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Ectomycorrhizal Community of Norway Spruce Stands with Different Degrees of Tree Decline

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Abstract: Dieback and decline of Norway spruce (*Picea abies* (L.) Karst.) tree stands on Mtn. Kopaonik are caused by a combination of abiotic and biotic factors. Ectomycorrhizal (ECM) fungi have stabilizing effects on forest trees that are under environmental stress. The aim of our study was to analyze the differences between ECM fungal communities, the number of fine roots, and the abundance of exploration types (ETs) in differently declined Norway spruce stands on Mtn. Kopaonik (Serbia). Three sites were selected: one with no tree decline recorded; one with a moderate decline of trees; and one with a massive decline of trees. Different degrees of tree decline in the studied spruce stands did not affect the number of ECM taxa, the diversity indices, or the percentage of vital fine roots. However, the number of old, non-turgescent, and nonmycorrhizal roots was higher in the spruce stands with tree decline. The ECM community composition differed between the studied sites, but the sites with tree decline had more ECM taxa in common. The ECM taxa of a long-distance ET were significantly more abundant in the spruce stands affected by tree decline.

Keywords: ectomycorrhizal diversity; exploration types; tree dieback; *Picea abies*; mountain Kopaonik

1. Introduction

Dieback and decline of Norway spruce (*Picea abies* (L.) Karst.) tree stands are among the greatest and long-standing issues in forestry throughout Europe [1]. The most frequently cited causes of the dieback are abiotic factors such as drought, high temperatures, and various forms of climatic stress [2–4], but also various biotic factors, primarily insects and fungi [5,6]. Also in Serbia, the dieback and decline of spruce, as the most widespread coniferous tree species, are a significant occurrence throughout the entire area. The most intense spruce dieback was recorded in Kopaonik National Park. According to Miletić et al. [7], a massive dieback occurred in pure natural spruce stands on an area of about 2400 ha in the last decade. They estimated a forest cover percentage decrease of 5.75% by 2022.

The causes of these degradation processes in Kopaonik National Park are numerous. Abiotic factors such as low amounts of precipitation in the growing season in combination with high temperatures are considered the primary factors which cause physiological weakening of the trees, especially in species with a shallow root system like spruce [8–10]. Such trees are susceptible to biotic factors like parasitic fungi and harmful forest insects that find suitable conditions for reproduction, after which their epiphytotia and mass reproduction occur [11]. Ectomycorrhizal (ECM) fungi which form a mutualistic association with forest trees are one of the essential components of the stability of the forest ecosystem. Moreover, the most important role of ectomycorrhizas in unfavorable conditions may be their stabilizing effects on forest trees under environmental stress [12,13]. During a drought, mycorrhizal fungi play significant roles in the mitigation of water and

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nutrient deficiency. Since the hyphae of ECM fungi have much smaller diameters compared to roots and root hairs, they can physically explore soil regions that could not be accessed directly by plant roots and root hairs, and overall provide a very large absorptive surface area [14].

Arnolds [15] noted a decline in the species diversity and sporocarp production of ectomycorrhizal fungi within Europe at the end of the 20th century, which was coincident with a decreased number of mycorrhizas. One of the possible causes of the decline was reduced tree vitality. In a review paper, Sapsford et al. [16] tried to answer the question "Are changes in communities of mycorrhizal fungi causing or predisposing trees to decline, is tree decline responsible for the decline or changes in communities of mycorrhizal fungi or is it a combination of both?" but they needed more empirical evidence for final conclusions. However, it was proved that changing environmental conditions such as soil moisture contribute to the morphological and functional changes of fine roots as well as to the degree of mycorrhizal colonization [17,18]. While severe drought might kill the mycorrhizal fine roots [19], moderate drought stimulates the formation and maintenance of mycorrhiza [20], and trees that experience intermediate levels of drought stress have a two-fold higher ECM colonization than trees at either ends of the stress continuum [21]. Also, Frymark-Szymkowiak et al. [18] noted that the forest with drier soil was characterized by significantly higher values of mycorrhizal colonization. However, mycorrhizal fungi differ in their efficiency of water regulation and tolerance to water deficit, which depends on the properties of the fungal mycelium [19].

Fungal morphological traits such as the amount and differentiation of extraradical mycelium are related to their ecology and are a basis for the classification of ectomycorrhizae into exploration types (ETs) [22]. Most ECM taxa in association with drought tolerant plants typically form rhizomorphs and hydrophobic mycelia and belong to long-distance ET, while ECM taxa in well-watered plants have a wider range of distinct ETs [23]. Furthermore, ECM fungi with longer ETs might play an important role in the survival and recruitment of seedlings in drought conditions [24].

The aim of our study was to analyze ECM fungal communities in three Norway spruce (*Picea abies* (L.) Karst.) stands on Mtn. Kopaonik (Serbia) that differ in the degree of decline, under the hypotheses that these communities differ significantly in their composition of ectomycorrhizal fungi [25,26], fine roots [17–19], and an abundance of ETs [23,24].

2. Materials and Methods

2.1. Study Sites

Kopaonik is the largest mountain massif in central Serbia with a highest peak elevation of 2017 m a.s.l. Kopaonik was declared a national park in 1981, covering an area of 11,969 ha, due to the rich diversity of flora and fauna and great cultural importance [27]. The dominant tree species is spruce, which grows in a very broad altitude range from 1100 to 1900 m a.s.l. At elevations from 1400 to 1700 m a.s.l., it most often forms pure mountain stands [8].

A moderate continental mountain climate dominates at lower and medium altitudes, while a subalpine climate prevails at higher altitudes. According to the data measured at the meteorological station on Mtn. Kopaonik from 1991 to 2020, the mean annual temperature in the area was 4.1 °C and the average annual amount of precipitation was 1040.1 mm [28]. According to the average monthly sum of precipitation in this area, the months with the highest and lowest precipitations were May and February, respectively [28].

After a detailed analysis of the decline of spruce forests on Mtn. Kopaonik that included evaluation of the existing condition, review of the records of planning documents, and estimation of the presence of fresh stumps, dry trees, and healthy trees with potential symptoms of dieback, three Norway spruce stands, differing in their stage of dieback, were selected for the study (Table 1). In two of the stands, significant dieback has been observed since 2013. The first is a natural stand of spruce with individual fir trees at the Jankove bare site, where massive dieback of spruce was determined, located in the center of a larger forest complex with massive dieback. The other is a mixed stand of spruce, beech and fir at the Vučačko brdo site, where significant dieback of spruce was noted, but the stand is located at the very edge of the complex with pronounced dieback. The third examined stand is a natural stand of spruce with individual fir and beech trees at the Metođe site, where no dieback of individual trees was observed. The stand is situated outside the forest complex where dieback was determined. At the Jankove bare and Metođe sites, spruce stands are situated on the slope, while at the Vučačko brdo site, the terrain is defined as flat. The biggest difference between the sites is reflected in the fact that the Metođe site is located near a stream which provides a constant source of water for the stand, compared to the other two sites (Jankove bare and Vučačko brdo).

Table 1. Site characteristics of studied Norway spruce stands from Mtn. Kopaonik, Serbia.

Site	**Metođe with No Decline**	**Vučačko Brdo with Moderate Decline**	**Jankove Bare with Massive Decline**
Coordinates	N 43°18′17.4″ E 20°50′37.6″	N 43°20′55.3″ E 20°47′44.9″	N 43°19′54.6″ E 20°46′59.2″
Altitude	1460 m	1530 m	1580 m
Climate	Temperately continental mountain, subalpine	Temperately continental mountain, subalpine	Temperately continental mountain, subalpine
Management type	Nature Reserve	Regularly managed stand	Nature Reserve
Slope aspect	North	North-northeast	West-northwest
Soil type *	Leptosol	Leptosol	Leptosol
Species	Natural forest of *Picea abies* with individual trees of *Abies alba* and *Fagus sylvatica*	Natural mixed forest of *Picea abies, Fagus sylvatica*, and *Abies alba*	Natural forest of *Picea abies* with individual trees of *Abies alba*

* According to IUSS Working Group WRB [29].

2.2. Soil Analysis

At all the sites, profiles were opened near the dominant tree species and soil samples were taken in the autumn of 2021. The origin of the litter was spruce. The positions of the soil profiles were different: at Vučačko brdo it was on the top of a hill or local sub-raising, flat to slightly convex, while at Jankove bare and Metođe the profiles were on the lower part of the slope close to the valley bottom, concave in shape. An analysis of both the organic layer and the mineral part of the soil was performed. The incline at Vučačko brdo was very low, while at the other two sites it was medium. The micro relief was uniform.

The physical and chemical soil properties were determined in the surface layer, i.e., the top 30 cm. The following soil characteristics were analyzed: particle size distribution (%) was determined by the international B-pipette method involving preparation in sodium pyrophosphate, soil textural classes were determined based on particle size distribution using the Atterberg classification, $CaCO_3$ percentage (%) was measured volumetrically using a Scheibler calcimeter (Royal Eijkelkamp, Giesbeek, The Netherlands) and pH in H_2O was determined with an electrometric method with a combined electrode on a Radiometer pH meter (InoLab® pH/ION/Cond 750, WTW, Wellheim, Germany) Carbon content was determined with a CHN element analyzer (Vario EL III, Elementar Analysen systeme, Langenselbold, Germany). The content of humus was measured by the Turin method, and soil and moisture content by the gravimetric method. All the analyses were performed in the laboratory of Soil Science in the Institute of Lowland Forestry and Environment in Novi Sad using the methodology described by Galić et al. [30] and the ICP Forests manual for soil sampling and analysis [31].

2.3. Sampling, Analyses of Fine Roots, and Morphotyping

Soil sampling for ECM analyses was performed on 27 October 2021 with a standardized soil corer of a total volume of 274 mL, a diameter of 4 cm, a measuring area of 12.57 cm^2, and a length of 18 cm at a distance about 1 m from the target tree trunk.

In every stand, five dominant neighboring Norway spruce trees of different ages were selected, and one soil sample was taken next to each tree. The soil samples were stored in a refrigerator at 4 °C, and before analyses were submerged overnight in tap water to loosen the soil structure. All fine roots were carefully washed from the soil using a sieve, then divided into vital ECM root tips, or old, non-turgescent, and nonmycorrhizal roots under an Olympus SZX 10 dissecting microscope (Olympus Corp., Tokyo, Japan) with magnifications 10–63× (light source: Olympus Highlight 3100, daylight filter, Olympus Corp., Tokyo, Japan). Based on their morphological and anatomical characteristics, vital ECM root tips were categorized into different morphotypes of ectomycorrhizae using a dissecting microscope and a microscope (Olympus BX 53®, Olympus Corp., Tokyo, Japan) with magnifications 100–1000×. All categories of fine root tips were quantified by counting under the dissecting microscope.

Morphotypes of ectomycorrhizae were described following the methodology given by Agerer [32] and Kraigher [33]. If possible, a fungal partner ECM fungus in morphotypes of ectomycorrhizae was determined by a comparison of the obtained descriptions with published descriptions in Agerer [34] or Agerer and Rambold [35]. Morphotypes of ectomycorrhizae were also classified into the ETs as proposed by Agerer [22].

2.4. Molecular Identification of Ectomycorrhizal Fungi

All of the morphotypes of ectomycorrhizae found in every soil sample were subjected to fungal partner identification by molecular methods. Determination of the ECM fungus from different morphotypes of ectomycorrhizae was based on PCR amplification of an internal transcribed spacer (ITS) region of fungal nuclear rDNA. The total genomic DNA was extracted from 2–5 ECM root tips of every ECM morphotype using a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The amplifications of DNA were performed using an ITS 1F [36] and ITS 4-primer pair [37] in an Eppendorf Master cycler (Eppendorf AG, Hamburg, Germany). Negative controls with no fungal DNA were run for each amplification reaction to check for any contamination. The PCR mixture for one sample was composed of 2.5 µL of 10× Gold Buffer, 2 µL of deoxynucleotide triphosphates (0.2 mM each), 0.6 µL of each primer (10 µM each), 2 µL of $MgCl_2$ (2.0 mM), 15 µL of sterile distilled water, 0.3 µL of Taq polymerase (5 U μL^{-1}), and 2 µL of a DNA extract. Thermal cycling conditions were: initial denaturation and polymerase activation at 95 °C for 5 min; 13 cycles at 94 °C for 45 s, 55 °C for 55 s and 72 °C for 45 s.; 13 cycles at 94 °C for 45 s, 55 °C for 55 s and 72 °C for 120 s; 12 cycles at 94 °C for 45 s, 55 °C for 55 s and 72 °C for 180 s and a final extension at 72 °C for 10 min. Purification of PCR products was performed using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) then amplified DNA fragments were sent to Macrogen Europe B.V. for sequencing. The ECM fungi were determined at the level of species or genus by comparison of the obtained sequences to those deposited in the GenBank [38] and UNITE [39] database. The threshold value applied to differentiate the OTUs based on the ITS sequence similarity was 97%. Morphotypes with more than a 97% sequence similarity were assigned to the species level, while those with less than 97% were assigned to the genus level. Morphotypes with less than 95% sequence similarity were assigned as unknown types of ectomycorrhiza and their identification was performed on the basis of the morpho-anatomical descriptions.

2.5. Data Analysis

Diversity indices, namely the species richness index (d), the Shannon–Weaver index (H), the Evenness index (e), Equitability (J), and the Berger–Parker evenness index (BP), were calculated per sample and per site (i.e., by pooling the ECM community data) following the formulas given by Atlas and Bartha [40] and Taylor et al. [41] and explained in detail by Milović et al. [42]. All calculations about the representation of taxa and about the diversity indices are based on the results of a combination of morphotyping and molecular analysis.

Data from an individual soil sample were used as a statistical unit. Fisher's LSD test was used to test the significance of differences between stands in the number of ECM fungal taxa; vital ECM root tips; old, non-turgescent, and nonmycorrhizal roots; the total number of fine root tips; and percentage of vital root tips. In order to fit the normal distribution, the data were transformed as follows: count data were transformed by square root transformation [43], while percentage values were transformed by arcsine transformation using the Bliss formula [44]. The multiple comparisons of mean ranks for all groups test [45] was used to determine the significance of the differences between stands in diversity indexes and in an abundance of ETs, respectively. All statistical analyses were performed using the package STATISTICA® version 12 (StatSoft Inc., Tulsa, OK, USA).

3. Results

The soil samples from all the sites were dominated by the fraction of total sand, which resulted in the textural classes loamy sand and sandy loam (Table 2). This indicates that the soils have a reduced capacity for binding easily accessible water and are largely dependent on rainfall. Mor humus, with a high proportion of humus and carbon, was determined as the type of humus at the studied sites. The content of humus, as well as carbon, varies slightly between the studied sites, but $CaCO_3$ content at the Vučačko brdo site was lower compared to the other two sites. Acidic soil reaction was determined at all the sites, while the highest values of pH and soil moisture were recorded at the Metođe site (Table 2).

Table 2. Physico-chemical properties of the soil samples from the studied Norway spruce stands on Mtn. Kopaonik.

Physical Properties of the Soil						
Site	**Physiologically Active Depth**	**Humus Accumulative Layer**	**Volume Mass ($g\ cm^{-3}$)**	**Texture Class**	**Total Sand (%)**	**Total Clay (%)**
Metođe with no decline	17 cm	25 cm	0.621	Loamy sand	81.2	18.8
Vučačko brdo with moderate decline	26 cm	26 cm	0.575	Sandy loam	80.7	19.3
Jankove bare with massive decline	30 cm	27 cm	0.794	Loamy sand	83.5	16.5
Chemical properties of the soil						
	pH	**Humus content (%)**	**$CaCO_3$ content (%)**	**Carbon content (%)**	**Soil moisture content (%)**	
Metođe with no decline	4.91	8.69	2.26	4.91	22.51	
Vučačko brdo with moderate decline	3.91	8.70	0.88	5.01	18.08	
Jankove bare with massive decline	3.47	8.72	2.76	5.06	18.29	

From the data in Table 3, it can be seen that the number of ECM fungal taxa, the percentage of vital ECM roots, and the diversity indices did not differ significantly among the stands. However, at the Vučačko brdo site (with moderate decline), a higher number of vital ECM roots was recorded compared to the other two sites. On the other hand, at the Metođe site (with no decline), a significantly lower number of old, non-turgescent, and nonmycorrhizal root tips was observed along with the total number of fine roots compared to the other sites.

Table 3. Total and average values (±standard error) of the number of ectomycorrhizal fungal taxa, number of vital ectomycorrhizal root tips, number of old, non-turgescent, and nonmycorrhizal root tips, total number of fine roots, % of vital ectomycorrhizal root tips, and diversity indices in different stands of Norway spruce (*Picea abies* (L.) Karst.) from Mtn. Kopaonik.

Site	**Metođe with No Decline**		**Vučačko Brdo with Moderate Decline**		**Jankove Bare with Massive Decline**	
Parameter	**Total Value per Site**	**Average Value per Soil Sample**	**Total Value per Site**	**Average Value per Soil Sample**	**Total Value per Site**	**Average Value per Soil Sample**
Number of ectomycorrhizal fungal taxa	8	3.0 ± 0.4 a*	9	3.2 ± 0.4 a	8	4.0 ± 0.4 a
Number of vital ectomycorrhizal root tips	1168	237 ± 59 b	2773	554 ± 129 a	1108	228 ± 46 b
Number of old, non-turgescent and nonmycorrhizal root tips	1508	301 ± 62 b	3732	782 ± 124 a	4247	910 ± 228 a
Total number of fine roots	2676	539 ± 66 b	6505	1337 ± 225 a	5355	1139 ± 247 a
% of vital ectomycorrhizal roots	44	44 ± 9 a	43	39.8 ± 7 a	21	22.0 ± 0.3 a
Species richness index (S)	2.28	0.93 ± 0.3 a	2.32	0.81 ± 0.1 a	2.30	1.29 ± 0.2 a
Shannon–Weaver index (H)	1.44	0.72 ± 0.2 a	1.41	0.60 ± 0.1 a	1.76	1.04 ± 0.2 a
Evenness (e)	1.59	1.51 ± 0.4 a	1.47	1.22 ± 0.2 a	1.95	1.71 ± 0.2 a
Equitability (J)	0.69	0.65 ± 0.2 a	0.64	0.53 ± 0.1 a	0.84	0.74 ± 0.1 a
Berger–Parker index (BP)	0.53	0.31 ± 0.1 a	0.43	0.28 ± 0.1 a	0.71	0.47 ± 0.1 a

* Differences among values of a particular variable marked with the same letter are not significant ($p > 0.05$), according to Fisher's LSD and multiple comparisons of mean ranks.

In three of the studied spruce stands, 19 ECM types were found in total, according to a combination of morphotyping and molecular methods. Ten ECM types were determined at the species and nine at the genus level (Tables 4 and S1). According to their relative abundance, the most abundant fungi on Mtn. Kopaonik were *Russula integra* and *Clavulina* sp. The most frequent fungus was *Imleria badia* which occurred in four out of the five soil samples at the Vučačko brdo site and in five out of five samples at the Jankove bare site. The second frequent fungus was *Russula integra* which was recorded at the same sites in four out of five and three out of five soil samples, respectively.

Table 4. ECM fungal taxa observed in three differently declined spruce (*Picea abies* (L.) Karst.) stands (Metođe (with no decline)—M, Vučačko brdo (with moderate decline)—VB, Jankove bare (with massive decline)—JB) from Mtn. Kopaonik with exploration types, frequency, and their absolute/relative (%) abundance.

Fungal Partner in Ectomycorrhiza Based on Morpho-Anatomical and Molecular Characterization	**Site**	**Exploration Type**	**Occurrence in Soil Samples (Frequency)**	**Absolute Abundance (Number of Ectomycorrhizal Tips)**	**Relative Abundance in % (Classification *)**
Amanita olivaceogrisea Kalamees	JB	LD	1/5	10	0.9 scattered
Cenococcum geophilum Fr.	JB, VB	SD	2/5, 2/5	59, 320	5.3 often, 7.2 often
Cortinarius delibutus Fr.	M	MD fringe	1/5	39	3.3 often

Table 4. *Cont.*

Fungal Partner in Ectomycorrhiza Based on Morpho-Anatomical and Molecular Characterization	Site	Exploration Type	Occurrence in Soil Samples (Frequency)	Absolute Abundance (Number of Ectomycorrhizal Tips)	Relative Abundance in % (Classification *)
Elaphomyces muricatus Fr.	JB	SD	3/5	188	17 frequent
Imleria badia Fr. (Vizzini)	VB, JB	LD	4/5, 5/5	271, 326	9.8 often, 29.4 frequent
Inocybe assimilata Britzelm.	VB	SD	1/5	13	0.07 rare
Russula firmula Jul. Schaf.	M	C	3/5	267	22.7 frequent
Russula integra (L.) Fr.	VB, JB	C	4/5, 3/5	1588, 216	57.3 numerous, 19.5 frequent
Sebacina epigaea (Berk. & Broome) Bourdot & Galzin	M	SD	2/5	179	15.2 frequent
Tomentella stuposa (Link) Stalpers	M, VB, JB	MD smooth	1/5, 1/5, 2/5	1, 33, 218	0.08 rare, 1.19 occasional, 19.67 frequent
Amphinema sp.	M, VB	MD fringe	1/5, 1/5	77, 320	6.55 often, 11.54 frequent
Clavulina sp.	M	C	2/5	554	47.11 numerous
Lactarius sp.	JB	C	1/5	55	4.96 often
Meliniomyces	JB	C	2/5	36	3.24 often
Pseudotomentella sp.	M	MD mat	2/5	55	4.68 often
Russula sp.	VB	C	1/5	321	8.33 often
Tomentella sp.	VB	SD	1/5	2	0.08 rare
Tomentellopsis sp.	VB	MD smooth	1/5	116	4.18 often
Tylospora sp.	M	SD	2/5	4	0.34 scattered

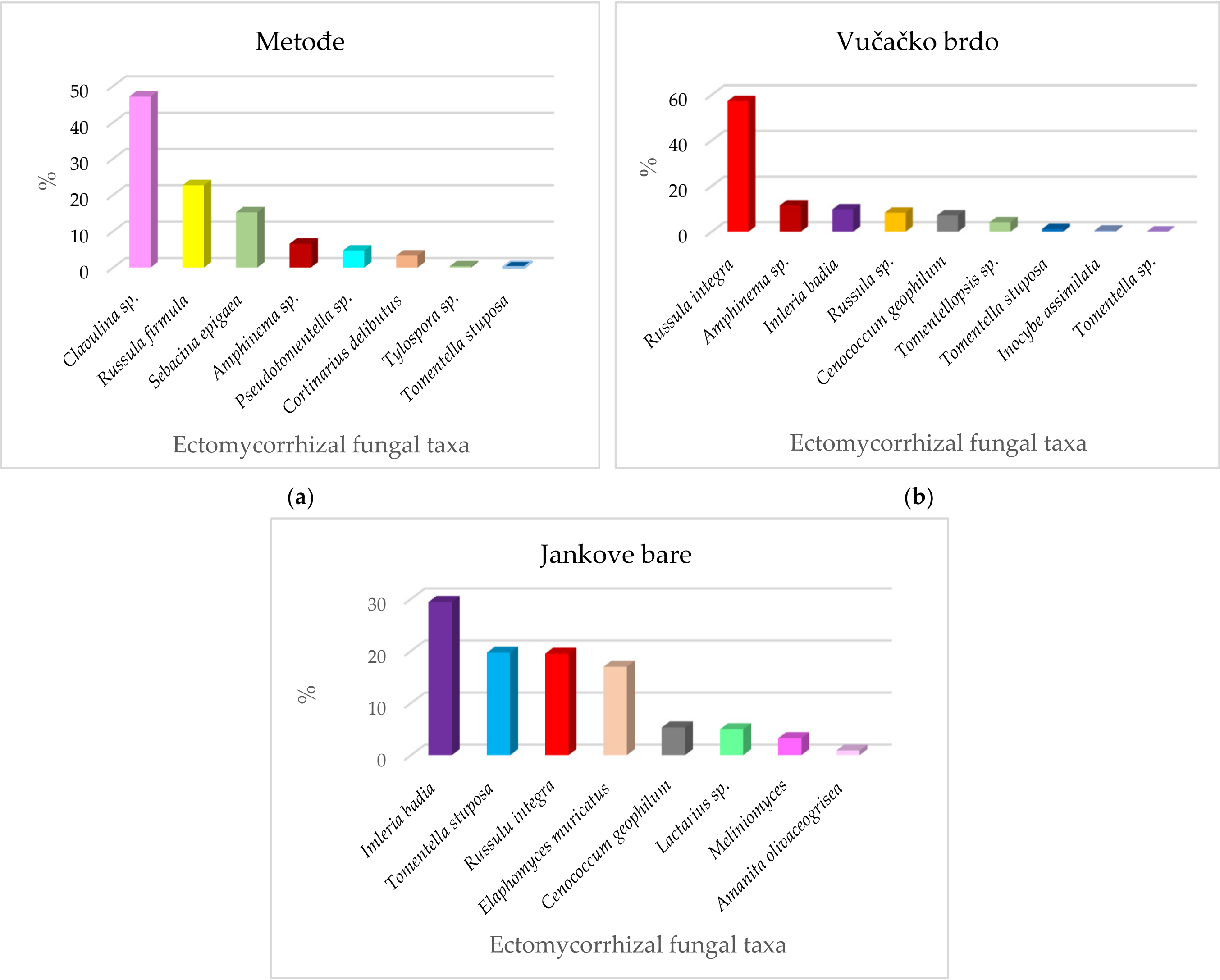
* According to their relative abundance types of the ectomycorrhizae were classified into six dominance classes [46]: (1) numerous (100%–32%), (2) frequent (31.99%–10%), (3) often (9.99%–3.2%), (4) occasional (3.1%–1.0%), (5) scattered (0.99%–0.32%), and (6) rare (0.31%–0%).

According to Figure 1 and Table 4, the most abundant ECM fungus at the Metođe site was *Clavulina* sp., which colonized almost 50% of all ECM root tips, while *Russula integra* formed more than a half of all the ECM root tips at Vučačko brdo. The Jankove bare site was dominated by ECM fungi *Imleria badia*, *Tomentella stuposa*, *Russula integra*, and *Elaphomyces muricatus*, which were more evenly distributed and made up more than 90% of all ECM roots.

T. stuposa was recorded at all three studied sites, but its relative abundance varied from rare (at the Metođe site) to frequent (at the Jankove bare site). The ECM taxa *R. integra*, *I. badia*, *C. geophilum*, and *T. stuposa* were recorded at both the Vučačko brdo and Jankove bare sites. *Amphinema* sp. and *T. stuposa* were observed at both Metođe and Vučačko brdo. Only *T. stuposa* was common for Metođe and Jankove bare, but its abundance differed to a large degree.

At the Metođe site, the ECM taxa from the Clavulinaceae and Russulaceae families made up 70% of all ECM roots, while at the Vučačko brdo site almost 70% of all ECM roots were formed by members of the Russulaceae family. On the other hand, at the Jankove bare site, the Boletaceae, Russulaceae, and Thelephoraceae families were the most abundant and colonized almost three quarters of all roots. They were evenly represented (Figure 2).

The most abundant ET at the Metođe site was contact ET, then short-distance and medium-distance ETs. Similarly, the Vučačko brdo site was dominated by contact ET which colonized more than 70% of all root tips and was followed with long-distance and medium-distance ETs. However, at the Jankove bare site all the studied ETs were more evenly distributed. The most abundant ET was contact, followed by short-distance and long-distance, and then medium-distance ET. The abundance of long-distance ET was significantly higher at the Vučačko brdo and Jankove bare sites compared to the Metođe site (Figure 3, Table 5).



(**a**) (**b**)

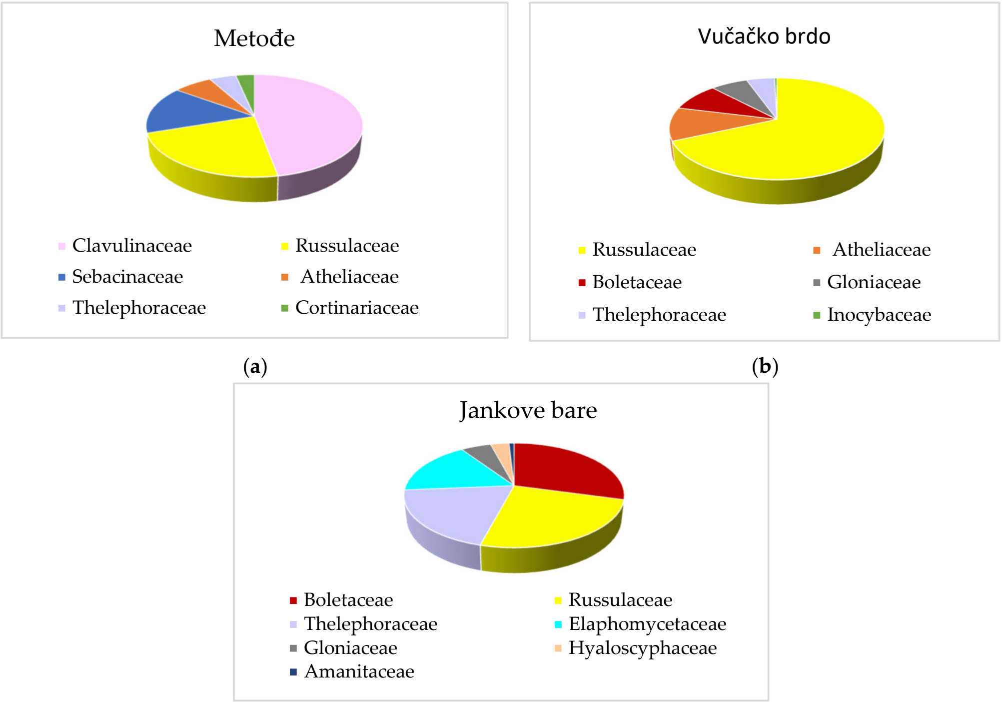
(**c**)

Figure 1. Relative abundance of ectomycorrhizal fungal taxa (based on the number of ectomycorrhizal root tips belonging to the particular ectomycorrhizal fungal taxon in relation to the total number of ectomycorrhizal root tips in Norway spruce (*Picea abies* (L.) Karst.) stands from Metođe with no decline (**a**), Vučačko brdo with moderate decline (**b**), and Jankove bare with massive decline (**c**) based on five samples per site.

Table 5. Average abundance (± standard error) (%) of ectomycorrhizal exploration types (C—contact, SD—short distance, MD—medium distance, LD—long distance) per soil sample in different stands of Norway spruce (*Picea abies* (L.) Karst.) from Mtn. Kopaonik and the significance of Fisher's LSD test for effect of the site.

Site	C	SD	MD Smooth	MD Fringe	MD Mat	LD
Metođe with no decline	61.4 ± 11.9 a*	15.8 ± 8 a	0.1 ± 0.1 a	8.4 ± 8.4 a	14.3 ± 11.9 a	0.0 b
Vučačko brdo with moderate decline	53.4 ± 11.5 a	7.8 ± 5.6 a	5.8 ± 4.6 a	7.1 ± 7.1 a	0.0 a	23.8 ± 15 a
Jankove bare with massive decline	37.1 ± 16 a	23.9 ± 11.6 a	12.8 ± 8 a	0.0 a	0.0 a	26.2 ± 12 a

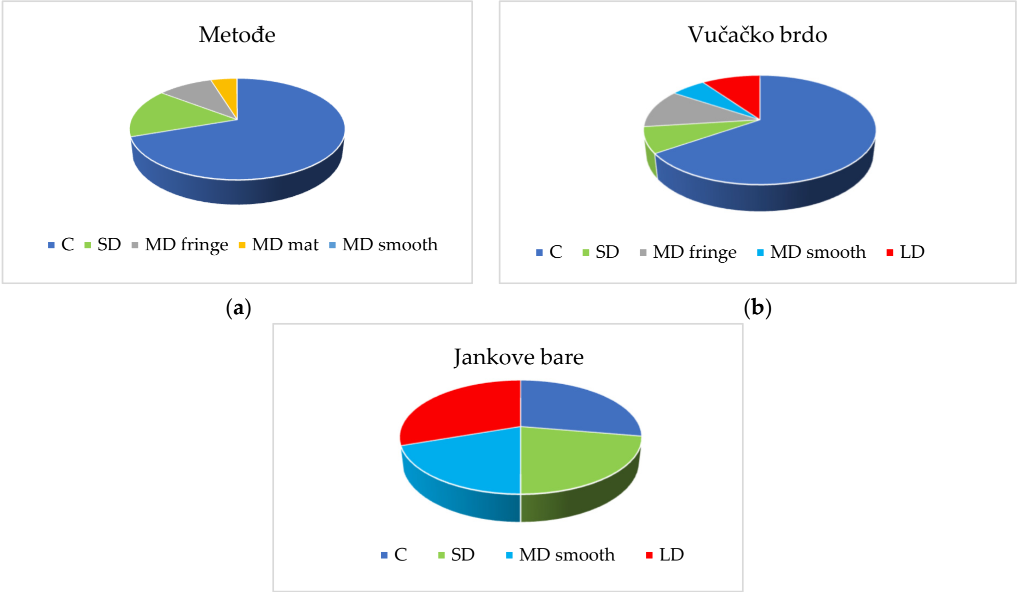
* Differences among values of a particular variable marked with the same letter are not significant ($p > 0.05$), according to Fisher's LSD.



(**a**) (**b**)

(**c**)

Figure 2. Relative abundance of taxonomic families of ectomycorrhizal fungi based on the number of ectomycorrhizal root tips belonging to a particular family in relation to all ectomycorrhizal root tips in Norway spruce (*Picea abies* (L.) Karst.) stands from Metođe with no decline (**a**), Vučačko brdo with moderate decline (**b**), and Jankove bare with massive decline (**c**) based on five samples per site.



(**a**) (**b**)

(**c**)

Figure 3. Relative abundance of ectomycorrhizal exploration types (C—contact, SD—short distance, MD—medium distance, LD—long distance) based on the number of ectomycorrhizal root tips belonging to a particular exploration type in relation to all ectomycorrhizal root tips in Norway spruce (*Picea abies* (L.) Karst.) stands from Metođe with no decline (**a**), Vučačko brdo with moderate decline (**b**), and Jankove bare with massive decline (**c**) based on five samples per site.

All three spruce stands were dominated by the fungal group Basidiomycota. This group made an association with all the ECM root tips at the Metođe site (site without decline of trees) as well as with 74% of all ECM root tips at the Jankove bare site (a site with severe decline of trees). On the other hand, Ascomycota fungi appeared only at sites where tree decline was recorded. The increase in the number of ECM taxa belonging to the Ascomycota group as well as the percentage of ECM root tips formed by this fungal group was related to the degree of tree decline (Table 6).

Table 6. Number of ECM taxa and percentage of ECM root tips belonging to the Basidiomycota and Ascomycota group in different stands of Norway spruce (*Picea abies* (L.) Karst.) from Mtn. Kopaonik.

Site	**Number of ECM Taxa**		**Percentage of ECM Root Tips**	
	Basidiomycota	**Ascomycota**	**Basidiomycota**	**Ascomycota**
Metođe with no decline	8	0	100	0
Vučačko brdo with moderate decline	8	1	93	7
Jankove bare with massive decline	5	3	74	26

4. Discussion

The analyzed physico-chemical characteristics of soils from three studied Norway spruce stands with different degrees of tree decline did not differ much. The soil samples from all three sites were dominated by sand which considerably limited their ability to bind easily accessible water. However, the Metođe site without a decline of trees was located near a stream and soil at this site contained more moisture compared to the other two sites which were dependent on precipitation.

The number of ECM fungal taxa and ECM diversity indices of the analyzed stands did not differ significantly. This is in accordance with Defrene et al. [47] who discovered that climate and soil fertility were not related to species richness or the diversity of ECM fungi. Furthermore, a comparison of the ECM fungal assemblage in mature spruce forests attacked and destroyed by bark beetles and in a mature non-attacked forest did not show significant differences in the number of ECM species, but ECM species composition changed [25]. On the other hand, Peter et al. [26] noted that ECM species richness on the roots of adult Norway spruce trees was significantly lower in the heavily damaged site compared with the other two less contaminated sites. At the Vučačko brdo site, with a moderate decline of trees, a significantly higher number of vital ECM roots was recorded compared to the other two sites. This parameter did not differ significantly between the Metođe site, without a decline of trees, and the Jankove bare site, where a massive decline of trees was observed. The obtained results are in accordance with previous studies [18–20], which revealed that moderate drought might have a stimulating effect on root colonization with mycorrhizal fungi. On the other hand, at the Vučačko Brdo and Jankove bare sites, significantly higher numbers of old, non-turgescent, and nonmycorrhizal root tips were observed compared to the Metođe site. A higher number of dead roots observed at sites with drier soils support the findings of Liese et al. [48] who documented that the life span of ECM fungi was reduced from 176 to 81 days in dry soil compared to moist soil. The percentage of vital ECM roots ranged from 20% at the Vučačko brdo site to 40% at the Jankove bare site and 44% at the Metođe site. Thus, it seems that stands with different degrees of tree decline had similar values of this parameter, suggesting that the percentage of vital ECM root tips cannot be related to the decline of trees. Obtained values are comparable with the findings of Katanić et al. [49] who, also on Mtn. Kopaonik, at the Metođe site eight years ago, noted 46% of vital ECM roots in autumn. Also, Kraigher [50] discovered that the percentage of ECM tips in an autochthonous altimontane spruce stand on Pokljuka and in an anthropogenic montane spruce stand in Zavodnje, both in Slovenia, was 40%.

At all the studied sites, only a few ECM fungal taxa dominated the ECM community. At the Metođe site, the most abundant ECM fungi were: *Clavulina* sp., *Russula formula*, and *Sebacina epigaea* which together colonized 84% of all ECM root tips; at Vučačko brdo, *Russula integra*, *Amphinema* sp., *Russula* sp., and *Imleria badia* formed almost 90% of all ECM root tips; and at the Jankove bare site *Imleria badia*, *Tomentella stuposa*, *Russula integra*, and *Elaphomyces muricatus* made up more than 90% of all ECM roots. Our observation that the ECM community consisted of a few abundant ECM fungal taxa and a larger number of rare taxa is in accordance with numerous studies [49,51–53].

T. stuposa was the only ECM fungus found in all three sites. ECM taxa *R. integra*, *I. badia*, *C. geophilum*, and *T. stuposa* were recorded at both Vučačko brdo and Jankove bare and *Amphinema* sp. and *T. stuposa* were observed at both Metođe and Vučačko brdo, while only *T. stuposa* was common for Metođe and Jankove bare. Our results are supported by the findings of O'Hanlon and Harrington [54] in that each forest type had a statistically distinctive ECM community. Moreover, most ECM taxa recorded in one forest type were confined to it and relatively few taxa were shared between the different forest types. As regards the ECM communities, the Sitka spruce plots showed the least similarity to each other [54]. Members of the genus *Russula* were well-represented at the studied sites on Mtn. Kopaonik. While *R. firmula* was abundant at the Metođe site, *R. integra* was dominant at the other two sites. According to Geml et al. [55], in Alaska, the genus *Russula* showed strong habitat preference and some preference for soil horizon. The highest number of ECM taxa shared between the Vučačko brdo and Jankove bare sites can be explained by the similar moisture content of the soils of these two sites. Moreover, the fungus *Cenococcum geophilum*, which is known to favor colder/drier climates and richer soils [47], was observed at these two sites, characterized by drier soil, while it was absent at the Metođe site with more humid soil.

In Norway spruce forests across the European climate gradient, Ostonen et al. [56] observed the following lineages: "russula-lactarius", "tomentella-thelephora", "amphinema-tylospora", "piloderma", "paxillus-gyrodon", and "boletus" as the dominant colonizers. In our study, representatives from the groups "russula-lactarius", "tomentella-thelephora", "amphinema-tylospora", and "boletus" were recorded while members of "piloderma", "paxillus and gyrodon" groups were not observed. Similar results were obtained in the work of Katanić et al. [49] on the ectomycorrhizas of Norway spruce from its southernmost natural distribution range in Serbia (sites Stara Planina, Kopaonik, and Tara). On Mtn. Kopaonik, the same authors observed the dominance of ECM type *Tomentella* sp. in the spring and *Russula firmula* in the fall.

In a 100-year-old Norway spruce forest in south Sweden, Dahlberg et al. [51] noted 25 ECM types among which *Cenococcum geophilum* Fr. and *Piloderma croceum* Erikss. & Hjortst. were the most abundant. In all three forest types of Norway spruce studied by Veselá et al. [25], the dominant ECM species were *Tylospora fibrillosa* and *Cenococcum geophilum*. On the other hand, in a young Norway spruce stand in the Bavarian Limestone Alps, Baier et al. [57] determined the dominance of ECM taxa *Cenococcum geophilum*, *Tomentella*, *Lactarius*, and *Sebacina*, which altogether colonized 60% of all ectomycorrhizal root tips within the plot. These genera were also recorded in studied spruce stands from Kopaonik and in a previous study by Katanić et al. [49], although some of them were less abundant. According to Veselá et al. [25] *Tylospora fibrillosa* (Burt) Donk, *Meliniomyces variabilis* Hambl. & Sigler, and *Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox were characteristic species in the spruce forest destroyed by bark beetles, whereas *Lactarius*, *Cortinarius*, and *Russula* were in the mature non-attacked forest. In our study, *Tylospora* sp. was recorded at the Metođe site without tree decline, while *Meliniomyces* was found at the Jankove bare site, in a stand with moderate tree decline.

At the Vučačko brdo site almost 70% of all ECM roots were formed by members of the Russulaceae family. On the other hand, ECM taxa from the Clavulinaceae and Russulaceae families made up 70% of all ECM roots at the Metođe site, while at the Jankove bare site families Boletaceae, Russulaceae, and Thelephoraceae colonized over 70% of all roots and they were evenly represented. Similarly, in the spruce stand from Kopaonik, the dominance of the Thelephoraceae family in the spring and Russulaceae in the fall was previously observed by Katanić et al. [49].

The dominance of the Basidiomycota fungal group observed at all three sites in our study is in accordance with previous studies [25,47,57]. However, the presence of the Ascomycota fungal group was recorded only at sites with a decline of trees, i.e., sites with drier soil conditions. This is in agreement with Defrenne et al. [47], who noted the occurrence of Ascomycetes exclusively in Douglas-fir forests in drier environments, and with Veselá et al. [25], who found members of the Ascomycota group only in forest destroyed by pest. Moreover, Peter et al. [26] recorded a higher percentage of mycorrhizal root tips colonized with Ascomycetes at a heavily damaged site compared to two other less damaged sites.

The relationship between different ETs and their ecological roles is well-known [22]. In European forests of beech, pine, and spruce, the most important factors shaping the ectomycorrhizal community, as well as the distribution of ETs, is host tree species and abiotic factors such as soil properties, N deposition, temperature, and precipitation [53]. Similarly, in the Douglas-fir forests of western Canada, Defrenne et al. [47] noted that abiotic factors (temperature, precipitation, and soil C:N ratio) filtered ECM fungal community composition and the abundance of ETs.

According to Rudawska et al. [58], the abundance of a particular ET is related to soil chemistry, while Baier et al. [57] observed that ETs have preferences towards particular soil horizons, which is caused by different amounts of humus. In all three studied spruce stands from Mtn. Kopaonik, contact ET dominated. A similar result was obtained in the work of Katanić et al. [49], who observed the dominance of the same ET at the Kopaonik site. Also, contact ET dominated the ECM community in the interior Douglas-fir forests of western Canada [47]. Moreover, in spruce forest types differently affected by bark beetles, short-distance ET was generally the most widespread ET [25]. In European forests, Rosinger et al. [53] observed higher mean abundance of contact and short-distance ETs than of medium- and long-distance ETs, and the highest abundance of the short-distance ET was recorded in the *Picea abies* stands. According to Baier et al. [57], contact and medium-distance ETs were proven to be associated with soil properties indicative of the mineral A-horizon, while short-distance ET preferred soil environments rich in humus. Moreover, the same authors observed that the majority of the ECM fungi preferred organic layers. Although in our study the percentage of humus in soil at all sites was higher than 8%, at the Metođe and Vučačko brdo sites ECM fungi with ETs which are considered to prefer mineral A-horizon prevailed, while at the Jankove bare site these ETs were less abundant, probably due to conditions that are less favorable to contact and fringe medium-distance ETs.

The growth and sustainability of forest trees depend a great deal on fine root acclimation to different environmental conditions. The efficiency of fungi in nutrient uptake and transfer is species-specific and may have functional importance in tree nutrition [59]. Relatively small changes in fine root biomass, morphology, or colonization with mycorrhizal fungi may result in a large change in forest cycles of carbon, nutrients, and water [56]. The same authors discovered different fine root foraging strategies in boreal and temperate forests and indicated the importance of an ECM symbiont foraging strategy in fine root nutrient acquisition [56].

ECM fungi with long-distance ET were significantly more abundant in spruce stands affected by a decline of trees at the Vučačko brdo and Jankove bare sites, which is most probably related to the lower content of moisture recorded in the soils from these two sites. Thus, it seems that plants exposed to drought may benefit by association with such ETs of ECM fungi which transport water more efficiently. Long-distance ETs that typically form rhizomorphs and hydrophobic mycelia dominated ECM fungal community of young pine trees surviving drought [23]. Similarly, García de Jalón et al. [24] found that ECM fungi with long-distance ET were more abundant on *Quercus ilex* L. under conditions of reduced precipitation and positively correlated with survival, which suggests the potential role of this ECM ET in seedling survival and recruitment.

5. Conclusions

This is one of few studies dealing with the influence of tree decline on ECM communities, fine roots, and ETs of spruce stands. The higher abundance of ECM fungi with long-distance ET in spruce stands affected with tree decline at sites Vučačko brdo and Jankove bare compared to an unaffected stand from Metođe may be related to the drier soil conditions prevailing at sites with tree decline. However, it is difficult to distinguish the correlation between mycorrhizal fungi and spruce decline from the influence of other environmental factors affecting both fungi and spruce health. Additionally, it is impossible to answer the question of which occurred first: changes in mycorrhizal fungi associations or the decline of spruce trees. In order to get a more thorough insight into the influence of tree decline on ECM fungal communities and fine roots, the number of repetitions or sampling scales should be increased and other parameters, such as tree species composition and management level, should be included.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15101838/s1, Table S1: ECM fungal taxa observed in three differently declined spruce (*Picea abies* (L.) Karst.) stands from Mtn. Kopaonik, identified on the bases of morpho-anatomical characteristics and molecular methods.

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