

Sharing rotting wood in the shade: ectomycorrhizal communities of co-occurring birch and hemlock seedlings

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Abstract Coarse woody debris (CWD) is an important nursery environment for many tree species. Understanding the communities of ectomycorrhizal fungi (ECMF) and the effect of ECMF species on tree seedling condition in CWD will elucidate the potential for ECMF-mediated effects on seedling dynamics. In hemlock-dominated stands, we characterized ECMF communities associated with eastern hemlock (*Tsuga canadensis* (L.) Carr) and yellow birch (*Betula alleghaniensis* Britt) seedling pairs growing on CWD. Seedling foliage and CWD were analyzed chemically, and seedling growth, canopy cover, and canopy species determined. Thirteen fungal taxa, 12 associated with birch, and 6 with hemlock, were identified based on morphology and ITS sequencing. Five species were shared by co-occurring birch and hemlock, representing 75 % of ectomycorrhizal root tips. Rarified ECMF taxon richness per seedling was higher on birch than hemlock. Nonmetric multidimensional scaling revealed significant correlations between ordination axes, the mutually exclusive ECMF *Tomentella* and *Lactarius* spp., foliar N and K, CWD pH, and exchangeable Ca and Mg. Seedlings colonized by *Lactarius* and *T. subulilacina* differed significantly in foliar K and N, and CWD differed in exchangeable Ca and Mg. CWD pH and nutrient concentrations were low but foliar macro-nutrient concentrations were not. We hypothesize that the

dominant ECMF are adapted to low root carbohydrate availability typical in shaded environments but differ in their relative supply of different nutrients.

Keywords Ectomycorrhizal fungi · Seedlings · Coarse woody debris · Foliar nutrients · Multivariate analysis · *Tsuga canadensis* · *Betula alleghaniensis*

Introduction

Symbiotic mycorrhizal associations between plant roots and fungi are estimated to occur with 90 % of terrestrial plant species (Smith and Read 1997). In exchange for plant carbohydrates, mycorrhizal fungi provide plants with access to limiting soil resources (Selosse et al. 2006). Variations in the effects of mycorrhizal symbioses on plant performance may alter plant community structure and developmental trajectories (Kernaghan 2005).

Yellow birch and eastern hemlock are native to northeastern North America and commonly co-occur (Erdmann 1990; Godman and Lancaster 1990). Both species are components of many forest types where the dominant trees can be arbuscular mycorrhizal (AM), ectomycorrhizal (ECM), or both (Erdmann 1990; Godman and Lancaster 1990; Molina et al. 1992). Yellow birch and eastern hemlock, which both form ectomycorrhizae, are important for enhancing forest biodiversity (Yamasaki et al. 2000; Holloway and Malcolm 2007). Historic timber harvesting practices, herbivory, pathogens, and strict germination and microsite requirements have led to declines in eastern hemlock and yellow birch (Rogers 1978; Erdmann 1990; Mladenoff and Stearns 1993). Knowledge pertaining to the sharing of ECMF communities between yellow birch and eastern hemlock would enhance our understanding of the potential for fungal mediation of plant interactions, such as the potential for common mycorrhizal

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networks (CMNs) that are believed to be important in structuring plant interspecific interactions (Newman 1988; Watkins et al. 1996; He et al. 2003; Egerton-Warburton et al. 2007; Booth and Hoeksema 2010; Philip et al. 2010) and might be helpful in guiding preservation and restoration efforts.

Coarse woody debris (CWD) is an important component of many forest understories that provides habitat for seedling and other organisms including mycorrhizal fungi (Harmon et al. 1986; Tedersoo et al. 2003). CWD exhibits a higher moisture holding capacity than soil, an important factor for eastern hemlock and yellow birch establishment (Harmon et al. 1986), yet CWD remains a nutrient poor medium for plant growth (Harmon et al. 1986; Marx and Walters 2006).

Environmental factors or stochastic effects can lead to variation in ECMF communities associated with individual microsites (Lilleskov and Parrent 2007). Given that lab experiments demonstrate functional diversity among ECMF species (e.g., Kennedy et al. 2007), it is likely that these community differences could influence seedling nutrition, performance, and growth. Yet, relatively little is known about the effect that differences in ECMF community composition has on seedling performance under natural conditions (van der Heijden and Horton 2009).

The factors structuring ECMF communities are not fully understood, in large part due to the difficulties in examining processes occurring underground without disturbing or altering the systems (Erland and Taylor 2002). Important factors known to play roles in structuring ECMF communities include the identity of the plant host (Murata et al. 2013) and environmental conditions, such as light and soil properties (Burke et al. 2009). Light levels affect the quantity of surplus C plants are able to allocate to mycorrhizal symbionts, and may alter ECMF community composition (Turner et al. 2009). Substrate characteristics, such as pH and N availability, have also been shown to influence ECMF community composition (Erland and Taylor 2002; Trudell and Edmonds 2004).

The primary objectives of this study were to determine the ECM associations of yellow birch and eastern hemlock seedlings in a northern hardwood-hemlock forest located in the western Upper Peninsula of Michigan. We assessed the community composition of paired eastern hemlock and yellow birch seedlings growing in CWD to determine whether ECMF taxa were shared between neighboring heterospecific seedlings. The specific questions we aimed to address with this research are: (1) what is the ECMF community composition of eastern hemlock and yellow birch seedlings growing on CWD in a northern hardwood-hemlock forest, (2) is ECMF community composition similar between individual pairs of eastern hemlock and yellow birch growing on the same piece of CWD, (3) does ECMF community composition have any effect on seedling growth, biomass, or foliar nutrient quality, and (4) is ECMF community variation correlated with substrate properties or canopy cover?

Methods

Study area

The 69-ha study area is located in a mature, managed, uneven-aged, northern hardwood forest owned by Michigan Technological University, and located in Baraga County in the western Upper Peninsula of Michigan (section 30, T49N, R33W, 46° 36' N, 88° 29' W). Seedlings were collected from hemlock inclusions located within the hardwood dominated study area. The dominant overstory species at the collection sites is eastern hemlock (*Tsuga canadensis* (L.) Carr; Appendix Table A1). Other species include yellow birch (*Betula alleghaniensis* Britt.), red maple (*Acer rubrum* L.), northern white cedar (*Thuja occidentalis* L.), black ash (*Fraxinus nigra* Marshall), sugar maple (*Acer saccharum* Marsh.), white spruce (*Picea glauca* (Moench) Voss), and balsam fir (*Abies balsamea* (L. Mill.); Appendix Table A1). Yellow birch, eastern hemlock, balsam fir, and white spruce form symbiotic relationships with ECMF (Brundrett et al. 1990; Peterson et al. 2004). Sugar maple, red maple, black ash, northern white cedar, and many shrub and herbaceous species form symbiotic relationships with AM fungi (Brundrett and Kendrick 1988; Brundrett et al. 1990; Peterson et al. 2004).

Soils are categorized as Champion-Net complex (five sites), Champion cobbly silt loam (two sites), and Witbeck muck (three sites; Berndt 1988). The terrain is level to rolling (slopes range from 0 to 15 %), with deep, moderately well-drained to poorly drained soils covering till plains and moraines (Berndt 1988). Temperatures have ranged from an average of -9 °C in January to 17 °C in July (National Oceanic and Atmospheric Administration (NOAA) 2012). Average yearly precipitation has been 84 cm (NOAA 2012). Snowfall averages have been 371 cm/year (Midwest Regional Climate Center 2012).

Field data collection

Ten separate CWD substrates, either logs or stumps (hereafter referred to as “sites”) with seedlings (<50 cm tall) of both yellow birch and eastern hemlock growing in proximity (<10 cm apart) were located early in the growing season. Sites were ≥ 25 m apart. During June–August 2012, 3- to 12-year-old seedlings and their roots were carefully excavated in order to collect as much of the root system as possible, with the goal of collecting the entire root system. Most of the root mass was collected for each of the seedlings, but some of the finer exploratory roots were severed in the extraction process. Seedlings were placed in plastic bags, watered, sealed, and transported back to the laboratory in a cooler. Two hemlock seedlings were collected at site M29. Basal area ($\text{m}^2 \text{ha}^{-1}$) at

the sites was calculated using a ten BAF prism. Basal area ranged from 11.5 to 41.3 m² ha⁻¹, with a mean±SE of 29.2±2.9 m² ha⁻¹. Eastern hemlock was the dominant canopy species surrounding the sites followed by yellow birch. Percent canopy cover at the sites was calculated using a concave spherical densiometer (Model-C, Robert E. Lemmon, Forest Densiometers, Bartlesville, OK). Canopy cover was uniformly high, ranging from 88 to 95 % (mean±SE, 93±0.68 %).

CWD samples were also collected. The CWD decay class ranged from 3 to 4.5, with a mean of 3.95±0.17 out of 5, with one being undecayed and five being highly decayed (Sollins 1982). Six of the CWD sites were cut stumps, and four were logs. Mean diameter of the CWD was 41.8±4.1 cm. CWD type, determined visually, was predominantly hemlock (seven pieces), but less certain for three pieces (two conifer, one unknown). Visual inspection indicated rot type was predominantly white (seven pieces) with a few pieces (three) inhabited by both brown and white rot types. Roots of canopy trees were present in the seedling rhizospheres at four of the sites.

ECMF identification methods

After collection, seedlings were stored in a refrigerator for no more than 3 days before processing. In the lab, seedling root systems were soaked in water. Roots were carefully washed to remove decayed wood, and other particulate matter. Once cleaned, roots were examined under a dissecting microscope. Healthy, intact ectomycorrhizae were counted and sorted into groups based on morphological characteristics (Agerer 1987–2006). The number of live nonmycorrhizal root tips was also tallied for each seedling.

DNA was extracted and purified from each morphotype on each seedling (except *Cenococcum geophilum*, which was identified morphologically) to identify all fungal symbionts associated with each eastern hemlock and yellow birch seedling. DNA extraction, dilution, and amplification were conducted using the Sigma-Aldrich REDExtract-N-Amp™ PCR Kit following their standard protocol, except extraction volume was scaled down to 20 µL (1:1 ratio of extraction solution and dilution solution). The ITS regions of the ectomycorrhizae were amplified by polymerase chain reaction (PCR) using the primers ITS1-F and ITS4. PCR reactions were run using a 20-µL volume (4 µL of DNA, 0.20 µL (50 µM) of ITS1-F and ITS4, 5.6 µL water, and 10 µL REDExtract-N-Amp PCR Ready Mix). PCR amplification cycling parameters were as follows: 1 cycle initial denaturation for 1.25 min at 94 °C; 35 cycles of denaturation for 35 s at 95 °C, annealing for 55 s at 55 °C, and extension at 72 °C for 45+6 s/cycle; and 1 cycle of final extension for 10 min at 72 °C. After PCR amplification, the DNA samples were run through gel electrophoresis to

determine whether extraction and amplification were successful. Successfully amplified single-banded amplicons were purified using the QIAquick PCR purification kit (Qiagen®), and quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Inc). Purified DNA was sequenced on an ABI Prism 3730 DNA Analyzer.

ITS sequence matching

CodonCode Aligner (CodonCode Corporation) was used to align forward and reverse complements of DNA sequences. The ITS sequences collected from ectomycorrhizae in this study were compared with known ITS sequences using the nucleotide BLAST search from the NCBI GenBank database (<http://blast.ncbi.nlm.nih.gov>). Similarity of 98 % and above between our ITS sequences and voucher ITS sequences in GenBank were considered to be matches to the species level. Similarities of 96–97 % were considered to be matches to genera. One fungal symbiont was only identified to the family level.

Seedling and substrate analyses

Growth rings of the seedlings were counted at the base of the stem under a 20× to 40× dissecting scope to determine age. Diameter (mm), height (cm), and shoot and root biomass (g) were determined. Seedling foliage and CWD samples were dried, ground to a fine powder and analyzed for %N and %C, using a Costech Elemental Combustion System 4010 connected to a Thermo Finnigan ConFloIII Interface and Deltaplus Continuous Flow-Stable Isotope Ratio Mass Spectrometer. Dried, ground seedling foliar tissue samples were chemically analyzed by inductively coupled plasma (ICP) using the dry ash method for nutrients phosphorus (P), K, Ca, Mg, Mn, Fe, Cu, B, Al, Zn, and Na (Miller 1998). Two of the yellow birch seedlings (M06 and M31) were not analyzed for trace elements due to insufficient quantities of leaf tissue.

CWD extracts (filtrate from 1.25 g wood in 20 mL 1 M ammonium acetate stock) were analyzed for exchangeable cations using a Perkin Elmer Optima 7000DV ICP-OES. Bray P-1 P analysis of the CWD was performed. CWD pH_{H2O} was determined by adding deionized water to 3 g of dried ground sample to bring the total volume up to 60 mL (20:1 ratio; Marx and Walters 2006).

Data analysis

To test for host species effects on taxon richness, rarefied fungal taxon richness (i.e., Chao richness) per seedling, which is a nonparametric species richness estimate, was calculated in the biodiversity analysis software package EstimateS (v 9.1.0; Colwell 2013) to compensate for differences in the number of root tips encountered. Seedlings were rarefied to four root tips,

which was the lowest number of ECMF root tips present on a single seedling. Fungal species accumulation curves were also calculated in EstimateS for eastern hemlock and yellow birch using actual root tip counts (Colwell 2013).

Paired student *t* tests were performed in R (R Core Team 2013) to evaluate whether significant differences occurred between seedling attributes (biomass, age, growth rate, foliar nutrients, ECMF root colonization rates, and number of rarefied ECMF taxa) associated with the yellow birch and eastern hemlock seedlings. Significance levels were set a priori for all statistical tests at $\alpha=0.05$. At site M29 where we sampled two hemlock seedlings, the average of both seedling attributes was used for the *t* tests.

Relative abundance was calculated as the number of individual tips of an ECMF taxon on an individual seedling divided by the total number of ECM tips found on the seedling (Horton and Bruns 2001; Mohatt et al. 2008). The relative frequency of taxa was calculated separately for eastern hemlock and yellow birch by dividing the total number of seedlings with each taxon present by the total number of seedlings. Importance values were calculated for each taxon associated with yellow birch and eastern hemlock by summing the relative abundance and relative frequency for each taxon (Horton and Bruns 2001; Mohatt et al. 2008).

Nonmetric multidimensional scaling (NMS) ordination was used to evaluate fungal community composition differences along measured environmental gradients (PC-ORD; McCune and Grace 2002; McCune and Mefford 2011). Relative abundance of ECMF taxa (% of ECM root tips) by seedling was used for analysis. In order to improve the data structure, singletons (fungal taxa with only one occurrence) were removed and species within the same genus were combined. Because leaf nutrient analysis was only performed on 19 of the seedlings (8 birch and 11 hemlock), NMS was run separately for leaf nutrients ($n=19$) and all other environmental variables ($n=21$). To normalize foliar nutrient data for multivariate analysis of fungal community effect on nutrition, the difference between the overall species mean (yellow birch minus hemlock) for each nutrient was added to the hemlock nutrient values for each seedling. For the foliar nutrient NMS analysis, 19 sample units (seedlings) were analyzed with 5 ECMF taxa in the main matrix and 13 foliar nutrients in the second matrix. Twenty-one sample units (seedlings) were analyzed in a separate NMS ordination with 6 ECMF taxa in the main matrix and 18 environmental variables (CWD, seedling, and environmental attributes) in the second matrix. A random starting configuration and autopilot mode were used for the NMS ordinations. The distance measure used was Sørensen (Bray-Curtis) with 250 runs of real data and 250 runs of random data with a maximum of 500 iterations possible. A Monte Carlo test was used to analyze data dimensionality. Pearson's correlation coefficient was used to evaluate the relationship between ECMF taxa, foliar nutrients,

environmental variables (CWD pH, C, N, P, K, Ca, Mg, Mn, Al, and Na content; seedling root/shoot ratio, height increment, diameter increment, and age; yellow birch and eastern hemlock basal area), and NMS axis scores (McCune and Mefford 2011). NMS solutions are based on observed ECMF community composition. Significant Pearson's correlations between measured environmental variables and axis scores signify potentially important factors structuring ECMF communities (McCune and Grace 2002).

To evaluate whether foliar nutrients that were significantly correlated with the first ordination axis also differed significantly between seedlings colonized by the dominant and mutually exclusive ECMF genera *Tomentella* and *Lactarius*, we used mixed effects models in R, lme4 package (Bates et al. 2013; R Core Team 2013). Seedling and fungal type were modeled as fixed effects and site was modeled as a random effect—nested within fungi (because each site contained only one of these fungal species). Type II Wald Chi-square tests were performed using the car package (Fox and Weisberg 2011; R Core Team 2013). The sites where foliar nutrient data was lacking for yellow birch (M06 and M31) were not analyzed in the model. Welch's two sample *t* tests were performed in R (R Core Team 2013) to evaluate whether significant differences occurred among the CWD samples with different ECMF dominants, testing only the CWD attributes that were significantly correlated with the first ordination axis.

Results

Fungal community composition

The majority of root tips were uncolonized, with colonization (mean \pm SE) only 19 ± 3.3 % for yellow birch and 10 ± 1.8 % for eastern hemlock and did not differ significantly between species ($p=0.060$, Appendix Table A2). Forty-two samples associated with the seedling pairs, 26 from yellow birch and 16 from eastern hemlock, were sequenced. Thirteen different fungal taxa, 12 growing on yellow birch root tips, and 6 growing on eastern hemlock root tips, were observed at the 10 sites (Table 1). Fungal taxon richness associated with yellow birch ranged from 1 to 4 with a mean of 2.1 fungal taxa per seedling. Species richness associated with eastern hemlock ranged from 1 to 2 ECMF taxa per seedling with a mean of 1.18. Rarefied fungal taxon richness was significantly greater for yellow birch than for eastern hemlock (1.53 vs. 1.17, t value = -3.74 , $p=0.005$). The species accumulation curves for ECMF taxa associated with yellow birch and eastern hemlock did not reach an asymptote after tripling the number of samples (Fig. 1; Colwell et al. 2004). However, estimated richness was relatively low for both

Table 1 Ectomycorrhizal fungal taxa associated with yellow birch (YB) and eastern hemlock (EH) seedling root tips by site

ECMF Taxa	Site ID									
	M03	M06	M07	M11	M13	M24	M29 ^a	M30	M31	M33
<i>Cenococcum geophilum</i> ^b	YB	YB	0	0	0	YB	0	0	0	0
<i>Cortinarius</i> sp.	0	0	0	0	YB	0	0	0	0	0
<i>Hebeloma</i> sp.	0	0	0	0	0	0	YB	0	0	0
<i>Lactarius chrysorrheus</i>	0	YB/EH	0	0	0	0	0	0	0	0
<i>Lactarius</i> sp.	0	0	0	0	0	0	0	YB	0	0
<i>Lactarius tabidus</i>	0	0	0	YB/EH	YB/EH	0	0	EH	YB/EH	YB/EH
<i>Paxillus involutus</i>	0	0	0	YB	0	0	0	0	YB	0
<i>Piloderma byssinum</i>	0	0	0	0	0	0	EH2	0	0	0
<i>Piloderma fallax</i>	0	0	0	YB/EH	0	YB/EH	0	0	0	0
<i>Russula</i> sp.	0	0	0	0	0	YB/EH	0	0	0	0
<i>Scleroderma citrinum</i>	0	0	YB	0	0	0	0	0	0	0
<i>Tomentella subtilacina</i>	YB/EH	0	YB/EH	0	0	0	YB/EH1	0	0	0
unknown Helotiaceae ^c	0	0	0	YB	0	0	0	0	0	0

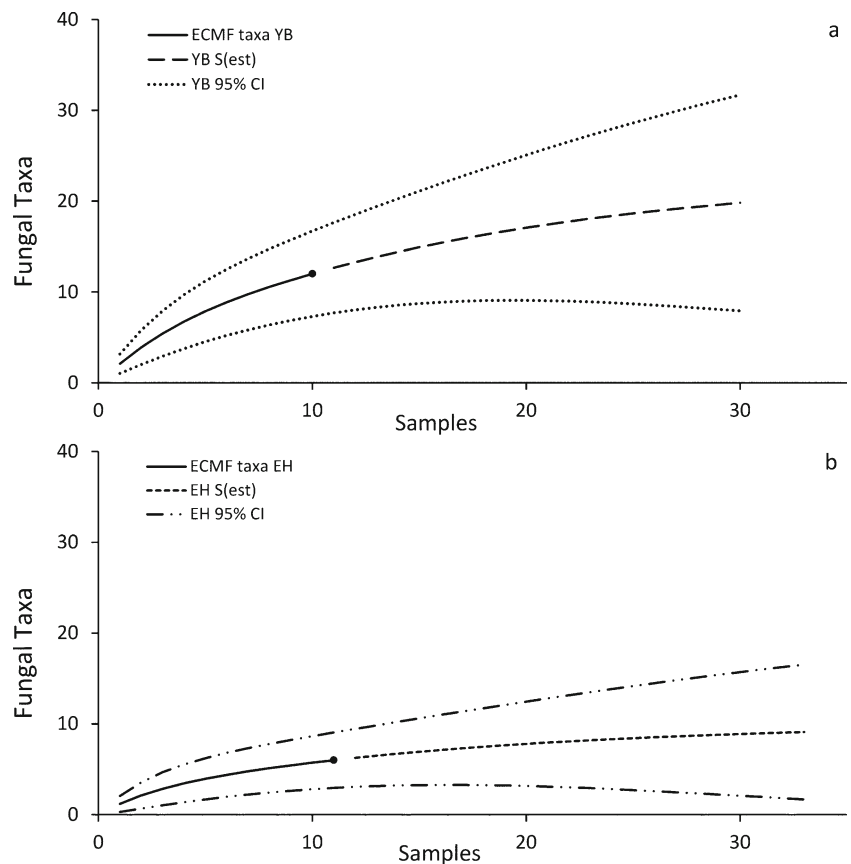
^a Two hemlock seedlings

^b DNA not sequenced

^c The three closest matches to the unknown Helotiaceae were root endophytes from a single study of boreal trees in eastern Canada (Kemaghan and Patriquin 2011; NCBI Genbank database)

host species (19.81 for yellow birch and 9.1 for eastern hemlock). Chao richness only increased slightly, to 23.02 and 10.9 ECMF species, respectively, by extrapolating to asymptote at 136 samples for birch and 142 for hemlock.

Fig. 1 Species accumulation curves for the ectomycorrhizal fungal taxa associated with **a** yellow birch (YB; $n=10$) and **b** eastern hemlock (EH; $n=11$) seedlings. Seedlings were considered samples and actual root tip count of ECMF taxa was used to calculate estimated fungal species richness ($S(est)$) and the 95 % confidence intervals (CI)



Fungal community similarity

ECMF taxa shared between pairs of eastern hemlock and yellow birch included *Lactarius chrysorrheus/vinaceorufescens* subgroup (hereafter referred to as *L. chrysorrheus*), *Lactarius tabidus* (=theiogalus), *Piloderma fallax*, *Russula* sp., and *Tomentella sublilacina* (Table 1). These taxa made up 95 and 70 % of hemlock and yellow birch ECM root tips, respectively. *T. sublilacina* and *L. tabidus* were the most important species associated with both eastern hemlock and yellow birch (Fig. 2).

Two unique ITS genotypes of both *L. tabidus* and *T. sublilacina* were present on the root tips and shared between yellow birch and eastern hemlock (Appendix Tables A3 and A4). Yellow birch and eastern hemlock shared 1–2 ECMF taxa on nine of the ten pieces of CWD. Sharing occurred 100 % of the time between yellow birch and eastern hemlock for *L. chrysorrheus*, *P. fallax*, *T. sublilacina*, and *Russula* sp. *L. tabidus* was shared 80 % of the time (four of five occurrences). ECMF unique to yellow birch included *Cortinarius* sp., *Hebeloma* sp., *Lactarius* sp., *Paxillus involutus*, and *Scleroderma citrinum*. *Cortinarius* sp., *Hebeloma*

sp., *Lactarius* sp., and *S. citrinum* were singletons. An unknown Helotiaceae was also associated with one yellow birch seedling. Morphological observations not included in our tabulation extended our insights regarding sharing. *C. geophilum* was observed on both yellow birch and eastern hemlock root tips (seven hemlock and six birch seedlings). However, healthy, live *C. geophilum* ECM root tips were only found on three yellow birch seedlings. Bright yellow mycelia and ECM (likely *P. fallax*) were also observed on pairs of yellow birch and eastern hemlock at three sites and only on eastern hemlock at another site, but ECM were only successfully sequenced at two of the sites. *Piloderma byssinum* was the only ECMF unique to eastern hemlock.

Seedling attributes

Both eastern hemlock and yellow birch had more biomass aboveground than below, with yellow birch having slightly, but not significantly, more biomass aboveground than eastern hemlock ($p=0.160$, Appendix Table A2). Total biomass was greater for eastern hemlock than for yellow birch by a factor of 1.9 ($p=0.220$) on average, and eastern hemlock was older than yellow birch by an average of 2.8 years ($p=0.007$). Mean annual biomass increment was also slightly greater in eastern hemlock, but not significantly so ($p=0.735$). However, mean diameter increment and height increment were both higher ($p=0.008$ and $p=0.006$, respectively) in yellow birch than eastern hemlock.

Foliar nutrient and substrate analysis

Significant differences occurred for concentrations of several foliar nutrients between eastern hemlock and yellow birch. Eastern hemlock needles contained significantly higher concentrations of C, and lower concentrations of N, K, Ca, Mg, Zn, and Na (Appendix Table A5). Wood pH_{H2O} ranged from 3.6 to 4.4, with a mean of 3.82 ± 0.08 . Mean \pm SE and range of CWD exchangeable cations and P concentrations are displayed in Appendix Table A6.

ECMF community and seedling foliar nutrient quality

The NMS ordination performed on the ECMF community from 19 of the 21 seedlings (for comparison with foliar nutrients) was two-dimensional with a final stress of 4.53, instability of <0.0001 , and cumulative r^2 of 0.945. Axis 1 explained 69.0 % and axis 2 explained 25.5 % of the variability in the ordination results. Axis 1 was most strongly correlated with *Lactarius* and *Tomentella* (Table 2; Fig. 3). The foliar nutrients N and K displayed the strongest correlations with Axis 1. Axis 2 was most strongly correlated with

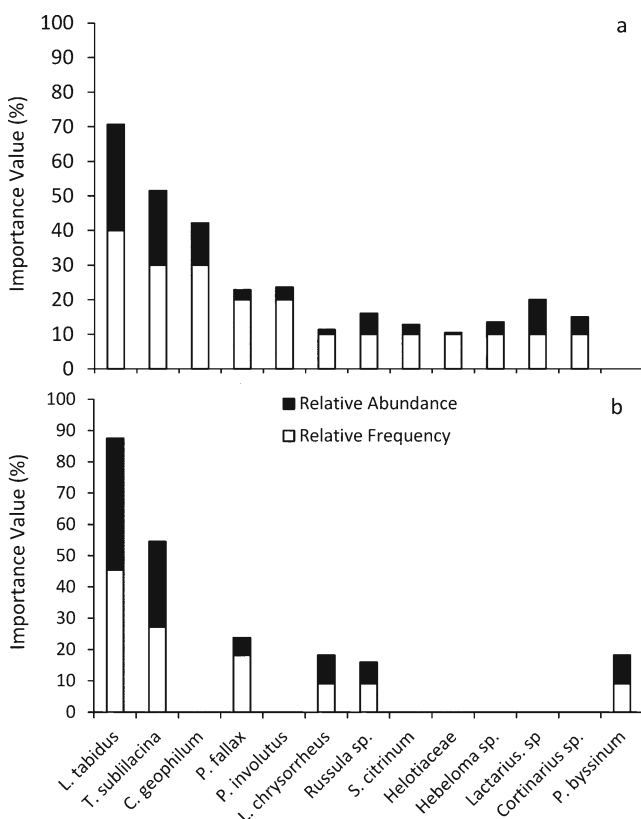


Fig. 2 Importance values (sum of relative abundance and relative frequency) of fungal symbionts associated with **a** yellow birch and **b** eastern hemlock seedling root tips growing in coarse-woody debris in a northern hardwood-hemlock forest. **C. geophilum* and *P. fallax* were observed on eastern hemlock root tips but not as live ectomycorrhizae

Table 2 Pearson's correlation between nonmetric multidimensional scaling ordination axes, genus level ECMF community composition, and foliar nutrient concentrations from 19 of 21 eastern hemlock and yellow birch seedlings growing on coarse woody debris ($n=10$) in a northern hardwood-hemlock forest

	Axis 1 ($r^2=0.690$)		Axis 2 ($r^2=0.255$)	
	r	p value	r	p value
ECMF taxa				
<i>Tomentella</i>	-0.908*	<0.001*	0.376	0.112
<i>Lactarius</i>	0.900*	<0.001*	0.386	0.103
<i>Cenococcum</i>	-0.251	0.299	0.134	0.584
<i>Piloderma</i>	-0.089	0.717	0.796*	<0.001*
<i>Russula</i>	0.021	0.933	0.749*	<0.001*
Foliar nutrient concentrations				
Carbon (%)	-0.223	0.358	0.046	0.853
Nitrogen (%)	-0.494*	0.032*	0.002	0.995
Phosphorus (%)	-0.325	0.175	-0.063	0.796
Potassium (%)	-0.469*	0.043*	-0.320	0.182
Calcium (%)	0.083	0.736	0.351	0.140
Magnesium (%)	0.282	0.242	0.442	0.058
Manganese (ppm)	0.092	0.709	0.127	0.605
Iron (ppm)	0.250	0.302	-0.212	0.383
Copper (ppm)	0.309	0.198	0.023	0.924
Boron (ppm)	0.105	0.670	0.344	0.149
Aluminum (ppm)	-0.045	0.853	-0.172	0.480
Zinc (ppm)	-0.375	0.114	-0.182	0.455
Sodium (ppm)	0.170	0.486	0.504*	0.028*

Two of the yellow birch seedlings lacked sufficient foliage for ICP nutrient analysis. Unit of measurement for the ECMF taxa is relative abundance (%ECMF root tips)

* $\alpha=0.05$, significant values

Piloderma and *Russula*. The foliar nutrient Na displayed the strongest and only significant correlation with Axis 2.

ECMF community and environment

The NMS ordination performed on the ECMF community for comparison with the environmental variables ($n=21$) was 3-dimensional with a final stress of 2.15, instability of <0.0001, and cumulative r^2 of 0.955. Axes 1, 2, and 3 explained 71.2, 16.3, and 8.0 % of the variability in the ordination results, respectively. Because axes 1 and 2 explain most of the variability, results from these axes are used for graphical display purposes. Axis 1 was most strongly correlated with the dominants *Lactarius* and *Tomentella* as well as wood pH, and wood exchangeable Ca and Mg concentrations (Table 3; Fig. 4). Axis 2 was most strongly correlated with *Piloderma* and *Russula*. *Cenococcum* and wood C content were significantly correlated with Axis 3. Canopy cover was not significantly correlated with any of the ordination axes.

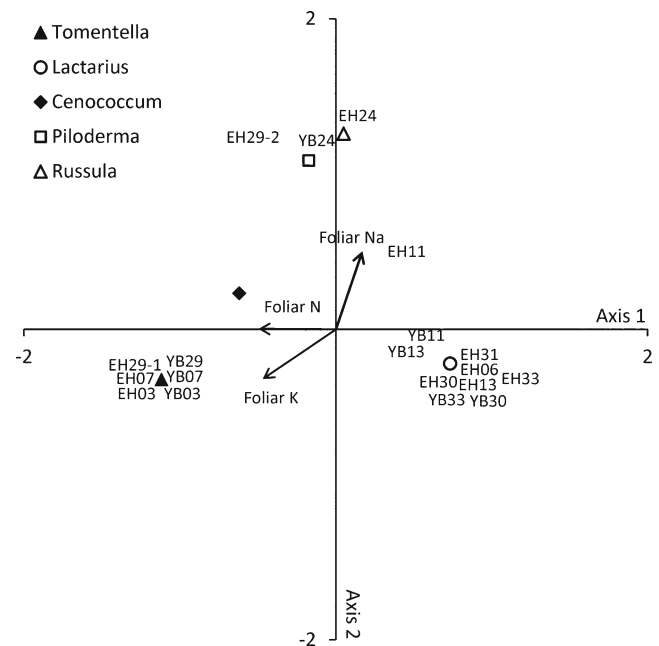


Fig. 3 Nonmetric multidimensional scaling ordination joint plot illustrating locations of ectomycorrhizal fungi and seedlings ($n=19$) along significant ($p \leq 0.05$) foliar nutrient gradients (N, K, and Na). Foliar N, K, and Na denote concentrations of nitrogen, potassium, and sodium in the seedling foliage. The yellow birch and eastern hemlock seedlings are denoted by seedling type (YB for birch and EH for hemlock) and site location (03, 06, 07, 11, 13, 24, 29, 30, 31, and 33)

Fungal dominants, seedling nutrition, and growth substrate

Because *Tomentella* and *Lactarius* were mutually exclusive, we compared significant foliar nutrient concentrations of seedlings and CWD attributes based on associations with *Tomentella* vs. *Lactarius* and the ordination results. Foliar K and N concentrations were significantly greater in seedlings associated with *Tomentella* vs. *Lactarius* (Table 4). CWD exchangeable Ca and Mg were significantly greater in CWD associated with *Lactarius*. CWD pH was greater in wood associated with *Lactarius* vs. *Tomentella* but not significantly so.

Discussion

ECMF community composition

The ECMF communities associated with yellow birch and eastern hemlock in this study were relatively simple. Tree species often differ in their ECMF community richness (Ishida et al. 2007) and ECMF taxon richness was always higher for yellow birch than eastern hemlock. *Cortinarius* sp., *Hebeloma* sp., *P. involutus*, and *S. citrinum*, all of which are ECMF taxa known to associate with both conifers and hardwoods (Arora 1986), only associated

Table 3 Pearson's correlation between nonmetric multidimensional scaling ordination axes, genus level ECMF community composition, and environmental variables for 21 eastern hemlock and yellow birchseedlings growing on coarse woody debris ($n=10$) in a northern hardwood-hemlock forest

	Axis 1 ($r^2=0.712$)		Axis 2 ($r^2=0.163$)		Axis 3 ($r^2=0.080$)	
	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value
ECMF taxa						
<i>Tomentella</i>	0.860*	<0.001*	0.442*	0.045*	-0.143	0.535
<i>Lactarius</i>	-0.914*	<0.001*	0.283	0.213	-0.138	0.550
<i>Cenococcum</i>	0.105	0.651	-0.012	0.960	0.839*	<0.001*
<i>Piloderma</i>	0.097	0.675	-0.800*	<0.001*	0.211	0.360
<i>Paxillus</i>	-0.271	0.236	0.079	0.733	-0.022	0.924
<i>Russula</i>	0.147	0.525	-0.725*	0.002*	-0.350	0.119
Environmental variables						
Wood characteristics						
pH	-0.524*	0.015*	0.108	0.641	-0.160	0.487
Carbon (%)	-0.063	0.787	-0.034	0.882	0.502*	0.021*
Nitrogen (%)	-0.136	0.558	-0.334	0.139	-0.309	0.173
Phosphorus (mg P/kg soil)	-0.066	0.775	-0.161	0.485	-0.343	0.128
Potassium (cmol _c /kg)	-0.256	0.262	-0.087	0.708	-0.212	0.355
Calcium (cmol _c /kg)	-0.569*	0.007*	-0.362	0.106	-0.208	0.365
Magnesium (cmol _c /kg)	-0.463*	0.035*	-0.268	0.240	-0.062	0.790
Manganese (cmol _c /kg)	-0.377	0.092	0.115	0.619	-0.078	0.738
Aluminum (cmol _c /kg)	-0.255	0.265	0.171	0.458	-0.207	0.368
Sodium (cmol _c /kg)	-0.341	0.131	-0.111	0.633	-0.318	0.161
Seedling attributes						
Root/shoot ratio	0.087	0.708	-0.153	0.507	0.379	0.090
Height increment (cm/year)	0.352	0.118	0.335	0.137	-0.079	0.734
Diameter increment (mm/year)	0.319	0.159	0.185	0.423	-0.055	0.814
Age (year)	-0.007	0.975	-0.355	0.115	-0.091	0.696
Environment						
Yellow birch RA (%)	-0.256	0.262	0.052	0.823	-0.411	0.064
Eastern hemlock RA (%)	0.211	0.359	-0.324	0.152	0.214	0.351
Canopy cover (%)	-0.350	0.120	-0.381	0.089	-0.003	0.988

Unit of measurement for the ECMF community is relative abundance (% ECMF root tips). Yellow birch and eastern hemlock (RA) is relative abundance of basal area

* $\alpha=0.05$, significant values

with yellow birch in this study. The absence of these species on eastern hemlock roots could possibly be due to chance or differences between root colonization rates of hemlock and birch. However, other factors must also contribute, because significant differences between eastern hemlock and yellow birch ECMF taxon richness remained when seedling root tips were rarefied to correct for the unequal number of root tips sampled. Although it appears that the full suite of ECMF taxa associated with both eastern hemlock and yellow birch seedlings growing in CWD was not sampled, the extrapolated richness of fungi on this substrate is relatively low compared with typical soil communities (Tedersoo et al. 2008).

Multi-host ECMF taxa were much more abundant than single-host taxa and the high level of interspecific sharing of ECMF establishes the potential for the existence of common mycorrhizal networks (CMNs; Newman 1988; Simard and Durall 2004). Although we did not analyze mature tree roots, studies have shown similarity in ECMF communities associated with canopy trees and understory seedlings (e.g., Kennedy et al. 2003; Bingham and Simard 2012), suggesting the potential for CMNs to connect canopy trees with seedlings. Persistence of hemlock and yellow birch seedlings in the highly shaded understory of these forests could be enhanced by carbohydrate subsidy to the mycorrhizal network from canopy trees (Kennedy et al. 2003).

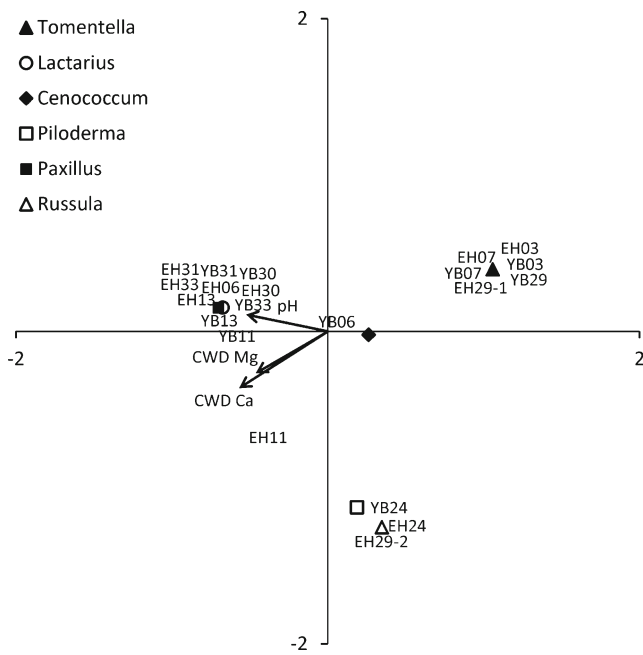


Fig. 4 Nonmetric multidimensional scaling ordination joint plot illustrating locations of ectomycorrhizal fungi (ECMF) and seedlings ($n=21$) along significant ($p \leq 0.05$) environmental gradients (pH, CWD Mg, and CWD Ca). Proximity of seedlings in the plot indicates similarity in ECMF communities. The environmental gradients are measured variables that are correlated with, and potentially structure, ECMF community composition. CWD Mg and CWD Ca denote magnesium and calcium concentrations of the coarse-woody debris growth substrate. The yellow birch and eastern hemlock seedlings are denoted by seedling type (YB for birch and EH for hemlock) and site location (03, 06, 07, 11, 13, 24, 29, 30, 31, and 33)

ECMF effects on seedling nutrition and physiology

Nutrient acquisition is more difficult for plants under low pH levels such as those encountered in the CWD at our sites. In acidic environments, P becomes less soluble, the availability of the cations Ca, Mg, and K is low, and these cations can be leached from the system (Mengel et al. 2001). Most foliar

macro-nutrient concentrations for both yellow birch and eastern hemlock were within a normal range (Jochner et al. 2013; Penn State University College of Agricultural Sciences 2014). However, N levels were slightly low in yellow birch, whereas, Mg, Cu, and B were excessive based on foliar values in silver birch (*Betula pendula* Roth; Jochner et al. 2013). Mean foliar K, Cu, B, and Zn were high for eastern hemlock (Penn State University College of Agricultural Sciences 2014). Mn and Fe were both excessive for hemlock and yellow birch (Jochner et al. 2013; Penn State University College of Agricultural Sciences 2014), consistent with mobilization of Mn and acid cations in these low pH conditions. The ECMF associated with the seedlings may counteract some of the nutrient imbalances that commonly occur in acidic environments (Finlay 1995). Ahonen-Jonnarth et al. (2003) observed that ECMF mycelia minimized the loss of base cations through leaching in soil.

It also appears from our analysis that the two dominant ECMF species differ in their effect on seedling nutrition, although we must be cautious because the study is correlative. Nevertheless, it seems unlikely that these patterns were driven by differences in substrate quality, because we did not see evidence that substrate differed in K or N, yet mean K and N concentrations were significantly greater in seedlings colonized by *Tomentella*, when compared with *Lactarius*. Other studies suggest that there are ECMF taxon-specific environmental (e.g., pH) optima for uptake of nutrients (Jongbloed and Borst-Pauwels 1992). Perhaps *Tomentella* is more efficient at foraging for K and N than *Lactarius* spp. Interspecific differences in effect on host stoichiometry have been observed under more controlled greenhouse conditions (e.g., Smith 2013).

Environment and ECMF community variation

Although we did not find significant effects of canopy cover, this may be due to the fact that the sites were uniformly shady. C limitation due to high levels of shade has been shown to

Table 4 Effect of dominant ECMF genus on mean foliar nutrient concentrations for eastern hemlock (EH) and yellow birch (YB) seedlings, and coarse woody debris (CWD) attributes

	<i>Tomentella</i>			<i>Lactarius</i>			Chi square	t value	p value
	EH (n=3)	YB (n=3)	CWD (n=3)	EH (n=4)	YB (n=4)	CWD (n=6)			
Nitrogen (%)	1.4	2.2		1.2	1.7		5.58		0.018*
Potassium (%)	0.95	1.35		0.73	0.99		5.44		0.020*
pH			3.67			3.90		-1.94	0.102
Calcium (cmol _c /kg)			5.3			10.5		-3.17	0.016*
Magnesium (cmol _c /kg)			0.71			1.17		5.56	0.031*

Seedling species effect was also significant for both foliar nutrients (see Appendix Table A5). Chi-square and accompanying p values for the foliar nutrients are from the type II Wald Chi-square test; t and p values for the CWD are from Welch’s two-sample t test

* $\alpha=0.05$, significant values

limit ECMF colonization (Zhou and Sharik 1997; Druebert et al. 2009) and may explain the low root tip colonization levels observed in our study. According to Zhou and Sharik (1997) ideal light conditions for ECMF development occur under approximately one-third canopy cover, a higher light environment than that found in the present study. Turner et al. (2009) also observed that ECMF diversity and percent colonization was significantly greater under high and intermediate vs. low light. In our study, both eastern hemlock and yellow birch had low root:shoot ratios, an indication that light was most limiting (Bloom et al. 1985).

In shaded environments, such as those observed in this study, we may expect that shade-midtolerant seedlings (yellow birch) are approaching their light compensation point resulting in less C to share with ECMF symbionts than shade-tolerant species (eastern hemlock). However, photosynthetic capabilities have been shown to be greater for shade-intolerant seedlings in both high and low light, compared with shade-tolerant seedlings (Kitajima 1994; Walters and Reich 1996). More light demanding species may be better able to use limiting resources when they become available, even in shady environments, due to their faster growth rates (Coomes and Grubb 2000). Therefore, eastern hemlock seedlings may be less responsive to ECMF symbioses or more conservative in C sharing.

Presence of *T. sublilacina* and the genus *Lactarius* (mostly *L. tabidus*) were mutually exclusive in this study. *T. sublilacina* occurred in CWD that contained significantly lower concentrations of exchangeable Ca and Mg than the CWD colonized by the genus *Lactarius*. The addition of Ca and Mg to forest soils has been shown to affect ECMF community structure (Qian et al. 1998; Rineau and Garbaye 2009). Alternatively, *T. sublilacina* has been shown to exclude other ECMF through direct competition (Lilleskov and Bruns 2003; Tedersoo et al. 2008; Burke et al. 2009) and may have prevented *Lactarius* spp. from colonizing seedling root tips when present.

Many of the common ECMF species from this study are tolerant of elevated nitrogen levels in the environment (Arnolds 1991; Lilleskov et al. 2002; Lilleskov et al. 2011). Furthermore, the two dominant species from this study, *T. sublilacina* and *L. tabidus*, respond positively to N deposition (Lilleskov et al. 2002). Shifts in ECMF community composition under elevated atmospheric N deposition may be related to the C allocation requirements of ECMF. ECMF with low C demands may be more successful in high N environments since plant hosts in these environments supply lower levels of C to fungal symbionts (Lilleskov et al. 2011). The CWD in this study was low in N, which suggests that the ECMF present were not responding to high N levels, but rather low levels of carbohydrates provided by the seedlings. ECMF with low C demands may also be the fungal symbionts most likely to associate with seedlings growing in shady forest

understorey environments. Although beyond the scope of the present study, analysis of ECMF communities on adult tree roots from the CWD would help to test the hypothesis that C supply is structuring these communities. Adult trees are likely less C limited, so if adult tree communities were represented by more C demanding species (i.e., mid- or long-distance exploration types like *Cortinarius* and *Piloderma* spp.), this would suggest that C supply may be an important agent structuring seedling ECMF communities in shaded CWD.

Conclusions

Eastern hemlock and yellow birch seedlings exhibited a high degree of similarity in their ECMF communities, providing a potential pathway for interactions mediated by fungal symbionts. Nutritional differences observed between seedlings colonized by *Tomentella* and *Lactarius* spp. suggest ECMF community mediation of plant nutrition that could be critical to seedling fitness in these nutrient-poor substrates. The dominant species *L. tabidus* and *T. sublilacina* should be investigated for tolerance to low carbohydrate supply. The above findings provide a strong impetus for experimental investigations into the role of ECMF communities in seedling survival, nutrition, and growth on CWD.

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