Ectomycorrhizal mats alter forest soil biogeochemistry

Laurel A. Kluber a,*, Kathryn M. Tinnesand b, Bruce A. Caldwell c, Susie M. Dunham d, Rockie R. Yarwood a, Peter J. Bottomley a, b, David D. Myrold a

a Department of Crop and Soil Science, Ag and Life Science Bldg, Oregon State University, Corvallis, OR 97331, USA
b Department of Microbiology, Nash Hall, Oregon State University, Corvallis, OR 97331, USA
c Department of Botany and Plant Pathology, Cordley Hall, Oregon State University, Corvallis, OR 97331, USA
d Department of Forest Ecosystems and Society, Richardson Hall 321, Corvallis, OR 97331, USA

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Dense hyphal mats formed by ectomycorrhizal (EcM) fungi are prominent features in Douglas-fir forest ecosystems, and have been estimated to cover up to 40% of the soil surface in some forest stands. Two morphotypes of EcM mats have been previously described: rhizomorphic mats, which have thick hyphal rhizomorphs and are found primarily in the organic horizon, and hydrophobic mats, which occur in the mineral horizon and have an ashy appearance. This study surveyed EcM mat and non-mat soils from eight early and late seral conifer forest stands at the H.J. Andrews Experimental Forest in western Oregon. EcM mats were classified by morphology and taxonomic identities were determined by DNA sequencing. A variety of chemical and biochemical properties, including enzymes involved in C, N, and P cycling were measured. Analysis was confined to a comparison of rhizomorphic mats colonizing the organic horizon with non-mat organic soils, and hydrophobic mats with non-mat mineral soils. Both the organic and mineral horizons showed differences between mat and non-mat enzyme profiles when compared on a dry weight basis. In the organic horizon, rhizomorphic mats had greater chitinase activity than non-mat soils; and in the mineral horizon, hydrophobic mats had increased chitinase, phosphatase, and phenoloxidase activity compared to the non-mat soil. The rhizomorphic mats had 2.7 times more oxalate than the non-mats and significantly lower pH. In the mineral horizon, hydrophobic mats had 40 times more oxalate and significantly lower pH than non-mat mineral soils. Microbial biomass C was not significantly different between the rhizomorphic mat and non-mat organic soils. In the mineral horizon, however, the hydrophobic mats had greater microbial biomass C than the non-mat soils. These data demonstrate that soils densely colonized by EcM fungi create a unique soil environment with distinct microbial activities when compared to non-mat forest soils.

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1. Introduction

In exchange for photosynthate, mycorrhizal fungi provide their host plant with a range of benefits (Smith and Read, 2008), including increased nutrient uptake. Although forest ecosystems have an abundance of organic matter, nutrients (e.g., N and P) are often in complex organic forms that are unavailable for direct plant uptake. Ectomycorrhizal (EcM) fungi can access these otherwise recalcitrant pools and provide their host plant with nutrients (Allen et al., 2003). Various EcM fungi have been found to form dense aggregations of hyphae known as mats (Griffiths et al., 1990; Agerer, 2001), which are prominent features in Douglas-fir forest ecosystems and have been found to cover as much as 25–40% of the forest floor in a given stand (Cromack et al., 1979; Griffiths et al., 1996; Phillips, 2009).

There are several ways that soils colonized with EcM mats differ from non-mat forest soils. Not only can the fungal rhizomorphs account for up to half the dry weight of the mat-associated soil (Ingham et al., 1991), but mats typically have higher microbial biomass and organic matter content, and high levels of oxalate, which likely contributes to their lower pH (Malajczuk and Cromack, 1982; Griffiths et al., 1994). Previous studies in the Pacific Northwest have demonstrated the prevalence of EcM mats in Douglas-fir forests (Cromack et al., 1979; Griffiths et al., 1996) and showed their ability to have increased enzymatic activities and litter decay rates (Entry et al., 1991; Griffiths and Caldwell, 1992), accelerate mineral weathering (Cromack et al., 1979; Griffiths et al., 1994), provide habitat for soil animals (Cromack et al., 1988), and possibly enhance seedling survival (Griffiths et al., 1991b). In this earlier work, mat identification
was limited to morphological descriptions and sporocarp identifications. EcM mats with rhizomorphic growth habits were often thought to be a *Hysterangium* species, whereas mats with an ashy, hydrophobic appearance were thought to be formed by *Gautieria* species (Cromack et al., 1979; Griffiths et al., 1996). More recently, a survey was done to determine the phylogenetic diversity of EcM mat-forming fungi in soils of Douglas-fir stands at the H.J. Andrews Experimental Forest (Dunham et al., 2007). By sequencing the fungal ITS region of EcM rhizomorphs and root tips, they found the diversity of mat-forming EcM fungi to be much greater than believed in the past. Previous studies in Douglas-fir forests focused on EcM mats in the mineral horizons; however, Dunham et al. (personal communication) found horizon-specific growth habits among the EcM mat-forming taxa and morphotypes. Although the majority of rhizomorphic mats are found only in the organic horizon, several taxa form rhizomorphic mats that colonize both the organic and mineral horizon. Hydrophobic mats, on the other hand, were found to colonize only the mineral horizon.

There has been increasing attention focused on the ability of ectomycorrhizal fungi to decompose organic material, enabling them to acquire N and P (Read and Perez-Moreno, 2003; Courty et al., 2010). Production of enzymes involved in nutrient cycling has been demonstrated for isolates of EcM fungi (Hutchison, 1990). EcM root tips (Pritsch et al., 2004; Buée et al., 2005; Courty et al., 2007; Collings et al., 2008), and EcM mats (Griffiths and Caldwell, 1992). Because of their ability to dominate soils locally, EcM mats have been hypothesized to be important players in forest nutrient cycling, and present a unique opportunity to examine the activities and properties associated with EcM hyphae in the soil. The survey of chemical and biochemical properties of EcM mat and non-mat soils presented herein provides an updated account of EcM mat properties and activities while accounting for the growth habits and taxonomy of EcM mat-forming fungi. Key extracellular enzymes involved in the C, N, and P cycles were analyzed in conjunction with soil chemical properties in EcM mat and non-mat samples from sites of varying stand ages throughout the H.J. Andrews Experimental Forest. The goals of this study were to: (1) determine whether the enzymatic activities and soil properties differ between EcM mat and non-mat soils, and (2) assess whether the taxonomic identity or morphotype of the mat-forming fungi corresponds to unique soil activities or properties.

2. Materials and methods

2.1. Site description

The study was conducted at the H.J. Andrews Experimental Forest located in the Western Cascade Mountains of Oregon (44° 13′ 25″N, 122° 15′ 30″W). Four old-growth and four second-growth stands dominated by Douglas-fir (*Pseudotsuga menziesii*) were selected to assess the diversity of EcM mats found at the HJA. A full description of the study location and soil properties can be found in Chaer et al. (2009).

2.2. Sampling

Eight sites (Chaer et al., 2009) were sampled, beginning on 22 June 2005 continuing through July, with one old-growth and 1 s-growth site sampled every other week. At each site, several people spread out over a 60 × 60 m area and gently raked away patches of the upper layers of moss, litter, and soils to expose the organic or mineral horizon. Four fungal mats were sampled from the organic horizon and four fungal mats were sampled from the mineral horizon. For the purposes of this study, fungal mats were defined as areas of densely aggregated fungal hyphae or rhizomorphs that covered an area with a minimum diameter of 20 cm, and non-mat soils are defined as patches of soil that were not heavily colonized with fungal hyphae or rhizomorphs. All mats were destructively sampled with the entire mat removed for analysis. Four organic and four mineral horizon samples were also taken from non-mat areas. Descriptive information on mat morphology, colonization, and horizon depth was recorded. All samples were transported on ice, sieved (4 mm for organic and 2 mm for mineral horizons) and stored at 4 °C until analysis (less than one week).

2.3. Molecular characterization of mats

Fungal mat samples were examined under a dissecting microscope to determine whether they possessed the characteristics necessary to be considered an EcM mat (Dunham et al., 2007; and personal communication). By examining the mats and EcM root tips under 10–40× magnification, we were able to determine whether the mat-forming rhizomorphs and hyphal material originated from the EcM roots found in the mat. If so, EcM root tip and rhizomorph material was selected for separate DNA extractions. If no EcM root tips were present in the fungal mats, the rhizomorph material alone was used for molecular identification of the mat-forming fungi. Selected root tips and mat-forming rhizomorphs were washed repeatedly with distilled water, then genomic DNA was extracted and the ITS region was amplified from each sample using the methods of Dunham et al. (2007). PCR products were purified using a QIAquick PCR purification Kit (Qiagen, Chatsworth, CA) and sequenced using direct dye-terminated, automated fluorescence methods performed at Oregon State University’s Center for Genome Research and Biocomputing, using an ABI Prism 3730 genetic analyzer (Foster City, CA). Sequences were viewed, cleaned, and aligned using Bioedit (Hall, 1999) and taxons were assigned according to the best BLAST matches from the GenomeBank database using the Entrez query option of “all [filter] NOT uncultured” to reduce the number of hits from poorly identified uncultured clones. Although we were able to identify some EcM mats to the species level, mats were grouped at the genus level for statistical analysis.

2.4. Soil enzyme assays

Standard assay procedures, modified to work with 1-ml soil slurries (Chaer et al., 2009), were used to measure: phenoloxidase (phenoloxidase) using t-DOPA (Sinsabaugh et al., 1999), β-glucosidase, N-acetyl-β-D-glucosaminidase (chitinase), and phosphatase using p-nitrophenol derivatives (Caldwell et al., 1999; Parham and Deng, 2000). Protease activity was determined by measuring casein degradation after a 24-h incubation with 1% sodium azide using the Folin–Ciocalteu phenol reagent method reported by Ladd and Butler (1972). Slurries for all enzyme assays were prepared by adding 60 ml deionized water to 6 g sieved soil in a 150-ml beaker. The mixture was vigorously mixed using a magnetic stir plate until the samples were homogeneously suspended in solution. While mixing continued, 1 ml of slurry was removed with a large diameter pipette tip and placed in a 15 × 85 mm glass test tube. Three aliquots were removed per soil sample for each enzyme assay, allowing for a control and two laboratory replicates. Samples used in the protease assay were covered and frozen until analysis, while the other assays were performed immediately. Five, 1-ml aliquots from each sample were dried overnight to determine the dry weight of soil in each 1 ml of slurry.

2.5. Soil analyses

Gravimetric water content was determined by drying the soils for 90 h at 54 °C. Soil pH was measured with a pH meter after 30 ml
deionized water was mixed with 10 g field moist soil and allowed to equilibrate for 60 min. Oxalate content of an acid extract was measured by ion chromatography using a modified method of Griffiths et al. (1994): 10 g of soil was extracted with 20 ml of 30 mM HCl and centrifuged, the supernatant was transferred to a fresh tube, centrifuged, filtered (0.2 μm), and diluted in deionized water for analysis on a Dionex Ion Chromatograph 2000 (Sunnyvale, CA). Soil organic matter (SOM) was determined by loss on ignition (430 °C for 24 h) of oven-dry soil. Inorganic N was measured by extracting 5 g soil with 15 ml 2M KCl and analyzing the filtrate for NH₄⁺ and NO₃⁻ on an Astoria-Pacific 300 series autoanalyzer (Portland, OR).

2.6. Microbial biomass C

Microbial biomass C (MBC) was measured by chloroform fumigation-extraction (Vance et al., 1987). Soil samples were fumigated for 24 h under chloroform vapor, extracted using 30 ml 0.05 M K₂SO₄, and total C was measured by combustion. Control soil samples were extracted and analyzed without exposure to chloroform.

2.7. Statistical analyses

Separate multiple analysis of variance (MANOVA) analyses were used to determine effects of mat presence on soil enzymes (phenoloxidase, β-glucosidase, chitinase, phosphatase, and protease) for each EcM mat morphotype and its corresponding non-mat horizon (hydrophobic mat vs. mineral non-mat; and rhizomorphic mat vs. organic non-mat). The soil properties (SOM, MBC, NH₄⁺, NO₃⁻, oxalate, water content, and pH) were tested using individual ANOVA analysis. All MANOVA and ANOVA models were blocked by site to account for variation among sites and to avoid any potential bias caused by spatial variation or sampling date. All MANOVA and ANOVA analyses were performed using SAS 9.1 (SAS Institute, Inc., Cary, N.C.). Variables were log transformed as necessary to meet the normality assumptions of the statistical procedures. Therefore 95% confidence intervals, rather than standard errors were reported with the means allowing for consistency between back transformed and untransformed means. Results were considered significant at \( p \leq 0.05 \), with significant trends considered to be \( p \leq 0.1 \).

Non-metric multidimensional scaling (NMS) using PC-ORD Software (MJM Software Design, Gleneden Beach, OR) was carried out to further assess differences in enzyme activities per g dry soil. Two “species” matrices were constructed, using the enzyme activity per g of soil; one with the organic horizon enzyme activities, and the other with mineral horizon enzyme activities. Environmental matrices were constructed to account for the site, sampling date, sample type (mat/non-mat), genus of mat-forming fungi, and morphotype, as well as the chemical properties of each soil sample. The enzyme data columns were relativized by the standard deviate to normalize the scale of the different enzyme activities. The Euclidean distance measure was chosen to calculate distance matrices to account for the negative values that resulted from relativization. Multi-response permutation procedures (MRPP) were used to test for differences between enzyme activity profiles of EcM mat and non-mat soils as well as among the genera of mat-forming fungi for each horizon. MRPP is a nonparametric procedure to test the hypothesis of no difference between groups and yields an A-statistic that describes the chance-corrected within-group agreement, and a p-value to evaluate how likely the observed difference is due to chance (McCune and Grace, 2002). A-statistic values range from zero to one, with higher values indicating greater within-group homogeneity, an \( A \geq 0.1 \) is considered strong grouping but values <0.1 are commonly observed in environmental data sets (McCune and Grace, 2002).

3. Results

3.1. Taxonomic identities of EcM mats

Visual inspection of EcM mat samples with a dissection microscope combined with the molecular characterization revealed that not all fungal mats samples collected met appropriate criteria to be considered an EcM mat for this study (insufficient hyphal density, necrotic hyphal material, absence of EcM root tips, or rhizomorphs were not from known EcM taxa). These samples were excluded from subsequent analysis, resulting in a total of 27 rhizomorphic mats from the organic horizon and 11 hydrophobic mats from the mineral horizon to be used for statistical analysis. Taxonomic designations were determined for 17 rhizomorphic and 5 hydrophobic EcM mats (Table 1) and their DNA sequences were deposited in GenBank under accession numbers HM234133–HM234154. The additional 10 rhizomorphic and 6 hydrophobic mats listed in Table 1 exhibited all the proper characteristics of an EcM mat, yet the sample resulted in a poor sequencing read due to mixed templates, humic contamination, or low DNA concentration.

3.2. Organic horizon

The blocked MANOVA for the combined enzyme activities expressed per g soil showed a significant difference between the enzyme profiles of organic horizon soils colonized with rhizomorphic mats compared to the non-mat organic soils (\( p = 0.007 \)). There was a consistent overall trend for mat enzyme activities to be greater than non-mat activities; however, chitinase was the only enzyme to have significantly greater activity in the mats, exhibiting 1.7 times the activity of non-mat organic soil (\( p = 0.007 \)). The other four enzymes showed no significant differences between rhizomorphic mats and non-mat organic soils (Fig. 1). When the enzyme activities were normalized to MBC (Fig. 1), the significance of the results remained (MANOVA \( p = 0.003 \)). Oxalate and pH were the only chemical properties that differed significantly between the rhizomorphic mats and the corresponding non-mat soils. Oxalate concentrations were 2.7 times greater in the mats while pH values were lower (Table 2).

MRPP results from the organic horizon enzyme activities per g soil agreed with the MANOVA results and confirmed differing enzyme activity profiles for rhizomorphic mat and non-mat samples (\( p = 0.01 \), but the effect size was low (\( A = 0.02 \)), possibly because of high spatial/temporal variability. The NMS ordination of organic horizon enzyme activities (Fig. 2) visually reveals the separation of mats from each other and their correspondents non-mat samples.
activities per g soil showed a significant difference in the enzyme profiles of the two sample types \( (p = 0.03) \). Hydrophobic mats expressed more than twice the chitinase \( (p = 0.04) \), phosphatase \( (p = 0.01) \), and phenoloxidase \( (p = 0.02) \) activity than the non-mat mineral soils \( (\text{Fig. 1}) \). Additionally, there was a consistent trend \( (p < 0.1) \) showing that nearly all the chemical soil properties differed between the two sample types \( (\text{Table 2}) \). Hydrophobic mats had strikingly higher oxalate content \( (40 \text{ times greater than the mineral non-mat soils}) \), higher NH\(_4\), and lower pH than non-mat mineral soils. SOM and MBC also differed, with hydrophobic mats tending to have greater SOM and MBC \( (p < 0.01) \). The enzyme profiles of hydrophobic mats were not significantly different than those for non-mat mineral soil \( (\text{MANOVA} \ p = 0.44) \) after normalizing for MBC \( (\text{Fig. 1}) \).

NMS ordination of the mineral horizon enzyme activities per g soil \( (\text{Fig. 3}) \) showed a significant separation between hydrophobic mat and non-mat soils, which was confirmed with the MRPP analysis \( (A = 0.12, p < 0.01) \). However, there was not a significant difference among the genera that form hydrophobic mats \( (A = -0.05, p = 0.77) \). Chitinase, \( \beta\)-glucosidase, and phenoloxidase activities are all strongly correlated with axis 1 \( (r = 0.84, 0.80, \text{and} 0.78 \text{respectively}) \), probably contributing to the separation of the mats from the non-mat soils seen on that axis.

4. Discussion

Ectomycorrhizal mats have been described as distinctive \( (\text{Griffiths and Caldwell, 1992}) \); however, we found that determining EcM status and distinguishing morphotypes of fungal mats was difficult in the field. Inspection under a dissecting microscope aided greatly in determining the status of fungal mats, by allowing us to visually determine whether the mat-forming hyphae and rhizomorphs emanated from an EcM root tip. Several of the fungal mats sampled had no obviously associated EcM roots and may have been formed by saprotrophic fungi. A number of variations of EcM mat growth habits were found with our initial sampling efforts, including “mixed mats” (presence of EcM tips and several colonizing EcM fungi), rhizomorphic mats directly above hydrophobic mats, and rhizomorphic mats that colonized both the O and A horizons. Furthermore, some taxa of EcM mats were present at only one or two sites. These variations of EcM mat morphotype and phylotype distributions can complicate sampling and limit data analysis if not properly considered. To overcome these limitations, our study focused on examining the dominant EcM mat morphologies: rhizomorphic mats colonizing the organic horizon, and hydrophobic mats colonizing the mineral horizon.

The phylogenetic diversity of our samples was greater than described in the early EcM mat studies of Douglas-fir forests undertaken before molecular analysis was widely available \( (\text{Cromack et al., 1979; Griffiths et al., 1991}) \), and better compares with the more recent survey by Dunham et al. \( (2007) \). Nearly all of the EcM mats we identified were comprised of genera matching those found by Dunham et al. \( (2007) \) in this same ecosystem. It is not surprising that exceptions were found \( (\text{Tricholomus sp. and Russula queletii}) \), given that the species accumulation curves constructed by Dunham et al. \( (2007) \) indicated that their sampling efforts were insufficient to capture the total richness of EcM mat-formers. Phylootyping offers the significant advantage of identification without sporicarps. We recommend that EcM mats be identified prior to sampling to assure proper replication between sites for robust statistical analysis.

Previous studies on the enzymatic capabilities of EcM fungi have examined activities associated with isolated cultures \( (\text{Hutchison, 1990; Burke and Cairney, 2002}) \), or EcM root tips \( (\text{Conn and Dighton, 2000; Pritsch et al., 2004; Buée et al., 2005; Courty et al., 2007}) \); however, little is known about the enzyme activity
associated with the extra-matrical hyphae of EcM fungi. The densely colonized EcM mats, allow us to examine how EcM hyphae and their associated microbial communities influence the soil enzyme activity. Our study follows earlier EcM mat studies with a more comprehensive examination of C, N, and P cycling enzymatic capabilities combined with a variety of soil properties, while taking into account the horizon-specific growth habits and taxa of EcM mat-forming fungi. NMS and MRPP results from our study demonstrated that hydrophobic mats colonizing the mineral horizon exhibited similar enzyme profiles regardless of the identity of the mat-former. Rhizomorphic mats colonizing the organic horizon appeared to show variation between the taxa sampled, with a significant grouping of the Hysterangium mats. Although these results are notable, the confounding variables of uneven distributions of taxa among sites and the progressive sampling design make it difficult to determine whether the grouping is due to a Hysterangium-specific activity profile, or because all but two *Hysterangium* mats were found at the first two sites sampled in the spring (data not shown). When the MANOVA model was used to block by site and analyze the activity profiles by morphotype, we were able to account for spatial and temporal variation resulting in more robust and quantitative differences between the EcM mat and non-mat activity profiles for each horizon.

The high chitinase activity that was found in the rhizomorphic mats is consistent with the work of López-Gutiérrez at the Holden Arboretum (personal communication), who found soil associated with *Piloderma* root tips to have increased chitinase activity. The rhizomorphic mats formed by *Piloderma* species have been found to be prominent at the H.J. Andrews Experimental Forest (Dunham et al., 2007), and accounted for nearly a third of the rhizomorphic mat-forming taxa identified in our study. In the mineral horizon, the activities of chitinase, phenoloxidase, and phosphatase per g soil were significantly greater in the soils colonized by hydrophobic mats, which is consistent with previous work that showed elevated enzyme activities within hydrophobic mats formed thought to be *Gautieria monticola* (Griffiths and Caldwell, 1992), although protease activity showed no increase in our study.

One would be prudent to resist assuming that the enzyme activities measured in the EcM mat soils reflect the activity of the EcM fungi alone. It has been well documented that there are a variety of organisms that live in conjunction with mycorrhizal fungi, ranging from the classic “helper bacteria” (Garbaye, 1994) to mycophasogens such as *Leveau and Preston, 2008*. In reality, many of the microorganisms associated with mats probably lie between these extremes (Bending et al., 2002). Several studies have examined bacterial communities (Timonen et al., 1998; Burke et al., 2008) associated with EcM root tips, and Warmink et al. (2009) found distinct bacterial communities associated with mygalal mats formed under EcM fruiting bodies. Although our study did not examine the microbial community composition associated with EcM mats, we hypothesize that the communities associated with mat and non-mat soils are distinct and likely contribute to the differing enzyme activities.

Griffiths et al. (1991a) suggested that EcM mats perform different functions in mineral and organic forest soils. We were unable to make any direct comparisons between the organic and mineral horizon mats in our study; however, we did observe differing trends for each horizon. Our findings mirror earlier work.
that found significantly greater oxalate concentrations in ECM mats, with the mineral horizon hydrophobic mats having the greatest amount of oxalate (Cromack et al., 1979; Griffiths et al., 1994). The ability of some ECM to excrete oxalate has been well documented (Lapeyrie et al., 1987; Rineau et al., 2008; Tuason and Arocena, 2009). It has been hypothesized that the abundance of oxalate in the mineral horizon mats is produced as a mechanism to acquire P through mineral weathering (Griffiths et al., 1994), similar to what has been described by other ECM fungi (Jongmans et al., 1997). Rhizomorphic mats had only slightly elevated concentrations of oxalate, perhaps because they are not in direct contact with weatherable minerals.

The enzymatic profiles of ECM mats were significantly different from their non-mat counterparts when analyzed on a dry weight basis. When enzyme activity was normalized by MBC, rhizomorphic mat activity remained significantly different than non-mat organic soils; however, the enzyme profiles of the hydrophobic mats were no longer significantly different than the non-mat mineral horizon. This suggests that the increased enzyme activity of hydrophobic mats could simply be due to the increased microbial biomass, whereas the rhizomorphic mat microbial community has distinct activities compared to the organic non-mat communities. In both horizons, we see that on a dry weight, or “per-area” basis, the ECM mats have greater enzyme activities than non-mats. Given that mats can cover 25–40% of the forest floor in some stands (Cromack et al., 1979; Phillips, 2009) ECM mats may have a significant influence on the overall nutrient cycling of forest soils.

Despite the expansive body of knowledge on ECM fungi, little is known about the activities and communities associated with their mycelial systems in forest soils (Anderson and Cairney, 2007). One approach to study the extra-matrical hyphae of ECM fungi is to examine aggregations of hyphae that form under fugitive bodies, as done by Warmink et al. (2009). However, because fugitive bodies generally are seasonal and temporary structures, this approach has limited utility for examining temporal dynamics of ECM activity. ECM mats offer an opportunity to examine the concentrated effects of ECM hyphae and their associated microbial communities, with the distinct advantage of having a soil that is continually occupied by the ECM fungus. The results presented herein demonstrate that ECM mats create unique soil conditions with distinct microbial activities in contrast to non-mat forest soils. This work furthers our understanding of the influence of ECM fungi on the soil environment, and lays the groundwork for a variety of future studies on ECM mat activities, communities, and temporal dynamics that will result in a better understanding of the role of ECM fungi in the soil environment.

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