. Quantitative analysis of abundance also showed that wood-residues-enriched soil has the biggest difference to control and to other types or organic supplements. The Bray–Curtis index for wood-residues-enriched soil ranged from 0.57–0.74, while for other organic supplements was in the range of 0.13–0.35.

The assignment of fungal ecological roles revealed that the number of mycorrhizal fungi OTUs increased from 11 in control to 24 and 21 after soil enrichment with bark compost or wood residues, respectively (Table 3). The addition of wood residues also increased the number of saprotrophs (from 20 to 31). This significant increase of mycorrhizal fungiand saprotroph in wood-residues-enriched soil was associated also with an increase of plant pathogens OTUs.

Table 3. Number of fungal functional group OTUs in different soil conditions.

	Functional Group		
Soil Condition	**Mycorrizal**	**Saprotroph**	**Plant Pathogen**
Control	11	20	4
Bark compost	24	15	3
Bark compost gravel	16	14	2
Sawdust	12	14	3
Wood residues	21	31	8

4. Discussion

4.1. Advantages of Adding Organic Substances

The inadequate state of biological properties of soils, especially the lack of naturally occurring antagonists of *H. annosum*, the cause of root and butt rot, is the main cause of stand health problems on former agricultural lands. Restoration of forests in such areas encounters numerous difficulties, and the death of pine stands is often caused by an infestation of the aforementioned fungal pathogen. Post-agricultural soils transferred for afforestation differ from forest soils mainly by the absence of an organic horizon [23]. In our experiment, we proved that we can positively modify the biodiversity of soil by adding organic matter that accumulated on the surface of forest soils (with varying degrees of decomposition), and determine its biological properties. We have showed that it provides a source of energy for the development of microorganisms, including mycorrhizal fungi.

Soils destined for reforestation after agriculture are generally completely degraded and deficient. The conversion of agricultural soils to the forest is a slow process, and without silvicultural measures such as ours, it is difficult to achieve stable stands. Our goal was to set these natural processes in motion to ensure sustainable and stable forest ecosystems for future generations. Forests grow best when the species composition of the stands is compatible with the forest habitat type, including relevant soil properties. This is favored by beneficial fungi (antagonistic and mycorrhizal). Similar beneficial effects of revitalizing soils with sawdust were described in 1999 [3]. Our experimental plots were established in 2001, applying shredded wood residues, bark compost, and sawdust to restore the organic horizon [24,25]. The initial results of these studies were already been presented [23,25–27], but we now have the opportunity to show the changes that have taken place after more than 20 years. The present study shows positive changes in the DNA of soil microbiota. It turned out that the earlier enrichment of afforested post-agricultural land with organic matter in the form of compost, wood waste, or sawdust improved the fertility of these poor soils and stimulated beneficial microbial processes in the soil environment, in particular increasing the proportion of antagonists, which increased the resistance of roots to pathogen attack.

It is known that post-agricultural soils lack organic matter, including lignin and hemicellulose tissues that make up the structure of wood, including roots, which are the main substrate and energy source for the development of microorganisms, including fungi, especially basidiomycetes, which degrade the cellulose–lignin complex [27].

Hydrolysis of cellulose requires synergism of several organisms, including bacteria, actinobacteria, filamentous fungi, plants, and animals [28]. Among these organisms, filamentous fungi stand out, with the genera *Penicillium, Trichoderma, and Aspergillus* known as models for cellulase production at laboratory and industrial scales [29]. In our study, *Penicillum* accounts for between 4% and 10% of sequence reads, and the highest abundance was found in soils enriched with wood residues (10%) (Table 2). *Aspergillus* is much less abundant, but after soil enrichment, the abundance of this genus increased from 0.02% to 0.3% (sawdust, bark compost gravel). Soil enrichment also increased the abundance of *Trichoderma* from 0.2% in control to 0.9% in compost and wood residues and it is fungi, bacteria, and other microorganisms that are involved in the processes of creating the specific structure of the forest soil, giving it its normal biological activity. These fungi have an antagonistic effect against pathogenic fungi, especially *H. annosum*.

Even in the first years after planting, the structure and proportion of arbuscular mycorrhizal symbionts and saprotrophic fungi in the soil after planting are important for the condition of future stands [30–32]. Saprotrophs, the main decomposers, produce a wide range of extracellular enzymes that allow them to degrade the recalcitrant fraction of tree biomass. Mycorrhizal fungi play a pivotal role in the mobilization and sequestration of nitrogen and phosphorus and are responsible for the significant transport of carbon [33]. In the juvenile life stage of forest trees, great importance is attached to the creation of conditions conducive to the establishment of mycorrhizal symbioses [34,35]. By using molecular biology methods (NGS), we were able to demonstrate the biodiversity of the mycobiomes. After a 20-year process of transformation of the mycorrhizal community, which was no longer present in the soils after agricultural use, its composition was typicalfor the forest environment. Although we are unable to determine exactly when this occurred, we believe that the fungal spores were transferred along with organic residues. Without our intervention, these processes would likely have continued, as evidenced by analyses of control soils (still resembling post-farming soil) or after the application of sawdust and/or compost (less-transformed soils).

4.2. Protective Effects of Identified Mycorrhizae against Heterobasidion spp. and Other Pathogens

Colonization with ectomicorrhizal fungi showed clear protective effects against *Heterobasidion* spp., but also against other root pathogenic fungi (*Rhizoctonia solani*, *Fusarium* damping-off, *Ilyonectria* destructans) in both pine and spruce [36–38]. Thus, even in root tips that were heavily covered with dense *M. bicolor* hyphae, fungal colonization may have

created a physical protective barrier around the roots in addition to direct antagonism of *M. bicolor* against *Heterobasidion* [39].

Among the numerous microorganisms found after treatment were those that support plant growth in poor soils, such as *Pezizella ericae*, which forms ericoid mycorrhiza. The hyphae of *P. ericae* secrete phosphatases that convert phosphorus from organic matter and polyphosphates into a plant-available state. Thanks to this phenomenon, fungi assimilate nitrogen from nitrates, ammonium, free amino acids, and various organic polymers through the release of proteases and chitin, which is decomposed by chitinases, and released to plants. They can also decompose pectins and lignins, releasing carbon [40]. We also found *Meliniomyces bicolor* and the closely related *Cadophora finlandica* [41], both colonizing the roots of many northern temperate forest trees, such as pine, spruce, and birch. Both form the well-defined and characteristic ectomycorrhizal morphotype *Piceirhiza bicolorata* on pine roots [42]. However, in northern temperate and boreal forests, *M. bicolor* can also form ericoid mycorrhizas with shrubs of the Ericaceae family, such as *Vaccinium* spp. Indeed, *M. bicolor* in vitro behaves similar to a typical ericoid mycorrhizal fungus by inducing a significantly higher growth rate in its Ericaceae host compared to uninfected seedlings and by causing a measurable mutual transfer of carbon and nitrogen [43].

Other fungal species *Rhizopogon* usually significantly affect the growth of bioassay seedlings [44]. It was found that the growth of seedlings colonized by *Rhizopogon* or *Meliniomyces* species was significantly improved compared to uncolonized seedlings [44]. In China, the positive effect of *Rhizopogon* on host plant growth and its crucial role in seedling establishment and forest regeneration of endangered Chinese Douglas-fir was established [44].

The analysis of soil microflora (bacteria and microscopic fungi) by the dilution method did not allow to separate healthy and infected stands [45]. However, using the soil clots' overgrowth method, it was found that more cellulose-degrading bacteria were present in sample plots with healthy trees. Antagonistic microflora of *H. annosum* was found in all analyzed soils. Both isolated ascomycetes and *Penicillium* spp. showed antagonism to *H. annosum*. Microscopic fungi of the genera *Verticillium*, *Aureobasidium*, and *Rhizopus* were found only in the soil of healthy spruce stands [45].

Another species, *Penicillium striatisporum* Pst10, was isolated from the rhizosphere of chili peppers [46]. An experiment was conducted in which this isolate showed very strong antagonistic effects on mycelial growth of *Phytophthora* spp., *Cladosporium cucumerium*, and *Sclerotinia sclerotiorum*. In vitro assays tested the toxicity of sterilized liquid culture filtrates (SLCF) of Pst10 grown in potato dextrose broth against mycelial growth of *Phytophthora capsici* and sporangia/spore formation or germination. The SLCF completely inhibited mycelial growth and resulted in abnormal mycelium even at a 100-fold dilution. A 20-fold dilution of SLCF inhibited the formation and germination of sporangia and spores [46].

Velmalas et al. [39] investigated whether root colonization by ectomycorrhizal fungi (EMF) can alter the susceptibility of spruce (*Picea abies*) seedlings to root rot or necrotic leaf disease pathogens. First, spruce seedlings were inoculated by different EMFs and challenged with *Heterobasidion* isolates in Triaxenix tubes. The ascomycete EMF *Meliniomyces bicolor*, which showed strong antagonistic properties against the root rot pathogen *Heterobasidion* in vitro, effectively protected spruce seedlings against root rot. Second, spruce seedlings inoculated with *M. bicolor* or the forest humus were exposed to necrotrophic foliar pathogens under conventional forest nursery conditions on peat substrates. Post-winter infection with *Botrytis cinerea* was mild, and the extent of needle damage was independent of substrate and colonization by EMF. The severity of needle damage caused by pathogen *Gremminiella abietina* was high in seedlings in substrates with high nutrient availability and seedlings with well-established EMF communities. These results indicate that *M. bicolor* can protect spruce seedlings in axenic cultures against *Heterobasidion* root rot, but cannot induce systemic protection against foliar pathogens. It was also noted that nonsterile inoculum sources, such as forest humus, should not be considered for use under greenhouse conditions because they may predispose seedlings to unintended needle damage.

It appears that an extract of *Oidiodendron truncatum*, the other species of fungus found, could be used against white-nose syndrome (*Pseudogymnoascus destructans*), a devastating disease of hibernating bats in the USA. Rusman et al. [47] isolated 14 terpenes and three anthraquinone metabolites. Ten of these compounds were already described in the literature, but the structures of seven analogous terpenes were new. In addition, this was the first report of 4-chlorophyscione from a natural source that had previously been identified as a semisynthetic product. The compounds PR 1388 and LL -Z1271α were the only inhibitors of *P. destructans* (MIC = 7.5 and 15 µg/mL, respectively). Of two nematicides, 4-hydroxyphenylacetic acid (4-HPA) (1) and oidiolactone D (2), isolated from cultures of the fungus *Oidiodendron* sp., compound 2 showed nematicidal activities against the root lesion nematode, *Pratylenchus penetrans*, and the pine wood nematode, *Bursaphelenchus xylophilus*. This finding is very important for the health of pines planted on former agricultural lands [48].

4.3. Protective Effect of Identified Mycorrhizal Fungi on Plants against Drought

Fungi of the genus *Oidiodendron*, which are abundant in the soils we studied (Table 2), form ericoid mycorrhizae (ErM) and are considered an important factor in increasing crop yield and drought resistance [49]. *Oidiodendron* has not been studied from the point of view of antagonism in relation to diseases of the root system, e.g., *Heterobasidion* sp. [50]. Antagonism of *Oidiodendron* sp. from Sitka spruce ectomycorrhiza in relation to the species pathogenic for plants *Phytophthora cinnamomi* was confirmed [51]. The benefits of ErM fungi were also investigated by inoculating well-watered and severely drought-stressed plants with *Oidiodendron maius*. The results showed that the fungi significantly increased the biomass of stems and roots of lingonberry [49]. In the face of climate change, such a strategy to help plants cope with water scarcity is very important. In the cited study, ErM also significantly increased chlorophyll content probably because of a better water supply. Similarly, inoculation with *Lachnum pygmaeum* improved drought resistance, promoted root growth, and increased root wet weight by 1157%. While drought reduced chlorophyll content and soluble sugar content in the plant, ErM significantly increased their content after inoculation. At the same time, inoculation with the fungus *L. pygmaeum* decreased the content of malondialdehyde (a marker of oxidative stress) but increased the activity of superoxide dismutase (an important antioxidant for protection against oxidative stress). Overall, these results suggest that the successful coexistence of ErM fungi and lingonberry mitigates the negative effects of drought stress through higher secondary metabolites and photosynthetic pigment synthesis [49]. Similar effects were obtained by inoculating plants with *Oidiodendron maius* and *Phialocephala fortinii* [52]. It promoted the growth of *Vaccinium corymbosum*. In conclusion, inoculation of plants with three root symbionts (*O. maius*, *Hymenoscyphus* sp., and *P. fortinii*) and with the endophytic fungus *Xylaria* sp. increased plant height in laboratory experiments. On a semiindustrial scale, inoculation improved plant biomass and growth performance. Supplementing root-associated fungal communities with a mixture of ericoid mycorrhizal fungi and endophytic fungi could therefore provide an alternative to conventional fertilization and pesticide use in large-scale blueberry production [52]. This confirms the extensive changes observed in our experiment with pine and organic material.

In addition, the species we identified, *Cenococcum geophilum* (Table 2), is a cosmopolitan ectomycorrhizal fungus known for its broad habitat range [53] and may be the dominant ectomycorrhizal fungus in both arctic and temperate and subtropical forests [53–55]. It is particularly notable for its drought resistance [56,57]. However, this fungus is not restricted to dry sites, as it has also been observed in moist, poorly drained soils [55]. *Cenococcum geophilum* has a pioneer ability, although it also occurs in mature stands [58,59]. The ability of sclerotia to survive for several years may provide sufficient inoculum for effective colonization of host species [60]. The ectomycorrhiza of this fungus has been demonstrated on over 200 tree species from 40 different angiosperm and gymnosperm genera [61].

Thanks to the mycorrhizae, biennial seedlings of *Tilia cordata* growing on sandy soils can survive periods when soil water potential drops to −18 to −55 bar [62]. Their mycorrhiza, formed by *Cenococcum geophilum*, were shown to remain alive. Water loss measurements of seedlings under similar conditions decrease from 16.0 to 26.2 mg/h when the soil is moist to 4.1 to 4.6 mg/h when the soil is dry. Calculation of the volume of mycorrhiza indicates that their water content would support transpiration for periods not exceeding 1 h. Survival of mycorrhiza over long dry periods is apparently related to the ability of the fungal partner to resist desiccation [62].

4.4. Identified Fungi as a Potential Source of Bioactive Molecules

Fungi are considered an important source of bioactive molecules, often effective against other fungi and/or bacteria, and are therefore potential candidates in the search for new antibiotics. The fruiting bodies of sixteen different fungal species of the *Basidiomycota* were collected in the Italian Alps. Fungal species were identified by internal transcribed spacer sequencing (ITS). Most of the species belong to the genera *Cortinarius*, *Mycena*, and *Ramaria*, whose metabolite content has hardly been studied so far. The crude extracts obtained from the above fungi were tested for their inhibitory activity against five human pathogens: *Candida albicans*, *C. glabrata*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Twelve crude extracts showed activity against *P. aeruginosa*. The highest activity was shown by some *Cortinarius* species, such as *C. nanceiensis* [63].

Six of the fungi [64] examined in this study [64] were known to produce pigments with antibacterial activity. The presence of emodin, physcion, torosachryson, hypericin, or skyrin would likely contribute to the antibacterial activity observed in *Cortinarius persplendidus*, *C. austrovenetus*, and *C. [D. canaria]*. However, nearly colorless extracts also showed antibacterial activity, suggesting that previously unidentified fungal constituents may also possess antibacterial activity. Australian fungi of the genus *Cortinarius* are promising sources of natural products for further drug discovery because of the high biological diversity and unique evolutionary lineages found only in this region. This is accompanied by a large proportion of bioactive species and a great diversity of chemical constituents [64].

4.5. Antioxidant Capacity and Antimicrobial Activities of Some Identified Fungi

In the study of Türkoğlu et al. [65], usingextracts obtained with ethanol, the antioxidant capacity and antimicrobial activities of *Russula delica* were demonstrated. The researchers used four complementary assay systems, namely, DPPH radical scavenging, β-carotene/linolenic acid systems, total phenolic compounds, and total flavonoid concentration. The inhibition levels of the ethanolic extract of *R. delica* and the standards (BHA and α-tocopherol) increased in parallel with the increase in the concentration in the linoleic acid system. The presence of the flavonoid quercetin and the phenolic compound pyrocatechol in the extract was detected. The antimicrobial activity of *R. delica* was tested in vitro by agar well diffusion method, and the study confirmed the antibacterial activity of *R. delica* extract. Therefore, the extracts could be suitable as antimicrobial and antioxidant agents in the food industry [65].

Similar results were obtained with another fungal species of this genus (*Russula griseocarnosa*) [66]. They proved that the mushroom contains bioactive substances such as phytochemicals, e.g., phenols, flavonoids, ergosterol, and β-carotene. The main constituent in (*R. griseocarnosa*) was quercetin (95.82 µg/g) [66]. Thanks to its properties and activity, the mushroom can be used as a natural immunostimulant and antioxidant [67].

4.6. The Role of Mycorrhizal Fungi in the Nutrient Cycle of Pine Trees

The fungal species we detected in the soil samples *Tricholoma matsutake* (Supplementary table OTU) forms a symbiotic association with conifers, forming mycelial aggregations called "shiro", characterized by different chemical and physical properties to the nearby forest soil. The fungal diversity that lives in shiro soils plays a key role in nutrient cycling [68]. Zhou et al. [68] combined phospholipid fatty acid (PLFA) analysis and NGS

sequencing to determine fungal biomass and community structure. It was found that *T. matsutake* dominated in the shiro, which had significantly lower saprotrophic fungal biomass compared to the non-shiro soil. Fungal diversity was negatively correlated with the relative abundance of *T. matsutake* in the shiro soil. The fungal community in the shiro was characterized by similar fungal species composition in most of the forest type samples. It appears that *T. matsutake* coexisted with a particular fungal community due to competition or nutrient interactions. However, *Oidiodendron* sp. was positively correlated with the abundance of *T. matsutake*, which is common in Shiro. In contrast, *Helotiales* and *Mortierella* were negatively correlated with *T. matsutake*, both of which commonly inhabit non-shiro soil but do not occur in shiro soil. Zhou et al. [68] concluded that *T. matsutake* produces a dominance effect that shapes the fungal community and diversity in shiro soils across different forest types [68]. In other studies with the genus *Tricholoma*, the active mycorrhizal zone of shiro was revealed, in which *T. matsutake* showed antimicrobial activities [69], and as a result, the abundance of bacteria and sporulating fungi decreased within the zone but increased outside the zone

The information on the mentioned fungus is inconclusive, as it was also considered a pathogen. This was due to inoculation studies in which it was found that the needles of pine seedlings inoculated with *T. matsutake* were yellow, while the needles of uninoculated pine seedlings were green [70]. It was concluded that *T. matsutake* was a parasitic species. Ogawa [70] also reported the parasitic tendency of the species, as the hyphae of this fungus penetrated the cells of the roots, while no fungal sheaths or Hartig nets were formed. Only Eto [71] reported that the vegetative hyphae of *T. matsutake* covered the surface and invaded the intercellular spaces of the roots when an isolate of this species was inoculated into sterile pine seedlings in a closed pot. The author also reported that the addition of iron citrate to the culture medium improved mycelial growth and mycorrhizal synthesis [72]. Yamada et al. [73] reported that a fungal mantle and a Hartig net developed on the roots of *Pinus densiflora* seedlings cultured on sterile vermiculite with nutrient solution after inoculation with *T. matsutake* culture. This was the first report of the formation of a typical EM by *T. matsutake*. The growth of pines was not changed, or was only slightly improved, after the formation of EM, indicating that this fungus is not parasitic. Improved growth of pines after inoculation with *T. matsutake* was also reported by [74], who did not observe a typical fungal coat but noted the presence of a Hartig net [75].

4.7. The Role of Micorrhizal Fungi in the Nutrient Cycle of Pine Trees

In our experiment, *Wilcoxina* sp., *T. terrestris*, and *Piloderma* sp. were the most effective colonizers, and in the treatments, they dominated the rhizosphere root systems and positively affected the growth of *P. sylvestris*. Some other studies are in line with our research. Velmala et al. [76] demonstrated that ECM colonization improved shoot biomass production. The shoot height of slow-growing seedlings was also greater with increasing ECM fungal colonization, and marginally improved with increasing richness. ECM improved aboveground growth, which appeared to be related to a high abundance of *Wilcoxina* sp. We also obtained similar results in our experiment in a fertile habitat. This is probably due to the chitinase activity of *Wilcoxina* sp. and also to the high potential activity of glucose-releasing cellulases and hemicellulases, but again not to the hydrolytic activity involved in the mobilization of P and the release of amino acids.

A high amount of *Wilcoxina* sp. resulted in relatively high production of chitinase, cellulases, and hemicellulases [76]. In the article in question, the high activity of chitinase and cellulases was strongly positively related to the nitrogen content of the needles. This was also consistent with the field study of Jones et al. [77], in which the abundance of *Wilcoxina* sp. was associated with high N accumulation in both shoots and roots. Based on the [76] measurements of potential enzyme activities, *Wilcoxina* sp. effectively degrades chitin, a structural fungal cell wall polysaccharide and a rich organic N reservoir in the soil of boreal forests. Endophytic fungi such as *Penicillium* spp. may play an important role in plant survival by enhancing nutrient uptake and producing growth-promoting metabolites

such as gibberellins and auxins. The plant-growth-promoting ability of this fungal strain could contribute to the maintenance and revegetation of rapidly eroding dune flora [45]. Recently mentioned studies showed that *Penicillium citrinum* can produce the mycotoxin citrinin and cellulose digesting enzymes such as cellulase and endoglucanase as well as xylulase. A new aspect of the above research is the discovery of the ability of this fungus to produce gibberellins (GA5).

The discovery of filamentous fungi in our soil samples is not surprising, as recent scientific reports based on DNA or RNA analysis demonstrated the widespread occurrence of *Mortierella* in various environments [78]. Some strains of this genus belong to the plant-growth-promoting fungi (PGPF) and are found in soil, the rhizosphere, and plant tissues. These microorganisms are often found even in extremely hostile environments and are responsible for improving access to bioavailable forms of P and Fe in soils, synthesizing phytohormones and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and, last but not least, protecting crops from pathogens. The association with crops confirms the specificity of these soils, and the identification of these fungi in our samples confirms the origin of the studied soils. In addition, previous reports classified *Mortierella* spp. as saprotrophic microorganisms isolated from agricultural soils, and today their status as very valuable decomposers of forest litter is confirmed. The ability to survive in very unfavorable environmental conditions and the use of carbon sources contained in polymers such as cellulose, hemicellulose, and chitin make these fungi efficient colonizers of different environments. The growing interest in the application of *Mortierella* spp. in agriculture is mainly due to the potential benefits of this genus in increasing nutrient uptake efficiency, beneficial effects in protecting plants from adverse conditions, and reducing the use of chemical fertilizers and pesticides. In addition, the activities of *Mortierella* species influence the soil microbiota and support the performance of beneficial microorganisms, which significantly increase crop yield [78].

4.8. The Role of Antagonistic Fungi in Biological Protection of Pines from Pathogens

Trichoderma spp. were found only in the rhizosphere of spruce in healthy stands [79]. From this literature, it appears that *Trichoderma* fungi, which constitute at least 70% of the total amount of microscopic fungi, protect roots of woody plants [79]. *Penicillium* spp. and *Mucor* spp. in soil were more abundant in stunted stands, while *Trichoderma* spp. was found mainly in healthy stands. Fewer cellulose-degrading bacteria were found in the soil of plots with dead spruce trees than in plots with healthy spruce trees. In our case, microflora antagonistic to *H. annosum* was found in all studied soils after treatment. The isolated ascomycetes, as well as *Trichoderma* spp. and *Penicillium* spp., showed strong antagonism, with 90% of the antagonists inhibiting the growth of both the S and P groups of *H. annusum* [79]. Microscopic fungi of the genera *Verticillium*, *Aureobasidium*, and *Rhizopus* were also isolated in our experiment. In the root rhizosphere, fungi of the genus *Trichoderma* were detected only in healthy spruce stands [79] and, in our case, after enrichment with organic material. It is commonly believed that the increase in the number and activity of *Trichoderma* occurs at a higher temperature (15–32°C), pH in the range of 5.5–8.5, moderate soil moisture, high content of organic matter, and no mechanical interference with the soil.

4.9. Fungi Identified as Pathogens for Animals and Humans

Pathogenic organisms were also found in the soil samples tested (Supplementary Table OTU), and not just for plants. A filamentous fungus that caused a fatal systemic infection in a dog was identified as a new species *Sagenomella chlamydospora* [80]. When the case was originally reported, the fungus was identified as *Paecilomyces* sp. This study highlights how difficult identifying the causative agent of infection can be when dealing with an unusual microorganism. This is the first time this genus has been implicated in infections in animals, including humans. Recently, Garcia et al. [81] reported and illustrated a case of disseminated mycosis in a dog (a German shepherd). The fungus was isolated on postmortem examination from lesions in numerous organs, such as the kidneys, mitral

valve, abdominal aorta, and intervertebral discs. The diagnosis was difficult because clinical symptoms were very nonspecific [80]. Thus, the presence of these fungi confirms the agricultural origin of the soils and the great potential of the NGS method to detect and identify them.

Pseudogymnoascus destructans causes white-nose syndrome (WNS) in North American bats, which has resulted in a dramatic decrease in the bat population in the United States and Canada. We found *Pseudogymnoascus pannorum*, which is the nearest fungal relative of *P. destructans*, with wider psychrophilic–physiological growth range, and ability to cause skin diseases in canines and humans [82,83].

Somewhat surprising is the presence of pathogens in our soil samples that cause the recent emergence of white-nose syndrome (WNS), a fungal disease that causes unprecedented mortality in hibernating bats [84]. Research in eastern North America has uncovered a gap in knowledge about fungal communities associated with bats and their hibernacula. American researchers used culture techniques to examine the diversity of fungi in soil samples collected from 24 bat hibernacula in the eastern United States [84].

Similar studies [85] were also carried out in Poland, and the occurrence of this species was found in the bats' hibernation sites. However, no disease symptoms were found in bats.

Fungi of the genus *Apiotrichum* identified in the soil samples are urease-positive, unencapsulated basidiomycetous yeasts characterized by the development of hyaline, septate hyphae that fragment into oval or rectangular arthroconidia. This makes morphological identification difficult, and we were able to detect their presence, probably thanks to molecular identification. All species are resistant to echinocandins and other classes of antifungal agents [86]; perhaps this is the reason why they have survived in post-agricultural soils. *Apiotrichum* species are frequently isolated from human and animal sources as well as from clinical samples, and some of them have been associated with opportunistic infections °C [87]. It is also worth mentioning that the pathogenic species all can grow at 37 °C.

Another fungal species identified in our research is *Exophiala jeanselmei* (Supplementary table OTU)—a dematiaceous hyphomycete commonly found in soil, decaying vegetation, and rotting wood [88,89]. Traumatic inoculation of *E. jeanselmei* can lead to a variety of subcutaneous infections, including mycetoma, chromoblastomycosis, or phaeohyphomycosis [90]. In immunosuppressed organ transplant recipients, *E. jeanselmei* is the most common dematiaceous fungus associated with skin infections, occurring in up to 32% of cases, with an associated mortality rate of up to 18%. *Exophiala jeanselmei* is the causative agent of maduromycosis [88], a usually asymptomatic disease manifested by black or brown macular lesions that enlarge by peripheral extension. The lesion is darkest at the periphery and has very distinct margins. *Exophiala jeanselmei* is clinically defined as a rare causative agent of subcutaneous lesions of traumatic origin that eventually cause eumycetoma [90].

4.10. Seedborne Fungi

The fungus we identified in the soil, *Caloscypha fulgens*, produces bulging infectious beads on the surface of the seed within 1 to 3 days after inoculation, and over the next 10 days, several straight penetration holes form in the hull just below each bead [91]. During the penetration period, the growth of the fungus is restricted to the penetration holes and tissue interstices until it reaches the endosperm and embryo and spreads its branches. Occasionally, it penetrates the seed through slits in the integument, but never through the micropyle. The fungus invades from infected seeds through intact or split seed coats and the micropyle [91].

4.11. Edible Ectomycorrhizal Fungi

Hydnum is a fungal genus with considerable ecological and economic importance, as it includes several edible ectomycorrhizal fungi. Different species of *Hydnum* can be found among plants from several families, such as *Pinaceae*, *Fagaceae*, *Dipterocarpaceae*, and *Myrtaceae*. Previous studies showed that they can form ectomycorrhizal relationships with these plant families, making them very important for maintaining forest ecosystems [92,93].

Hygrophorus species are distributed worldwide and are mostly found in forests with pines or with ectomycorrhizal (ECM) angiosperms [94]. *Hygrophorus* are essential components of ECM communities in temperate regions of the Northern Hemisphere [95]. Recently, a new edible species, *H. parvirussula*, was described from southwestern China [96], belonging to the *Hygrophorus* section Pudorini [97]. It is not entirely clear why it was found in Poland (initial report); perhaps it was imported, or perhaps it already existed but was not yet discovered without NGS testing.

4.12. Identified Fungi Occurred as Pathogenic to Various Plant Species

Karadžić et al. [98] consider *Cenangium ferruginosum* as one of the most dangerous pathogenic fungi on pines. Pathogenicity tests conducted with mycelial plugs in summer confirmed that *C. ferruginosum* is not only a saprophyte but also a pathogen. Once infected and colonized by pathogens that cause bark necrosis in the crown, trees are severely stressed and may soon be attacked by secondary invaders, such as *Ips acuminatus* Gyll., *Ips sexdentates* Börn, *Tomicus piniperda* L., *Tomicus minor* Htg., and *Buprestidae*, or by other pathogens, such as *Armillaria* sp. or *Ophiostoma* sp. *Cenangium ferruginosum* is an important pathogen in some countries of Southern Europe [98,99]. In Central Europe, it used to be considered a saprophyte rather than a pathogen [100].

4.13. Other Identified Fungi Specific for Different Environments

Solicoccozyma terricola M, the yeast strain isolated from a soil sample from blueberry cultivation in Miedzyrzec Podlaski in Poland, can cleave phosphorus–nitrogen and nitrogen–carbon bonds into N-phosphonomethylglycine (PMG, glyphosate) [101]. This was the first report of PMG degradation as a phosphorus and nitrogen source by a yeast strain isolated from glyphosate-contaminated soil, and in this way, the above studies filled a gap in PMG biodegradation research between bacteria and filamentous fungi. The release of inorganic phosphate into the culture medium confirmed the progress of xenobiotic biodegradation, suggesting that the tested strain degrades this compound independently of phosphate cell status and may contribute to the development of effective methods of soil and water bioremediation in the future [101]. If so, it was also likely involved in improving soil properties to support pine growth in our experiment.

Compounds obtained from another fungal species we identified, *S. terricola* (it grows on lignocellulose), could be suggested as an additional source of oleochemicals [102]. Given the rising prices of fossil fuels and the environmental problems associated with their use, the use of compounds produced by *S. terricola* on lignocellulose could be a promising option as an additional oleochemical source, especially for biodiesel production [102].

The presence of the genus *Collarina* Jullien, 1886 (Cribrilinidae Hincks, 1879) [103] was surprising, since only two species were previously known from the Atlantic–Mediterranean region, *C. balzaci* [104], synonym of *Collarina cribrosa*. It is exclusively epiphytic (mainly on *Posidonia oceanica* (L.) Delile, 1813 and brown algae), its lifecycle is adapted to ephemeral hosts, and it can reproduce dramatically under unusual environmental conditions (Gulf of Gabes, chemical disturbances) on Posidonia leaves in association with diatoms [105].

5. Conclusions

Wood waste, sawdust, or bark compost in the form of organic material can be successfully used to accelerate the natural processes of formation of mycobiota characteristic for forest soils.

The wood substrate in degraded post-agricultural soils rebuilt the organic layer after 20 years, which had a positive effect on the composition of mycobiota.

The substrates used provided suitable growth conditions (moisture content, nutrient availability) for many fungal species beneficial to planted pines.

Fungal biodiversity was restored, which (as in forest soils) became antagonistic to pathogens, e.g., *Heterobasidion annosum*, which is dangerous to pines growing on former farmland.

Beneficial fungi included those that form mycorrhizal associations with pine roots, e.g., from the genera *Amphinema*, *Inocybe*, or *Rusula*, thus protecting them (mechanically and chemically) from pathogens and drought (as they increase the surface area of the fungal roots many times over). Fungi with antagonistic properties to *H. annosum* included those of the genera*Penicilium* or *Trichoderma*.

In general, the rhizosphere of treated pines was dominated by fungi of the genus Basidiomycota, and the control area by Ascomycota. The latter was dominated by pathogens of, e.g., pine needles and shoots, such as *Cenanglium ferruginosum*.

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