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# Effect of Deadwood on Ectomycorrhizal Colonisation of Old-Growth Oak Forests

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**Abstract:** Although the importance of coarse woody debris (CWD) for species diversity is recognized, the effects of coarse woody debris decay class on species composition have received little attention. We examined how the species composition of ectomycorrhizal fungi (ECM) changes with CWD decay. We describe ectomycorrhizal root tips and the diversity of mycorrhizal fungal species at three English oak (*Quercus robur* L.*)* sites. DNA barcoding revealed a total of 17 ECM fungal species. The highest degree of mycorrhizal colonization was found in $CWD_{advanced}$ (27.2%) and $CWD_{early}$ (27.1%). Based on exploration types, ectomycorrhizae were classified with respect to ecologically relevant soil features. The short distance type was significantly correlated with soil $P_2O_5$, while the contact type was correlated with soil C/N. The lowest mean content of soil $C_{org}$ was found in the $CWD_{absent}$ site. The difference in total soil N between sites decreased with increasing CWD decomposition, whereas total C/N increased correspondingly. In this study we confirmed that soil CWD stimulates ectomycorrhizal fungi, representing contact or short-distance exploration types of mycelium.

**Keywords:** Białowieża; coarse woody debris; *Quercus robur*; ectomycorrhizae; exploration type

## 1. Introduction

Ectomycorrhizal (ECM) symbiosis is a mutualistic association between soil fungi and the roots of the majority of temperate and boreal forest trees [1]. The fungi are of particular interest because they are important in nutrient cycling [2] and can have a strong effect on the growth and health of their plant hosts [3,4]. Usually, communities of ECM are characterised by high species richness, comprising a few common species and many rare ones [5,6]. Most likely, the maintenance of such richness is the result of niche partitioning, because fungal species differ in their abilities to exploit soil resources and therefore display unique habitat preferences [7–10].

Nowadays, one of the most important issues regarding European oaks is forest decline. Differences in the degree of mycorrhizal colonization are shown in *Quercus robur* L. stands in relation to disturbances caused by abiotic and biotic factors (health status) [11]. The oak decline phenomenon is a complex disease, caused by several biotic and abiotic factors, often acting synergistically [12,13].

Kovacs et al. [14] and Mosca et al. [15] showed that the Shannon index of diversity of ECM was higher in the root systems of vital trees. The lower diversity of ECM fungi in the more damaged stand is the result of the reduction of photosynthetic activity, due to crown defoliation [16,17].

One kind of nutrient resource is coarse woody debris (CWD), which consists of pits, stumps, root mounds, and logs, that are formed as a result of tree fall [18]. CWD performs many physical, chemical, and biological functions in forest ecosystems, also affecting the composition and function of ECM communities [9,19] by stimulating their activity [20]. Biologically, CWD is a key factor in imparting resilience and is a part of the above-ground litter fraction, and its accumulation leads to considerable interaction with below-ground components of the soil [21]. CWD is an important contributor to soil organic matter (SOM) and provides input for long-term nutrient cycling, ensuring nutrients for beneficial soil organisms and for the formation of ectomycorrhizal root tips [22,23].

Some ECMs produce enzymes such as extracellular laccases and peroxidases, that cause available nutrients to be trapped inside CWD. ECM enzyme activity often correlates with mycelial morphology. For example, many *Russula* species have very little extraradical mycelium and often have the highest laccase activity [24,25]. The correlation of hyphal exploration type with CWD was seen in a tropical forest [24] and was more strongly related to exploration type than host phenology in a boreal forest [25]. ECM fungi can explore the surrounding substrate by extramatrical mycelia, which are either concentrated close to the mycorrhizal mantle or form far-reaching rhizomorphs. Based on the distribution and differentiation of mycelium into so-called "exploration types", different foraging strategies have been distinguished [26].

The goal of this study was to assess how the structure of ectomycorrhizal fungi in old growth oak forests was affected by the stage of CWD decay—early decay and advanced decay stages—with an emphasis on elucidating the modes of exploration employed by ectomycorrhizae. We hypothesised that the presence of CWD in the soil substrate stimulates ectomycorrhizal fungi that produce contact or short-distance exploration types of mycelium.

## 2. Materials and Methods

### *2.1. Study Area*

The study was carried out in the Bialowieża old-growth forest (BF) (52°70′ N, 23°85′ E), which is the last remaining primaeval forest in lowland Europe. The forest covers an area of 1450 km$^2$ on the Polish–Belarusian border (northeastern Poland) and has been designated a World Heritage Site and a European Commission (EU) Natura 2000 Special Area of Conservation. The climate has features of both a continental and an Atlantic character. The mean annual temperature is 7.0 °C and mean annual precipitation is 550–600 mm. The growing season lasts, on average, 190 days, and snow cover lasts 92 days [27]. The study area is situated on the Precambrian East European platform and is dominated mainly by sands, gravels, and glacial boulders [28]. Soils of the BF represent various types, from poor sands through to loam and peat soils. In the western part of the forest, loam soils overgrown with deciduous forest predominate, while in the eastern part, poor soils with coniferous and mixed forest are most abundant. The soils of the BF belong to the divisions of autogenic soils, semi-hydrogenic soils, hydrogenic soils, alluvial soils, and antropogenic soils [29].

The forest consists of continuous multi-species stands, which have been classified into the following five forest types: deciduous (54%, dominant species: *Q. robur*, *Tilia cordata* Mill., *Carpinus betulus* L.), mixed deciduous (23%, *Picea abies* (L.), *Q. robur*, *T. cordata*, *C. betulus*), black alder bog (14%, *Alnus glutinosa* (L.), *Fraxinus excelsior* L.), mixed coniferous (13%, *Pinus sylvestris* L., *P. abies*, *Q. robur*), and coniferous (9%, *P. sylvestris*, *P. abies*) (21). The shrub layer consists of *Vaccinium myrtillus* L., *Oxalis acetosella* L., and *Rubus saxatilis* L. [29].

There were three study sites selected in the 150-year-old oak forest: two with CWD in two stages of wood decay (early and advanced) and one without CWD. The area of each study site was 500 m$^2$ with a radius of 12.62 m, within which all samplings were performed. The $CWD_{early}$ was represented by logs and stumps in early stages of wood decay, while $CWD_{advanced}$ was represented by deadwood

in an advanced stage of decomposition. In the $CWD_{absent}$ site, decaying logs and snags were not present. Details of deadwood characteristic in the three study sites are presented in Table 1.

### 2.2. Deadwood Measurements

We used a modified classification of dead wood decomposition from Renvall [30] to classify each selected piece of CWD on our study sites into early (I, II, and III decay classes) or advanced (IV and V decay classes) stages of wood decay, based on the presence or absence of branches (if originally present), hardness of wood, wood appearance, and bark intactness, in the following way: I—wood hard, without marks of decomposition; II—peripheral parts are mostly soft, inner section hard, share of soft rot less than 40%; III—peripheral parts are mostly soft, inner section partially soft, share of soft rot 40–80%; IV—wood soft, share of soft rot more than 80%, contour partially deformed; V—wood soft, contour deformed or absent, wood covered with soil [30].

The volume ($v$) of CWD was calculated using Huber's equation [31], according to the formula:

$$V = (L) \times (dob)^2 \times (C), \quad (1)$$

where $V$ = the cubic volume of the log (cubic feet), $L$ = the length of the log (feet), $dob$ = the diameter outside of the bark (inches) at a point midway from the ends of the log, and $C$ = 0.005454 (unit conversion factor) (Table 1).

**Table 1.** The volume ($m^3$) of coarse woody debris in various decay classes at study sites in the Białowieża old-growth forest. The five-class scale of decomposition of fallen logs was based on Renvall [30]. CWD: coarse woody debris.

| | | $CWD_{absent}$ ($m^3$/500 $m^2$) | $CWD_{early}$ ($m^3$/500 $m^2$) | $CWD_{advanced}$ ($m^3$/500 $m^2$) |
|---|---|---|---|---|
| Decay class | I | - | 16.45 | - |
| | II | - | 0.05 | - |
| | III | - | 0.90 | - |
| | IV | - | - | 11.99 |
| | V | - | - | 13.61 |

### 2.3. Root Sampling and Morphotyping of Mycorrhizae

In spring 2018, to estimate the ectomycorrhizal colonisation of English oak (*Q. robur*), a total of 60 oak trees were investigated (20 per study site). For mycorrhizal analysis, one soil subsample from the northern and one from the southern side of the tree bases were collected. Soil samples were extracted to a depth of 20 cm using a 5 cm diameter soil corer. Separate soil samples were collected at distances of 0.5–1 m from the base of selected oak trees. The soil-root samples were stored at −20 °C for a maximum of four weeks before analysis. All fine roots from each soil sample were collected and separated from soil particles on a sieve under running tap water. Ectomycorrhizal morphotypes were analysed under a dissecting microscope with magnification 10–50x (Delta IPOS-808, Delta Optical, Warsaw, Poland), according to Agerer [32]. Each ectomycorrhizal morphotype was described in detail (ramification system, colour, shape, texture, the thickness of the mantle, presence and organization of emanating hyphae, rhizomorphs). Additionally, the ECM morphotypes were classified into the following exploration types: contact type—represented by a smooth mantle and only a few emanating hyphae; short distance type—characterized by a voluminous envelope of emanating hyphae and lacking rhizomorphs; and medium smooth distance type [26]. Two to three representative mycorrhizal root tips of each morphotype were photographed and stored in a plastic tube at −10 °C until molecular analysis.

According to Olchowik et al. [33], the degree of mycorrhization of oak roots can be determined by classifying and counting the vital mycorrhizal (VM—turgid ECM tips with an ECM mantle), vital non-mycorrhizal (NM—well-developed, turgid fine root tips, mantle lacking) and non-vital (NV—non-turgid, with a scurfy surface and easily detachable cortex, with or without the remnants of an ECM mantle) root tips. The relative abundance of individual ectomycorrhizal fungal taxa was

calculated as the number of ectomycorrhizal root tips of each morphotype/taxon divided by the total number of ectomycorrhizal roots tips. The abundance was calculated as the number of tips of given ectomycorrhizal species per total number of all root tips. The frequency of ectomycorrhizal taxa was expressed as a percentage of colonized plants. Estimates of species richness were calculated with the EstimateS program version 7.51 [34].

### *2.4. Molecular Identification of Ectomycorrhizal Root Tips*

To confirm the identity of previously collected and preserved morphotypes, molecular analyses of two to three mycorrhizal tips of each unique morphotype were performed. The fungal internal transcribed spacer (ITS) region was amplified using the ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers [35]. Direct PCR was performed using the Phire Plant Direct PCR Kit (ThermoFisher Scientific, Waltham, MA, USA). Each primer was added to 10 μL of 2X Phire Plant PCR Buffer, 0.4 μL of Phire Hot Start II DNA Polymerase, and 2.0 μL of 5.0 μM, along with a mycorrhizal tip and additional $H_2O$, to reach a total volume of 20 μL. Cycling was performed using a Veriti 96-Well Thermal Cycler as follows: an initial denaturation step of 98 °C for 5 min, followed by 35 cycles of 94 °C for 5 s, 55 °C for 5 s, and 72 °C for 20 s, and a final extension step at 72 °C for 5 min. Amplicons were visualised with UV illumination after ethidium bromide (Sigma-Aldrich, Milwaukee, WI, USA) staining. Excess dNTPs and unincorporated primers were removed from the PCR product using the Clean-Up Purification Kit (A&A Biotechnology, Gdynia, Poland). DNA was eluted in 40 μL $H_2O$.

Sequencing PCR reactions were performed with 1 μL BigDye Terminator v. 3.1 Ready Reaction Mix (ThermoFisher Scientific, Waltham, MA, USA), 2 μL BigDye sequencing buffer (ThermoFisher Scientific, Waltham, MA, USA), 1 μL (5 μM), ITS4 primer, and $H_2O$ to bring the total volume to 10 μL. The thermal profile for sequencing reactions consisted of 25 cycles of 96 °C for 1 min, 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 105 s. The rDNA region was sequenced with an ABI 3500 xL genetic analyser (ThermoFisher Scientific, Waltham, MA, USA). For species identification, 97% alignment threshold over at least 450 base pairs was applied. The assignment of sequences obtained to ECM species was performed by the BLASTSYSTEMS Identification Engine [36] and BLAST [37].

### *2.5. Chemical Analysis*

Five soil cores were collected from each study site. Samples of soil were air-dried, passed through a mesh screen, and stored for further analysis. The soil analyses were performed in the laboratory of the Polish Centre for Accreditation (No. AB740). The soil pH and essential nutrient contents were measured according to ISO 10390 [38] and PB-14 ed.2 of 1 January 2010 (using inductively coupled argon-plasma spectrometry following mineralisation in chloric (VII) acid). The percentage of nitrogen (N) and total organic carbon ($C_{org}$) were measured according to ISO13878 [39] and PN-ISO 10694 [40]; phosphorus ($P_2O_5$) was determined for all samples with 1% citric acid extraction, according to Schlichting et al. [41], and exchangeable cations (Ca, Mg, K, Na) were evaluated following ISO 11260 [42].

### *2.6. Data Analysis*

To evaluate significant differences between the study sites for the abundance of VM, NM, and NV root tips and the abundance of different ectomycorrhizal exploration types, generalized linear models (GLMs) with binomial distributions were used. Tukey's linear contrast was used to test a pairwise comparison between the study sites for the GLM model. Statistical analyses of soil properties were conducted using the one-way ANOVA and the Tukey test was used for "post hoc" comparisons. This data set satisfied the assumptions of ANOVA, based on the homogeneity of variances (Levene's test) and checked the data normal distribution (Shapiro-Wilkinson test). The principal component analysis (PCA) was used to explore potential relationships and correlations between soil variables and abundance of taxa with different exploration types. Statistical analyses were performed using R version 3.5.1 [43] and with the accepted level of significance set at $p < 0.05$.

## 3. Results

### *3.1. Soil Substrate Properties*

Details of soil characteristics are summarized in Table 2. The physical and chemical properties of the soils varied between study sites. The soil in all sites was acidic and varied between the stages of CWD decomposition and $CWD_{absent}$ ($pH_{KCl}$ = 4.10 and 3.68 for $CWD_{early}$ and $CWD_{advanced}$, respectively). The lowest mean content of $C_{org}$ was found in $CWD_{absent}$ ($C_{org}$ = 2.81%), while higher $C_{org}$ content was found in $CWD_{advanced}$ and $CWD_{early}$ ($C_{org}$ = 4.47% and 6.79%, respectively). The highest total nitrogen value was recorded in $CWD_{early}$ (0.51%), followed by $CWD_{advanced}$ and $CWD_{absent}$, with 0.22% and 0.11% N, respectively. Furthermore, K and Mg concentrations were lower in $CWD_{absent}$, whereas $CWD_{early}$ was characterized by the lowest $P_2O_5$ content and the highest Ca, Mg, and K contents.

**Table 2.** Mean values of selected physical and chemical properties of soils in the study sites ($n$ = 10), with different conditions of coarse woody debris (absence of coarse woody debris ($CWD_{absent}$)) and early ($CWD_{early}$) and advanced ($CWD_{advanced}$) stages of decomposition. Different letters indicate significant differences between values in columns.

| | $pH_{KCl}$ | | $N_{total}$ (%) | | $C_{org}$ (%) | | C/N | | $P_2O_5$ (mg/100g) | | Ca (cmol/kg) | | Mg (cmol/kg) | | K (cmol/kg) | | Relative Soil Humidity (%) | | OM (%) (Organic Matter) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $CWD_{absent}$ | 3.1 | b | 0.1 | c | 2.8 | c | 25.3 | a | 7.72 | c | 0.7 | c | 0.1 | c | 0.1 | b | 13.1 | c | 4.8 | c |
| $CWD_{early}$ | 4.1 | a | 0.5 | a | 6.8 | a | 13.5 | c | 8.2 | b | 13.1 | a | 0.9 | a | 0.3 | a | 25.8 | a | 12 | a |
| $CWD_{advanced}$ | 3.7 | b | 0.2 | b | 4.5 | b | 20.4 | b | 16.6 | a | 5.32 | b | 0.6 | b | 0.2 | a | 20.6 | b | 7.7 | b |

### *3.2. Mycorrhizal Colonisation*

Oak trees on all sites had a low degree of ECM colonisation, ranging from 23.3% to 27.2% of the root tips categorized as vital ECM (VM). The highest degree of mycorrhizal colonization was found in sites with $CWD_{advanced}$ (27.2%) and $CWD_{early}$ (27.1%). The abundance of non-vital and the proportion of non-mycorrhizal root tips were significantly different between sites (Figure 1). The largest proportion of NV root tips was observed in $CWD_{absent}$ (35.3%, $p < 0.0001$), while 19.0% and 13.7% were observed in $CWD_{early}$ and $CWD_{advanced}$, respectively. The lowest abundance of NM root tips was recorded at the $CWD_{absent}$ site (41.4%, $p < 0.0001$).

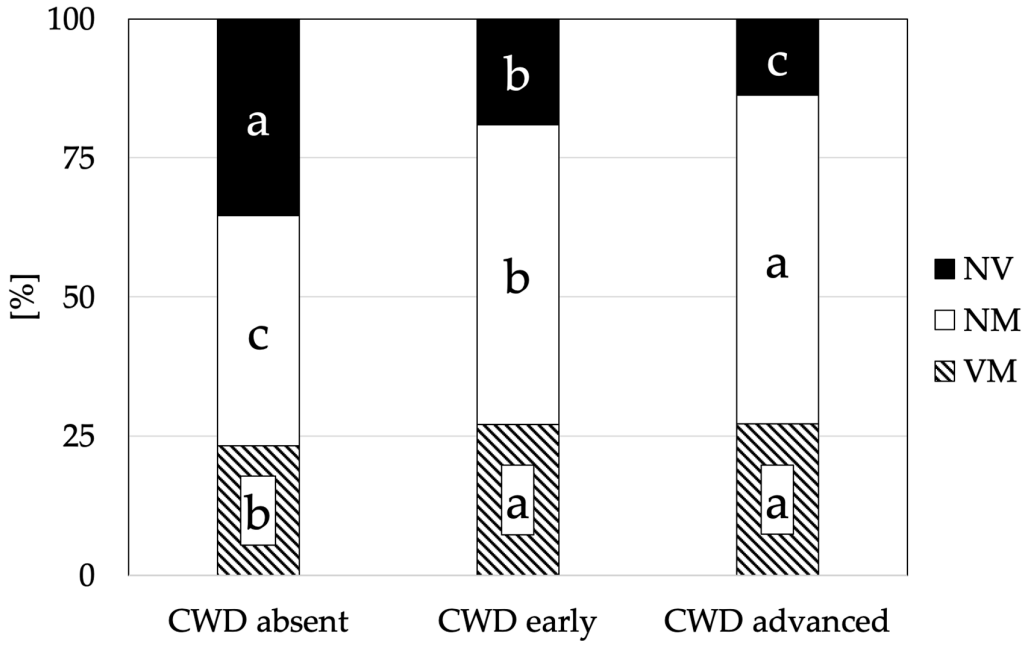

**Figure 1.** The abundance of vital mycorrhizal (VM), non-vital (NV), and vital non-mycorrhizal (NM) root tips (%) from oak trees in three study sites, based on the absence of coarse woody debris ($CWD_{absent}$) and early ($CWD_{early}$) and advanced ($CWD_{advanced}$) stages of wood decay. Within bars of each root tip classification, different letters indicate significant differences (Tukey's contrast, $p = 0.05$).

After regrouping and combining on the basis of the results of the molecular analysis, a total of 18 fungal taxa were detected, 17 at species level and 1 at genus level (Table 3, Figure 2). ECM fungal species richness ranged from 12 taxa for $CWD_{absent}$ to 13 and 14 taxa for $CWD_{early}$ and $CWD_{advanced}$, respectively. *Tomentella* sp. was found to be the most abundant (8.1%) and frequent (45%) ECM fungal species in $CWD_{early}$. The Shannon–Wiener and Simpson diversity indices of molecularly identified ECM fungi were higher for $CWD_{advanced}$ and $CWD_{early}$ than for $CWD_{absent}$ (Table 3).

**Table 3.** Estimated species richness, diversity, and occurrence of fungal taxa associated with the roots of oak trees in the three study sites, characterized based on the absence of coarse woody debris ($CWD_{absent}$) and early ($CWD_{early}$) and advanced ($CWD_{advanced}$) stages of wood decay. Data are the frequency (Freq.; percent of colonized plants) and abundance (Abun.; percent of mycorrhizal roots colonized) of fungal taxa on root tips. Abbreviations: ECM—ectomycorrhizal fungus, Endoph.—endophyte. Different letters indicate significant differences.

| Identification | BLAST Top-Hit | | | Putative Ecology | $CWD_{absent}$ | | $CWD_{early}$ | | $CWD_{advanced}$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Closest Match | NCBI | Identity (%) | | Freq. | Abun. | Freq. | Abun. | Freq. | Abun. |
| Basidiomycota *Tomentella* sp. | *Tomentella* sp. | MK583527 | 95 | ECM | 30 | 7.8 | 45 | 8.1 | 55 | 5.4 |
| *Xerocomellus* sp. | *Xerocomellus* sp. | MK583530 | 95 | ECM | 5 | 2.6 | 5 | 2.6 | 5 | 0.4 |
| *Laccaria* sp. | *Laccaria* sp. | MK583519 | 95 | ECM | 15 | 2.3 | 20 | 1.1 | 15 | 1.1 |
| *Clavulina* sp. | *Clavulina* sp. | - | 96 | ECM | 10 | 1.7 | 5 | 1.5 | 10 | 0.9 |
| *Scleroderma citrinum* Pers. | *Scleroderma citrinum* | MK583525 | 97 | ECM | 5 | 1.1 | 5 | 0.2 | 5 | 0.2 |
| *Thelephora terrestris* Ehrh. | *Thelephora terrestris* | MK583526 | 99 | ECM | 5 | 0.6 | 10 | 0.4 | 5 | 0.4 |
| *Tylospora asterophora* (Bonord.) Donk. | *Tylospora asterophora* | MK583528 | 98 | ECM | 5 | 0.3 | 5 | 1.1 | 10 | 2.8 |
| *Xerocomellus porosporus* (Imler ex G. Moreno & Bon) Šutara | *Xerocomellus porosporus* | MK583529 | 99 | ECM | - | - | 5 | 0.2 | 5 | 1.1 |
| *Russula ochroleuca* (Pers.) Fr. | *Russula ochroleuca* | MK583524 | 99 | ECM | 5 | 0.6 | - | - | 15 | 1.5 |
| *Russula nigricans* (Bull.) Fr. | *Russula nigricans* | MK583523 | 97 | ECM | 5 | 1.1 | - | - | - | - |
| *Russula amoenolens* Romagn. | *Russula amoenolens* | MK583522 | 98 | ECM | | | | | 20 | 4.3 |
| *Inocybe lanuginose* (Bull.) P. Kumm. | *Inocybe lanuginosa* | MK583518 | 97 | ECM | - | - | - | - | 10 | 1.1 |
| *Amanita citrina* Pers. | *Amanita citrina* | MK583515 | 97 | ECM | - | - | 5 | 0.4 | - | - |
| Ascomycota *Pachyphlodes nemoralis* Hobart, Bóna & Conde | *Pachyphlodes nemoralis* | MK583521 | 100 | ECM | 25 | 4.0 | 10 | 0.9 | 40 | 6.6 |
| *Cenococcum geophilum* Fr. | *Cenococcum geophilum* | MK583516 | 98 | ECM | 10 | 0.6 | 10 | 0.7 | 15 | 1.1 |
| *Humaria hemisphaerica* (F.H. Wigg.) | *Humaria hemisphaerica* | MK583517 | 97 | ECM | 5 | 0.3 | 15 | 3.7 | 5 | 0.4 |
| *Hyaloscypha bicolor* (Hambl. & Sigler) Vohník, Fehrer & Réblová | *Hyaloscypha bicolor* | MK583520 | 97 | ECM | - | - | 20 | 2.2 | - | - |
| *Phialocephala fortinii* Wang & Wilcox | *Phialocephala fortinii* | - | 98 | Endoph. | - | - | - | - | 15 | 4.7 |
| Mycorrhizal fungal species richness (*n*) | | | | | 12 | | 13 | | 14 | |
| Degree of mycorrhization (%) | | | | | 23.27 | | 27.07 | | 27.19 | |

| | | | |
|---|---|---|---|
| Estimated species richness: | | | |
| Chao1 | 1.3 | 1.7 | 2.2 |
| Diversity | | | |
| Shannon–Wiener (H') | 0.23 [b] | 0.41 [ab] | 0.66 [a] |
| Simpson 1/D | 0.26 [b] | 0.40 [ab] | 0.55 [a] |

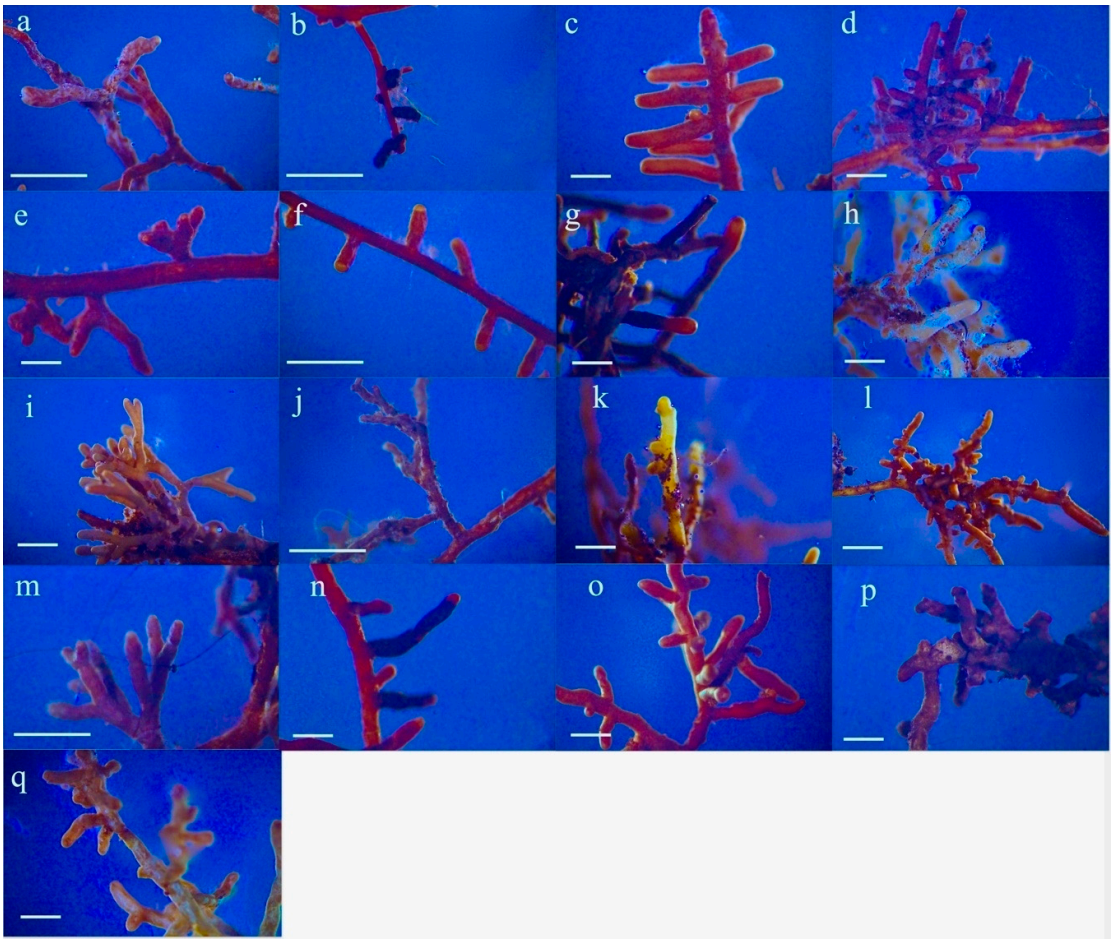


**Figure 2.** Ectomycorrhizas observed on *Q. robur* (*Quercus robur* L.) trees in the Bialowieża old-growth forest: (**a**) *Amanita citrina* Pers., (**b**) *Cenococcum geophilum* Fr., (**c**) *Clavulina* sp., (**d**) *Humaria hemisphaerica* (F.H. Wigg.), (**e**) *Inocybe lanuginose* (Bull.) P. Kumm., (**f**) *Laccaria* sp., (**g**) *Hyaloscypha bicolor* (Hambl. & Sigler) Vohník, Fehrer & Réblová, (**h**) *Pachyphlodes nemoralis* Hobart, Bóna & Conde, (**i**) *Russula amoenolens* Romagn., (**j**) *Russula nigricans* (Bull.) Fr., (**k**) *Russula ochroleuca* (Pers.) Fr., (**l**) *Scleroderma citrinum* Pers., (**m**) *Thelephora terrestris* Ehrh., (**n**) *Tomentella* sp., (**o**) *Tylospora asterophora* (Bonord.) Donk., (**p**) *Xerocomellus porosporus* (Imler ex G. Moreno & Bon) Šutara, (**q**) *Xerocomellus* sp. Bars in each photograph indicate 1 mm length.

All three study sites shared 11 of the ECM taxa: *Tomentella* sp., *Laccaria* sp., *Clavulina* sp., *Scleroderma citrinum* Pers., *Thelephora terrestris* Ehrh., *Tylospora asterophora* (Bonord.) Donk., *Xerocomellus* sp., *Pachyphlodes nemoralis* Hobart, Bóna & Conde, *Cenococcum geophilum* Fr. and *Humaria hemisphaerica* (F.H. Wigg.). *Russula amoenolens* Romagn. and *Inocybe lanuginose* (Bull.) P. Kumm. were detected exclusively at the site with $CWD_{advanced}$; *Amanita citrina* Pers. and *Hyaloscypha bicolor* (Hambl. & Sigler) Vohník, Fehrer & Réblová were found exclusively at the $CWD_{early}$ site (Figure 3).

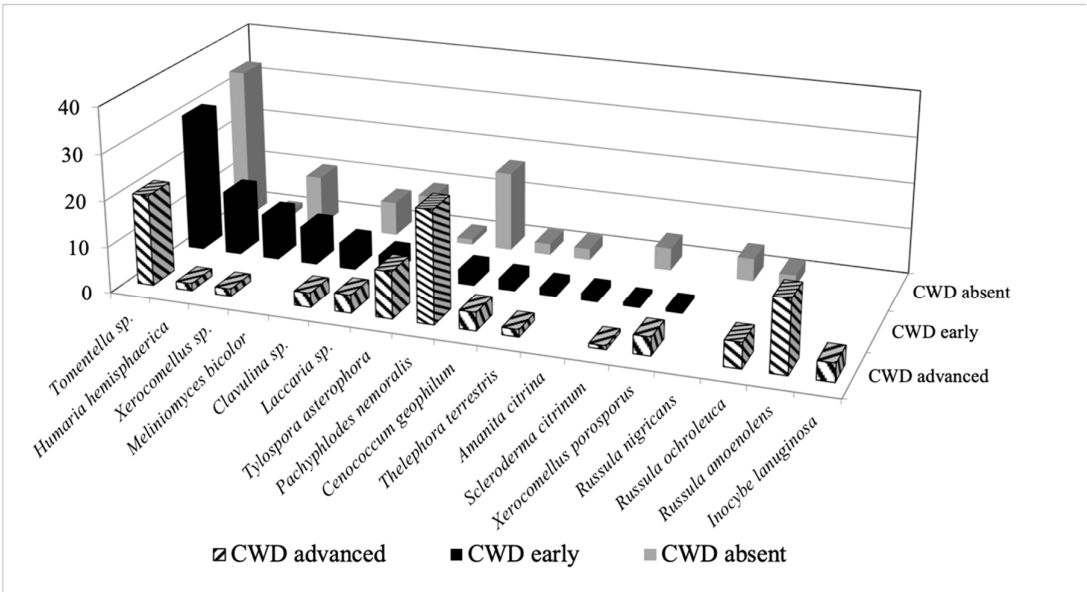


**Figure 3.** Relative abundance (%) of each mycorrhizal fungal taxa associated with *Q. robur* trees in the three study sites, characterized by the absence of coarse woody debris ($CWD_{absent}$) or coarse woody debris in the early ($CWD_{early}$) or advanced ($CWD_{advanced}$) stages of wood decay.

The ECM community in $CWD_{absent}$ was dominated by the medium distance smooth (50.6%, $p < 0.0001$) and long distance (16.0%, $p < 0.0001$) exploration types (Figure 4). In contrast, $CWD_{advanced}$ had the highest percentage of short distance (18.1%, $p < 0.0001$) and contact (50.4%, $p < 0.0001$) exploration types of ectomycorrhizae.

The contact type was significantly correlated with soil C/N, while the short distance type was correlated with soil phosphorus ($P_2O_5$) (Figure 5).

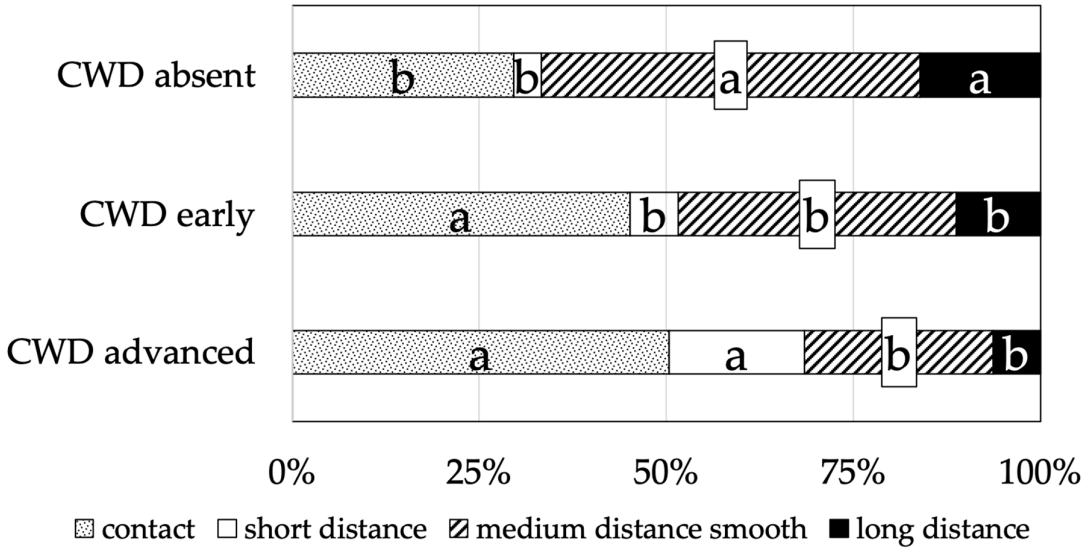


**Figure 4.** Mean relative abundance of taxa with different exploration types associated with *Q. robur* trees in the three study sites, characterized by the absence of coarse woody debris ($CWD_{absent}$) or coarse woody debris in early ($CWD_{early}$) or advanced ($CWD_{advanced}$) stages of wood decay. Within each tip classification, different letters indicate significant differences between sites (Tukey´s contrast, $p = 0.05$).

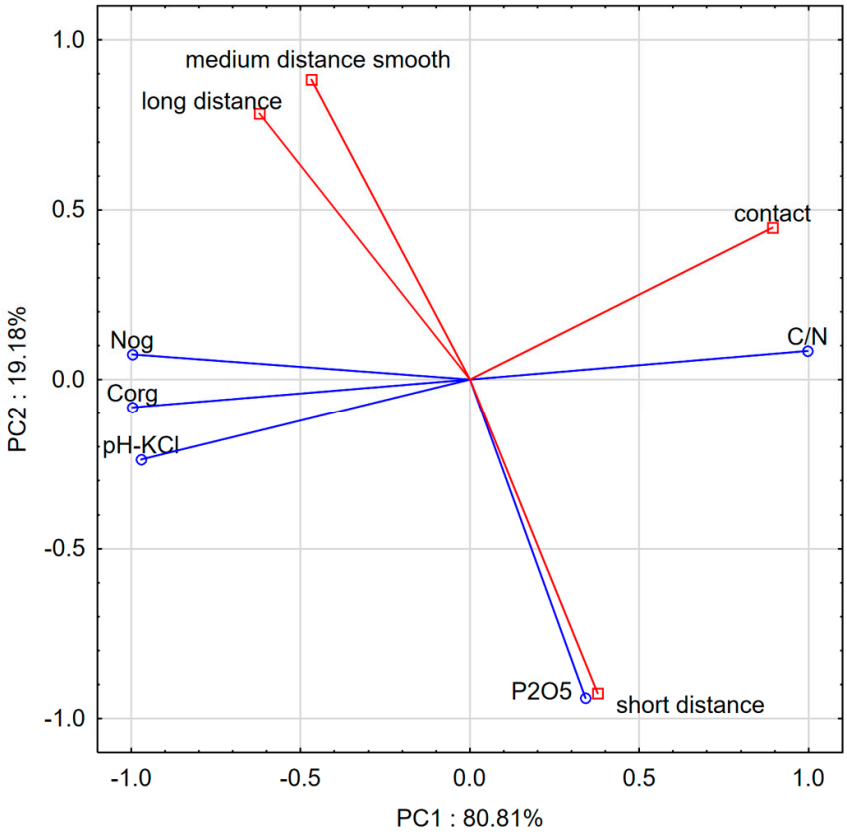


**Figure 5.** Principal component analysis (PCA) of different ectomycorrhizae exploration types observed on *Q. robur* roots, and selected soil properties.

## 4. Discussion

CWD supported the development of ectomycorrhizae, mainly those with contact or short-distance exploration types of mycelium with more NM root tips, observed more on oaks growing in both $CWD_{advanced}$ and $CWD_{early}$ than in $CWD_{absent}$. An increased amount of NM roots might be caused by higher nitrogen and phosphorous concentrations, as well as greater soil moisture where dead wood was present. Pregitzer et al. [44] demonstrated that additions of water plus ammonium–nitrate resulted in much greater root elongation in natural, second-growth, mixed hardwood forests. Similarly, Drew [45] showed that a localized supply of nitrate, ammonium, and phosphorus stimulated root elongation and increased the number of lateral roots of barley. Although Drew [45] studied a non-forest tree species, the importance of mycorrhiza is similar for cereal and tree species.

Several studies have shown that the fungal community changes during the wood decomposition process [30,46,47]. In our study, the stages of wood decay did not alter the structure or quantity of vital mycorrhizal root tips, but did have a major effect on the composition of the ECM fungal community. The differences in species composition might also suggest that the substrate [48] at different stages of wood decay has an important effect on the ECM fungal community. Species of ECM fungi differ strongly in their potential enzymatic capacity, which is a function of both the substrate and climatic conditions [49]. This study showed that CWD contributed to ectomycorrhizae fungal diversity, with the most abundant ectomycorhizae being *Tomentella* sp., with its greatest abundance in the $CWD_{early}$ site. The high occurrence of fungal species may be due to the large amount of organic matter in this site. According to Rasmussen et al. [19], increased organic matter is conducive to mycorrhizae formed by fungi from Thelephoraceae. Moreover, *Tomentella* species have been found to occur more often in dead wood in intermediate decay phases than in late decay phases

[50,51]. CWD supports a relatively high abundance of ECM fungi from the lineages *Tomentella-Thelephora* (Thelephorales) [9,52], which are dispersed by soil microarthropodes [9,11,53–55].

The second most numerous mycorrhizae were formed by *P. nemoralis*, belonging to the phylum Ascomycota. This fungus forms hypogeous fruiting bodies and, to the best of our knowledge, the first record of this fungus in Poland was noted in our recent study [11]. Due to very limited data in this context [56,57], we can only presume that this species is a short-distance exploration type of mycelium, like the majority of ectomycorrhizal ascomycetous species [26]. Indeed, we consider this likely, since the highest abundance of these mycorrhizae was in $CWD_{advanced}$, where phosphorous content was high and mycorrhizae of fungi with short or medium distance exploration type mycelia dominated.

Differences in substrate preference was often correlated with the morphology of ECM fungi. Species of *Russula* and *Inocybe* form very little extraradical mycelium and form contact exploration or short types of mycelia [26]. In our study, *R. amoenolens* and *I. lanuginosa* were present exclusively at the $CWD_{advanced}$ site. The ectomycorrhizae of both genera are mostly hydrophilic, which is crucial for successfully exploring nearby substrates [26,58]. ECM fungi from the genera *Russula* or *Laccaria* have very little extraradical mycelium and often have the highest enzyme activity [19,24], which enables them to break down a variety of substrates, giving them greater spreading strategies and increased functionality [7,26,59]. The high abundance of *Tomentella* sp. mycorrhizae in $CWD_{advanced}$ supports this view. Velmala et al. [60] found that *Tylospora* sp. exhibit lignocellulose-degrading laccase enzyme activity, which, according to the authors, indicates that apart from mycorrhizal functions, it also has wood-decaying capabilities. Similarly, Wallander et al. [61] showed that *Tylospora* sp. are very efficient in colonising soil substrates, especially when the amount of available carbon is high. As *Tylospora* sp. is often found as an ECM symbiont of coniferous trees, especially Norway spruce [62], we presumed that old spruces in the vicinity of our Białowieża forest study sites may have been the source of this symbiont in the oak trees we studied. However, detailed environmental variables critical for this fungus remain unknown at this time.

Long-distance exploration ectomycorrhizae types (e.g., *S. citrinum*) were primarily found in the site lacking CWD, whereas the contact (e.g., *R. ochroleuca*) and short-distance types (e.g., *C. geophilum*) preferentially colonised sites, where CWD was present. Given that the highest phosphorous content occurred at the $CWD_{advanced}$ site, the presence there of fungi with lower ability to uptake nutrients (in comparison with long-distance exploration ectomycorrhizae types, e.g., *Scleroderma* spp.) seems logical. These results are in accordance with the distribution of different ECM exploration types in spruce seedlings growing in organic, mineral, or dead wood substrates [63]. A lower share of ECM fungi with a long-distance exploration type of mycelium (here represented by *S. citrinum*) was found in sites with CWD, both early and advanced decay classes, than in the site without CWD. This may be due to lower soil N content in the soil in the absence of coarse woody debris, a hypothesis made based on the fact that the long-distance exploration types of fungi usually show low enzyme activity [19]. Although we did not measure enzyme activity, we consider future research to address whether changes in ECM composition and enzyme activities may drive changes in N cycling to be a worthwhile endeavour.

Mycorrhizal fungi have the ability to translocate nutrients to plant roots [64]. Indeed, ECM fungi may colonize dead wood and compete with saprotrophs for nutrients [9]. Differences in the enzymes of ECM exploration types play a role in the release and transport of nutrients [26]. Our data revealed that at the $CWD_{advanced}$ site, where short-distance ECM were most abundant, soils had the highest phosphorus contents. Rousseau et al. [65] showed that *Pisolithus tinctorius* (Pers.) Coker & Couch, classified as a long-distance exploration type, stimulated the development of the surface area of fine roots and consequently improved phosphorus absorption in comparison to *C. geophilum,* classified as a short-distance exploration type. The relationship of the short exploration type and phosphorus uptake may be explained by differences in the total mycelial surface area and mycelial length.

Some authors found a positive relationship between contact mycorrhizae and N-related variables, which was confirmed by our results [66,67]. ECM taxa (e.g., members of the Russulaceae), due to their mycorrhizal features that allow nitrate to pass directly into the host tissue by diffusion,

produce a lower carbon cost to the host plant for nitrate assimilation and avoid host plant nitrate toxicity [68].

Although ECM species richness differed slightly between sites with early versus advanced coarse woody debris decomposition, there were no significant differences. This result is in accordance with findings by Mäkipää et al. [69], who observed that fungal species richness in the soil of Norway spruce stands did not vary.

## 5. Conclusions

In summary, in this study we confirmed our hypothesis that a soil substrate with incorporated CWD stimulates ectomycorrhizal fungi, representing contact or short-distance exploration types of mycelium. However, other studies in similar ecosystems are necessary to generalize our results on a wider scale. We are of the opinion that research into the environmental conditions favouring different ectomycorrhizal species could improve the understanding of their ecological functioning.

**Author Contributions:** J.O. and Z.B. generated the data. Statistical analysis was done using R version 2.15.0 (R Core Team 2012) with a vegan package for multivariate analysis: M.S. Molecular analysis: T.M. and J.O. Chemical analysis: D.H. The manuscript was written by J.O., D.H. The general conception: Z.B. All authors analysed and discussed the data.

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