ORIGINAL RESEARCH PAPER

Biomass of external mycelium of ectomycorrhizal fungi in Norway spruce stands in Poland

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Abstract

Biomass of extramatrical mycorrhizal mycelium (EMM) was examined under canopies of mature Norway spruce trees grown in different forest stands in Poland. Two mountain forest sites (Brenna and Salmopol), one upland site (Zwierzyniec) and one lowland site (Mirachowo) have been investigated, using sand-filled mesh-bags method. The in-grow mesh-bags were buried in the soil for 12 months (since October up to the next October) or for 4 months (since June up to October) at four depths at each site: 5, 15, 30 and 45 cm (Brenna and Salmopol) or 60 cm (Zwierzyniec and Mirachowo). The mycelium biomass was estimated from the ergosterol content determined in the mesh-bags. The results indicated significant differences in EMM production and their vertical distribution between the mountain and the upland and lowland forest sites. The lowest EMM biomass was found at the experimental plot in the mountainious site Brenna. Considerable decrease of EMM biomass with the soil depth was recorded after 12 months of the mesh-bags incubation in soil in the upland and lowland sites, while in the mountain forests decrease of the EMM biomass in the lower soil depths diminished more gradually EMM biomass determined in the mesh-bags placed in soil at the upper 5 and 15 cm tended to be higher after 4 months than after 12 months of incubation period. Such results suggest that the time necessary for evaluation of EMM biomass in soil may be limited to the summer–autumn months, when the production of EMM is the highest. Variable stress factors can influence decreased ectomycorrhizal mycelium production and/or their destruction. Further research in different forest types and regions are needed for better understanding factors determining EMM biomass production and surviving in soil.

Keywords

Picea abies; ergosterol; soil depth; mesh-bags

This issue of Acta Mycologica is dedicated to Professor Maria Lisiewska and Professor Anna Bujakiewicz on the occasion of their 80th and 75th birthday, respectively.

Introduction

Norway spruce is the second most common coniferous forest tree in Poland after Scots pine and one of the most important timber species, with a high commercial and ecological value. As do most boreal and temperate forest tree species, Norway spruce live in symbiotic association with ectomycorrhizal fungi (EMF) [1]. For Norway

spruce, this association is obligate, and no proper growth and development occur when ectomycorrhizas are lacking [2]. Apart from the mantle and Hartig net, an integral part of fungal ectomycorrhizal (ECM) structures is the extramatrical mycorrhizal mycelium (EMM), which emanates from the mantle and grows into the surrounding soil [3]. The EMM is characterized by high-absorption surfaces and has a higher capacity than do root hairs for mobilizing and absorbing water and nutrient elements, thus influencing plant production under limiting conditions [4].

In return, the EMM associated with ectomycorrhizas accounts for allocation of photoassimilates from the host plant to the mycelial structures of ectomycorrhizas. Wallander et al. [5] have developed a specific approach to sampling EMM from soil, based on fine-mesh nylon bags filled with sand and buried underground. The in-growth mesh-bags exclude roots but allow ingrowth of hyphae and are preferentially colonized by ectomycorrhizal fungi [5,6]. This method makes it possible for researchers to quantify the biomass of EMM produced during the period of time during which the bags are in the soil. To measure amounts of fungal mycelium in a substrate, the fungal-specific biomarker ergosterol has been frequently used. Ergosterol (22E)-Ergosta-5,7,22-trien-3β-ol ($C_{28}H_{44}O$) is a membrane lipid found almost exclusively in fungi, the principal sterol of Ascomycota and Basidiomycota, abundant in membranes of living fungal cells [7]. The level of ergosterol in soil is a reliable indicator of fungal biomass [8–10].

Several factors were found to influence the growth of EMM in soils, e.g., availability of water and/or main nutrients [11–14], forest fertilization [15,16], tree defoliation [17], season [18], age of the host plant [19], and presence of soil fauna [20]. However, the number of studies addressing external mycelial biomass production under field conditions is still limited, and most works concern coniferous trees in the boreal forests in Europe, chiefly in the Fennoscandia countries (see review papers Wallander et al. [21] and Ekblad et al. [18]).

Up to now, external mycelial biomass production in Poland has not been determined. Therefore, the aim of this study is to examine the biomass of the EMM of ectomycorrhizal fungi in different Norway spruce stands in Poland, using the sand-filled in-growth mesh-bag method. We hypothesize that site conditions will influence the mycelial biomass of EMM accompanying Norway spruce trees. In addition, we examined the effects of soil depths on extramatrical mycelial biomass, as past work (e.g., [22]) has indicated some degree of influence of soil depths on this parameter. For this reason, we hypothesized that the biomass will decline with soil depths. The present contribution also compares two different incubation periods of the mesh-bags in soil.

Material and methods

Study sites

Studies were conducted at four permanent study plots (50 × 50 m) representing mature forests in Poland, differed in climatic conditions, soil, and forest type, and in the degree of anthropogenic pressure [23]. The two mountainous study plots, i.e., Brenna (49°43′ N 18°56′ E; 679 m a.s.l.) and Salmopol (49°41′ N 18°58′ E; 1000 m a.s.l.), were located in the Silesian Beskid Mountains and were represented by homogenous Norway spruce forests (Brenna: 115-year-old trees with 15–25% crown canopy; Salmopol: 75-year-old trees with 50–85% crown canopy). The upland plot Zwierzyniec (50°36′ N 22°58′ E; 240 m a.s.l.) was located at the area of Roztocze National Park and was represented by a 70-year-old moist mixed forest consisting of Norway spruce, Scots pine, silver fir, and some European beech or black alder trees in the neighborhood. The tree canopy ranged between 20 and 75%. The lowland plot Mirachowo (54°24′ N 18°02′ E; 150 m a.s.l.) was situated at the Kaszuby Protected Landscape Area and was represented by 66–100-year-old Norway spruce and Scots pine coniferous forest with up to 75% crown canopy.

In the '70s and '80s of the last century, the mountainous regions of Brenna and Salmopol were influenced by elevated levels of air pollution by SO_2, NO_x, and heavy

metals. However, the concentration of pollutants has gradually declined since the end of the '80s [24]. Zwierzyniec belongs to forest sites moderately influenced by SO_2 and NO_x pollutants, and Mirachowo is an environment free of direct pollutants [23,25].

Sampling procedure

Samples of the external mycelium of ectomycorrhizal fungi were obtained from the soil using the in-growth mesh-bag method described by Wallander et al. [5]. The mesh-bags of size $2 \times 5 \times 10$ cm were made of fine nylon mesh with a pore size of 50 μm (Sefar Nitex, Switzerland), which allows for fungal mycelium in-growth without the roots. The mesh-bags were filled with 120 g of quartz sand. The sand was acid washed (0.1N HNO_3) and sterilized prior to use to match the pH of the sand to the pH values of soil in the forest study sites. The in-growth mesh-bags were horizontally placed in the soil under canopies of Norway spruce at different depths (from 5 to 60 cm). To minimize disturbance of soil structure, the holes for the mesh-bags were bored with a soil corer.

The sand-filled mesh-bags were intended to capture mainly the external ectomycorrhizal hyphae connected to ectomycorrhizas and receiving carbohydrates from plants, as quartz sand is theoretically too poor environment to be colonized by saprotrophic fungi [5]. At each study site, a PCV tube (Ø16 cm, length 70 cm) was forced down into the soil to isolate the soil from the roots. Mesh-bags placed at different depths inside this tube were the controls for mesh-bags located directly in the soil in the vicinity of the roots. Harvested mesh-bags were placed in plastic bags and stored at a temperature of −20°C until analysis.

The mesh-bags placed inside control tubes revealed the presence of negligible amounts of ergosterol, which was taken into account in the calculation of the biomass of EMM in non-trenched mesh-bags.

The sand-filled mesh-bags were buried in the soil in two separate experiments. The first one was started in the autumn (October), and samples were harvested after 12 months (the next October). The in-growth mesh-bags were placed at four depths at each site: 5, 15, 30 and 45 cm (Brenna and Salmopol) or 60 cm (Zwierzyniec and Mirachowo). Because of thin and rocky soil layers at the mountain sites, the range of depths had to be reduced to 45 cm.

The second experiment began in June of the next year and was finished after four months (October). In this experiment, the in-growth mesh-bags were placed in soil only at depths of 5 and 15 cm. On the basis of experience obtained from the first experiment, the range of soil depths analyzed was reduced to the upper soil layers accessible at each of the study sites. Also, the time of the mesh-bags' incubation in the soil was reduced to the second half of the growing season determined by Wallander et al. [5] and Nilsson and Wallander [15] as the time of highest production of external ectomycorrhizal mycelium.

Ergosterol analysis

Samples of sand from the mesh-bags were observed under a stereomicroscope to control the presence of fungal mycelium, carefully mixed and freeze-dried prior to analysis of the total ergosterol content. The extraction (5 g dry weight) was performed using the MAE (microwave-assisted extraction) procedure described by Montgomery et al. [9]. Extracts dissolved in methanol were separated and quantified by a HPLC (high-performance liquid chromatography) device (Waters) fitted with a stainless steel Nova-Pack C18 3.9×150 mm (WAT 086344) and an absorbance detector (Dual Wavelength Absorbance Detector Waters 2487).

The HPLC was monitored with Waters Millennium32 software. Ergosterol was eluted for 10 minutes with 100% methanol at a flow rate of 1 mL min^{-1} and detected at 280 nm wavelength. The ergosterol peak was identified by comparing the retention time of the sample with the external standard synthetic 5,7,22-ergostatrien-3β-ol (provitamin D2, Sigma) and by coinjection of a sample and the standard. Ergosterol content was determined by comparing the sample area peak with a model curve based

on a number of external standard solutions. For calculations of fungal biomass, we used the conversion factor of 250 µg of dry fungi biomass per 1 µg of ergosterol and a correction factor for average percent recovery [9].

Statistics

Data from the 12-month and 4-month experiments were analyzed separately. A two-way analysis of variance (ANOVA) was used to examine the level of significance ($P < 0.05$) of the site and depth factors and their interaction. In each of the experiments, the significance of differences between study sites and soil depths were tested using Tukey's test. Also, the results of both experiments (12 and 4 months) for two soil depths (5 and 15 cm) were compared to analyze the effect of incubation time and site and soil depth overall (three-way ANOVA). Statistical analysis was performed using Statistica 9.0 (StatSoft) software.

Results

12-month experiment

The analysis of variance (ANOVA) revealed a significant impact of soil depth and study site on the ergosterol concentration in the mesh-bags kept in soil for 12 months (Tab. 1). At each of the study sites, a decrease of ergosterol concentration with soil depth was found. At the lowland/upland sites (Mirachowo and Zwierzyniec), the concentration of ergosterol in the mesh-bags decreased rapidly under a soil depth of 5 cm, whereas at the mountain sites (Brenna and Salmopol), the decreasing of the ergosterol content was more gradual (Fig. 1). The highest ergosterol concentrations were recorded at Mirachowo and Zwierzyniec at a depth of 5 cm (5.56 and 4.42 µg g^{-1} d.w. of sand, respectively, what is the equivalent of fungal biomass (*FB*) 2.24 and 1.78 mg g^{-1}). At Salmopol, the concentration of ergosterol decreased only slightly with the depth and presented relatively higher values in the deeper soil layers in comparison to other study sites. The lowest ergosterol content was found at Brenna (1.19–0.22 µg g^{-1} d.w. of sand; *FB* = 0.48–0.09 mg g^{-1}; Fig. 1).

4-month experiment

The analysis of variance (ANOVA) in the 4-month experiment showed a significant influence of the study sites and their interaction with soil depth on the ergosterol content in the mesh-bags. However, no significant effects for soil depth only were recorded (Tab. 1). The sites in Mirachowo and Zwierzyniec presented significantly higher ergosterol concentrations at a soil depth of 5 cm (6.86 and 6.0 µg g^{-1} d.w. of sand, respectively; *FB* = 2.76 and 2.42 mg g^{-1}) than the mountain sites Brenna and Salmopol (2.27 and 3.44 µg g^{-1} d.w. of sand; *FB* = 0.91 and 1.39 mg g^{-1}; Fig. 1). At a level of 15 cm, the mountain sites also revealed a reduced ergosterol concentration (2.18 and 4.05 µg g^{-1} d.w. of sand, respectively; *FB* = 0.88 and 1.63 mg g^{-1}) compared to the upland and lowland sites (7.23 and 4.64 µg g^{-1} d.w. of sand; *FB* = 2.91 and 1.87 mg g^{-1}). However, a significant difference was found only between Brenna and Zwierzyniec (Fig. 1).

Taking into account the soil depths of 5 and 15 cm in both experiments (12 and 4 months), the content of the EMM in the mesh-bags, estimated based on ergosterol content, was significantly influenced by soil depth as well as by interaction between the

Tab. 1 Results of ANOVA to evaluate the influence of site and soil depth on biomass of external mycelium of ectomycorrhizal fungi and mean values for 12- and 4-months experiments.

Factor	12-months experiment		4-months experiment	
	F	*P*	*F*	*P*
Site	8.02	0.0002*	64.56	0.0000*
Depth	10.37	0.0002*	0.07	0.7882
Site × depth	1.98	0.0871	10.18	0.0000*

* ANOVA significant effect.

12-months experiment
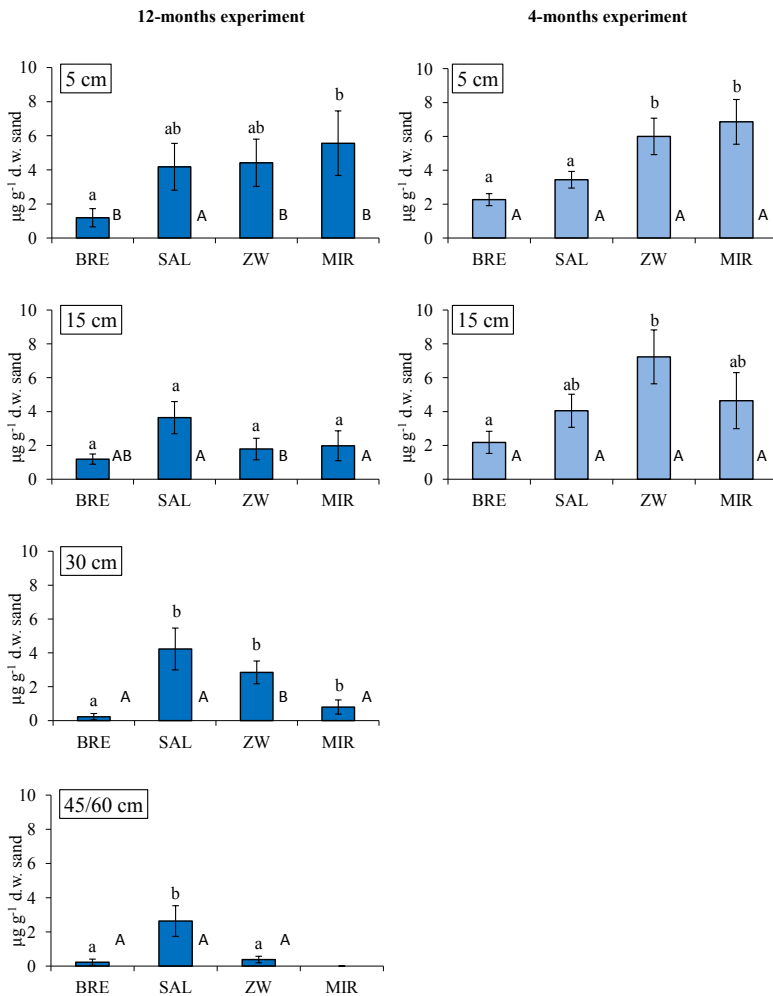
4-months experiment

Fig. 1 The concentration of ergosterol in mesh bags at four study sites (BRE – Brenna; SAL – Salmopol; ZW – Zwierzyniec; MIR – Mirachowo) and at four soil depths (left: 12-months experiment) and at two soil depths (right: 4-months experiment). Lower-case letters a–b indicate significant differences between sites at separate soil depths; Upper-case letters A–C indicate significant differences between soil depths at separate study sites ($P < 0.05$, Tukey's test).

Tab. 2 Results of ANOVA testing the influence of the incubation time of in-growth mesh bags in soil (12- and 4-months experiment), site and soil depth (5 and 15 cm) on biomass of external mycelium of ectomycorrhizal fungi.

Factor	F	P
Time	3.04	0.0853
Site	0.77	0.5126
Depth	4.57	0.0355*
Time × site	5.32	0.0021*
Time × depth	0.17	0.6817
Site × depth	0.61	0.6083
Time × site × depth	1.09	0.3596

* Significant effect.

incubation time of the mesh-bags in soil and the study site (Tab. 2).

Discussion

In the present study, extramatrical mycorrhizal mycelium (EMM) was detected in all but one of the soil depths of the forest sites studied. Significant differences in the EMM biomass, estimated from the ergosterol concentration between the four study sites were recorded, regardless the period of incubation of the mesh-bags in the soil (12 or 4 months). The results revealed also a significant role of soil depth in the EMM biomass production in the mesh-bags. In our study the highest concentrations of ergosterol were recorded at the upland site Zwierzyniec (up to 7.23 µg g^{-1} dry weight of sand), and the lowest concentrations were found at the mountainous site Brenna (ranged between 0.22 and 2.27 µg g^{-1} dry weight of sand, depending on the soil depth and the period of incubation of the mesh-bags in soil). A similar range of ergosterol concentrations in mesh-bags located in the soil interface between organic and mineral layers in the Norway spruce forests in southern Sweden was reported by Hageberg et al. [26], who found a large variation in concentration of ergosterol (0.63–1.30 µg g^{-1}) between different years. In general concentrations of ergosterol in mesh-bags located in soils of various Scandinavian forests represented lower values (within the range from 0.05 µg g^{-1} [16,19] to 0.4 µg g^{-1} [27]) than found in our studies. Concentration of ergosterol and resulting fungal biomass can be affected by different factors like heterogenous soil substrates, environmental biotic and abiotic conditions and efficiency of used analytical methods etc. In our case the variance between ergosterol concentration measured in the present study and the results of Scandinavian researchers may arise due to variable methods of ergosterol extraction [21,28]. We used the method of Montgomery et al. [9], that has been shown to be 2.6 to 8.8 times more efficient than a classical refluxing saponification method. Additionally recovery of this method was independent of soil properties [9]. More research are needed for better understanding factors determining EMM biomass production and surviving in soil in different forest types and regions. The production and turnover of ectomycorrhizal mycelium in soil is regulated, at least in part, by the availability of carbohydrates produced and delivered to mycorrhizas and EMM by the aboveground half of the tree. Any factor that can alter carbohydrate allocation to roots has a potential to influence EMM biomass. The growing season, longer in Poland than in Scandinavia, can promote a higher biomass of EMM than can Scandinavian forest sites. Similarly, the lower EMM biomass on

the mountain than in the lowland/upland sites can be partly related to the shorter growing season and specific mountain climate in the Silesian Beskid Mountains when compared to upland (Zwierzyniec) and lowland (Mirachowo) ecosystems [29]. Another possible explanation of the lower EMM biomass at the mountainous site Brenna can be a lower, than in the other study sites, density of trees (15–25% crown canopy) due to anthropogenic factors (elevated levels of air pollution by SO_2, NO_x and heavy metals) significantly influencing the Silesian Beskid Mountains in the second half of the 20th century [30,31]. Negative effects of these antropogenic factors on development of EMM biomass cannot be excluded (e.g., [32]).

The EMM in northern latitudes and higher altitudes is exposed for a long period to soil freezing, which is a direct factor influencing the mycelium decline [18]. In the present study data from the upper 5 cm and 15 cm soil layers present a slightly higher content of fungal mycelium in the mesh-bags after 4 months of incubation than after 12 months. Probably the incubation of the mesh-bags during the winter caused mycelium degradation due to winter froze. This result is in agreement with the findings of Wallander et al. [5], who investigated the influence of season on colonization of mesh-bags by fungal mycelium and found the highest values in autumn, which corresponded with an increasing abundance of ectomycorrhizas during that period of the year. The results suggest, that instead of the relatively long, 12-months incubation of the mesh-bags in the soil, the better time for determining EMM production in forests is shorter summer–autumn incubation period when the productivity of mycelium is most abundant and fast.

Lower concentration of EMM biomass in mesh-bags after 12 months of incubation could be explained by the limited longevity of hyphae as well as by temperature and soil moisture in the years of the experiments [18].

EMM biomass production is also largely influenced by the community structure of ectomycorrhizal fungi associated with the forest trees [18], as different fungal species produce variable amounts of extramatrical mycelium, which builds a mycelial network in the soil and may have variable tolerance to natural and anthropogenic stress factors. The ECM fungi composition can be determined by geographic location [33], local site conditions [34], and soil depth [35,36]. Our previous studies describing community structure [23], completed in 2006 on these same four forest sites revealed that the upland site Zwierzyniec and the lowland site Mirachowo had a higher percentage of ECM types with abundant extramatrical mycelium (medium- and long-distance exploration types, according to Agerer [37]) than the mountainious sites (Brenna and Salmopol). The inconsistency in ECM communities could be one reason of differences in EMM between the study sites. Moreover, fungal species and strains have been shown to differ in ergosterol concentration [8,9,38]. This inter- and intraspecific variability as well as variable ergosterol content during stationary and expotential phases of growth [9] can affect large differences in ergosterol content between forest sites and between soil depths.

The biomass of EMM might also be attributable to the development of the fine root system and ectomycorrhizas in the soil profile, generally more abundant in the topsoil layers containing humus than in underlying layers of mineral soil [39]. For instance, Schmid and Kazda [40] observed a higher concentration of roots of Norway spruce in the upper soil layers of mixed beech-spruce forest in comparison to a homogenous spruce forest. As well, Wallander et al. [22] revealed a higher concentration of EMM in the top soil in mixed than in homogenous spruce stands.

In literature, a common approach is to estimate EMM biomass production per hectare. In the upper 10 cm layer of soil, the EMM biomass in both our (4 and 12-months) experiments varied in range: for Brenna, 576–1095 kg ha^{-1}; for Salmopol, 1663–2018 kg ha^{-1}; for Zwierzyniec, 2133–2899 kg ha^{-1}; and for Mirachowo, 2688–3314 kg ha^{-1}. The content of the extramatrical mycelium of ectomycorrhizal fungi in Scots pine forests of northern Sweden was estimated to be 32% of the soil microorganisms' biomass [41], which was the equivalent of 145 kg ha^{-1}. In southern Sweden, the biomass of external mycelium was calculated to be in the range of 700–900 kg ha^{-1} for a 35-year-old Norway spruce forest [5]. The comparison of our data (even if the range is higher) and the results from Sweden suggest a longitudinal increase of EMM biomass in soil from the north to the south. But this and other factors regulating EMM biomass production in European forests awaits further research.

Conclusions

The results indicated significant differences in EMM production and their distribution according to the soil depth, between mountain forests and the forests located in lowlands and uplands. The EMM biomass relates to the distribution of the roots of trees in the soil profile and the abundance of ectomycorrhizas colonizing roots of Norway spruce. The time of incubation of mesh-bags needed to establish an EMM biomass in soil may be limited to the period of summer–autumn, when the production of EMM is highest. Further research efforts in different forest types and regions (especially in Central and Southern Europe) are needed to better characterize EMM biomass production and the factors determining its concentration in soil.

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