

Fertility-dependent effects of ectomycorrhizal fungal communities on white spruce seedling nutrition

Alistair J. H. Smith II¹ · Lynette R. Potvin² · Erik A. Lilleskov²

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Abstract Ectomycorrhizal fungi (EcMF) typically colonize nursery seedlings, but nutritional and growth effects of these communities are only partly understood. To examine these effects, *Picea glauca* seedlings collected from a tree nursery naturally colonized by three dominant EcMF were divided between fertilized and unfertilized treatments. After one growing season seedlings were harvested, ectomycorrhizas identified using DNA sequencing, and seedlings analyzed for leaf nutrient concentration and content, and biomass parameters. EcMF community structure–nutrient interactions were tested using nonmetric multidimensional scaling (NMDS) combined with vector analysis of foliar nutrients and biomass. We identified three dominant species: *Amphinema* sp., Atheliaceae sp., and *Thelephora terrestris*. NMDS+envfit revealed significant community effects on seedling nutrition that differed with fertilization treatment. PERM ANOVA and regression analyses uncovered significant species effects on host nutrient concentration, content, and stoichiometry. *Amphinema* sp. had a significant positive effect on phosphorus (P), calcium and zinc concentration, and P content; in contrast, *T. terrestris* had a negative effect on P concentration. In the unfertilized treatment, percent abundance of the *Amphinema* sp. negatively affected foliar nitrogen (N) concentration but not content, and reduced foliar N/P. In fertilized seedlings, *Amphinema* sp. was positively related to foliar concentrations of N, magnesium, and boron, and both concentration and content of

manganese, and Atheliaceae sp. had a negative relationship with P content. Findings shed light on the community and species effects on seedling condition, revealing clear functional differences among dominants. The approach used should be scalable to explore function in more complex communities composed of unculturable EcMF.

Keywords Stoichiometry · Ectomycorrhizal fungal community effects · Nitrogen · Phosphorus · Micronutrients · *Amphinema* · Atheliaceae · *Thelephora terrestris* · Greenhouse

Introduction

Seedlings regenerating naturally or artificially are influenced by both soil fertility and the root symbionts that mediate nutrient supply. Fertilization of tree seedling stock is a common practice in nursery and greenhouse settings (e.g., Timmer 1997; McAlister and Timmer 1998; Hawkins et al. 2005). Even under these high nutrient conditions, trees form symbioses with ectomycorrhizal fungi (EcMF; e.g., Croghan 1984; Richter and Bruhn 1993; Menkis et al. 2005). EcMF often aid in the accumulation of essential nutrients such as nitrogen (N), phosphorus (P), and other nutrients (Landeweert et al. 2001; Smith and Read 2008; Courty et al. 2010). Although many EcMF species often decline in environments with high nutrient availability, in particular N (Arnolds 1991; Wallenda and Kottke 1998), some EcMF are less affected by such conditions (Lilleskov et al. 2011). Additionally, the frequent seedling turnover associated with nurseries favors pioneer EcMF species (Danielson and Visser 1990; Kranabetter 2004). The combination of these factors creates favorable growing conditions for common greenhouse colonists such as *Thelephora terrestris* and *Amphinema byssoides* (e.g., Menkis et al. 2005).

Nutrition effects of ectomycorrhizal fungi depend on nutrient availability. EcMF communities adapted to high nutrient

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✉ Erik A. Lilleskov
elilleskov@fs.fed.us

¹ Michigan Technological University, 1400 Townsend Dr, Houghton, MI 49931, USA

² Northern Research Station, US Forest Service, 410 MacInnes Drive, Houghton, MI 49931-1199, USA

conditions in greenhouses (Flykt et al. 2008) do not necessarily always function as mutualists, and may actually act as conditional parasites, leading to reduced growth or nutrient uptake (Johnson et al. 1997; Kummel and Salant 2006; Karst et al. 2008). Furthermore, once the seedling and fungi are outplanted, available nutrients typically decline greatly (Danielson and Visser 1990; Krasowski et al. 1999; Kranabetter 2004). This transition has potential implications for both the EcMF community and the host. Whether the same species can act as mutualists in both the greenhouse and in the field is unclear, so understanding the function of the community under both high and low fertility conditions is critical.

Only in the last decade have greenhouse studies begun to use DNA-based molecular methods to identify specific EcMF species (e.g., Kernaghan et al. 2003; Menkis et al. 2005; Flykt et al. 2008). These methods provide greater species resolution and better comparability among studies. In addition, these methods often uncover formerly unrecognized species that may differ functionally. Better species characterization is an essential prerequisite for community functional analysis.

In this study, we addressed two questions. Does the composition of the EcMF community affect the foliar nutrient concentration and content, stoichiometry, growth, and biomass allocation of both fertilized and unfertilized white spruce seedlings? How do individual EcMF species affect host seedling nutrition and biomass parameters? We examined the potential nutritional, growth, and allocation effects of the naturally occurring greenhouse EcMF community of white spruce seedlings under fertilized and unfertilized conditions. Examination of the effects of naturally occurring (versus inoculated) EcMF communities provides greater realism in mycorrhizal fungal functional analyses because EcMF typically occur in mixtures of genotypes and species. There are two challenges in analyzing community and species effects on greenhouse seedlings when the community composition is uncontrolled: first, identifying significant community effects, and second, characterizing the role of individual species in community function. To address the first challenge, we used multivariate ordination methods to determine whole community impacts, and for the second challenge, we analyzed whether EcMF species relative abundance on root tips affected nutrition and biomass parameters.

Methods

Greenhouse conditions and seedling selection

White spruce seedlings were grown using standard greenhouse procedures at the USDA Forest Service Toumey Nursery (Watersmeet, MI, USA) as follows (C. Makuck, personal communication). Seeds from the Oconto River Seed Orchard were germinated in April 2010 and sown into

SunGro Forestry Mix (70 % peat/30 % vermiculite) in Styroblock containers (160 cavities/block; cavity volume, 90 cc). During 2010, they received a 9–45–15 (N–P–K) starter fertilizer, followed by treatment with a high N fertilizer (Scott's Champion 21–8–18), and finally, a 4–25–35 finisher fertilizer. Seedlings were also treated with Banrot 40 % WP and Cleary 3336 WP fungicides. Thiophanate-methyl is an ingredient of both of these fungicides and has been found to suppress ascomycetes relative to basidiomycetes (Bollen 1972), so the ectomycorrhizal community was likely biased toward the latter. Ectomycorrhizal mycelium was observed growing from the base of cavities in Styroblocs by the greenhouse staff during the growing season of 2010 while seedlings were still in the greenhouse. Seedlings were overwintered on the ground outdoors under the snowpack, and our seedling collection for the present study occurred shortly after snowmelt.

One-year-old container-grown white spruce seedlings for the present study were collected by the authors on April 29th, 2011. Seedlings were chosen from widely dispersed locations in many Styroblocs to obtain independent genets. We collected seedlings in a targeted fashion to sample replicates of the apparent dominant fungal diversity present on the roots, as evident by readily visible fungal mycelium of different colors and morphology at the bottom of the containers. Three major mycelial types were present, which we described as brown, yellow, and white (Supplemental Fig. 1). Examination of these seedlings with a dissecting microscope indicated that mycelium arose from ectomycorrhizal root tips. The seedlings were placed in open plastic bags in a cooler and brought to the Northern Research Station Forestry Sciences Laboratory in Houghton, MI, USA, where they were kept in climate-controlled growth chambers until morphotyped several days later. EcMF were morphotyped under dissecting and compound microscopes, using color, rhizomorph presence and type, mantle color and texture, and hyphal characteristics (Agerer 1987–2008). Only the surface of the plugs was examined to minimize disturbance of the mycelium and contamination. The goal was not complete characterization but rather rapid characterization of the dominant morphotypes (morphological phenotypes evident at the root-tip level under a dissecting microscope) to permit relatively even distribution among treatments. The three dominant morphotypes were *Thelephora*-like, *Amphinema*-like, and a white cottony type (which turned out to be *Atheliaceae* sp.). Given our goal of an analysis of the community effects, having perfect control over the composition of the community was not as important as having representation of the dominant taxa in both treatments.

Experimental treatments

After initial morphotyping was completed, 73 seedlings were transplanted to Stuewe and Sons Deepots with 1050 cm³ of

pasteurized peat moss (Sunshine Organic Genuine Canadian Sphagnum) added surrounding the original plug. We used peat because thick organic horizons and moss are a common regeneration niche for white spruce seedlings (Nienstaedt and Zasada 1990), and we wanted to provide an organic-rich but available-nutrient poor matrix to support any taxa with ability to access organic nutrients. Morphotypes were assigned evenly to the two fertilization treatment groups and distributed randomly on racks holding 20 seedlings. To minimize contamination by airborne fungal spores, pots were capped with a 2.5-cm thick layer of fibrous synthetic filler (Poly-Fil 100 % Polyester Fiberfill). The outside of the Deepot container rack was covered with rigid insulating blue foam to keep roots cool. In early May 2011, the racks were placed outdoors in full sunlight on a concrete pad. At each watering, fertilized seedlings received 100 ml of DI water and soluble fertilizer mix (Scott's Champion 21–8–18, trace elements included; see supplemental Table 1), at a rate of 100 ppm of nitrogen. Unfertilized seedlings received 100 ml of DI water. Seedlings were watered with these solutions every day for the first week of establishment, then as needed for the remainder of the growing season. Regular precipitation was also allowed into the pots.

Sample harvest and tissue analysis

Seedlings were harvested in early October 2011, 5 months after morphotyping and initiation of fertilizer treatments. For the present study, 21 of the original 73 seedlings (10 fertilized and 11 unfertilized) were selected in a stratified random fashion to ensure representation of the dominant initial morphotype groups in both treatments. Root-tip colonization was estimated using the methods of Giovannetti and Mosse (1980) with modifications. Briefly, root tips were placed on a 1-cm grid. Root tips that aligned with the grid were morphotyped. Six hundred root tips were characterized per seedling, divided equally between roots outside the original peat plug and roots within the original peat plug. Both areas had large populations of healthy roots, and we wanted to ensure that our sample included both initial colonists from the root plug and any new colonists on the roots growing out from the plug. From each seedling, 10 root tips per morphotype were randomly selected from each of these two regions of the rooting environment and reserved for DNA analysis. The root tips were freeze-dried and stored in 1.5-mL microcentrifuge tubes. The stem, foliage, and remaining roots were placed into a 45 °C oven and dried. Once dry, all tissues were weighed, and foliage was ground for chemical analysis. N concentrations were determined on an elemental analyzer (Fisons NA1500). Foliar tissue was analyzed for K, Ca, Mg, Mn, Fe, Cu, B, Al, Zn, Na, and S concentration and content using acid digestion followed by inductively coupled plasma

spectroscopy (ICP) (Huang and Schulte 1985) at the Penn State Agricultural Sciences Laboratory.

Fungal identification

Identification via PCR and sequencing was carried out on individual root tips from each morphotype from both the old and new substrate on each seedling. DNA extraction and PCR were carried out using a REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich Corp., St. Louis, MO, USA) following manufacturer's instructions, except that root tips were digested in 10 µL of extraction solution and treated with 10 µL of dilution solution. The internal transcribed spacer region was amplified with primers ITS1F and ITS4 (White et al. 1990; Gardes et al. 1991) following the PCR program of Gardes and Bruns (1993), except that the extension time started at 45 s and was increased by 6 s per cycle.

PCR products were visualized using gel electrophoresis. Single-banded PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands). DNA concentration was determined on a NanoDrop 3300 (Thermo-scientific, Waltham, MA, USA). Cleaned and quantitated samples were sent to the Nevada Genomics lab (University of Nevada, Reno) for sequencing on an ABI3730 (Applied Biosystems, Foster City, CA, USA). Amplified root tips (81 %; 61 of 75) were successfully sequenced. Sequences were manually trimmed to remove low quality regions, then run through nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for identification.

Two species of Atheliaceae were common on tree roots. To better understand their relationship to known Atheliaceae species, we compared our isolates to their closest matches among previously sequenced *Amphinema* and *Tylospora* species. DNA sequences were aligned with related sequences from GenBank and UNITE using Muscle in Unipro Ugene v1.92, manually trimmed, and both percent similarity matrices and a neighbor-joining tree generated. The tree was edited in TreeView (v.1.6.6). These species were also assigned “species hypotheses” based on the new UNITE algorithm for assigning cluster of sequences to stable provisional names (Kõljalg et al. 2013).

In 19 % of root tips (14 of 75), DNA sequencing was not successful. For those samples, remaining root tips were examined for detailed morphological features (especially hyphal diameter and ornamentation, cystidia, clamp connections, and presence and anatomy of rhizomorphs) and compared to successfully sequenced samples to determine identity.

Statistical analysis

There was no difference in the species composition in the old and new parts of the peat plug, and no significant trend in community composition between these regions, so the tips

from the old and new regions were pooled for the following analyses. To test for the effects of the EcMF communities on white spruce seedling foliar nutrient status, biomass, and biomass allocation, NMDS and the envfit function were used in the R package *vegan* (Oksanen et al. 2012, Oksanen 2013). NMDS analyses were carried out separately for fertilized and unfertilized seedlings, based on the rationale that the fertilization effects would confound our effort to see the EcMF community impacts on nutrition, NMDS does not test for interactions, and our goal in the analysis was to relate community to nutrition while holding all else constant. NMDS was done on the percentage data using Wisconsin double standardization and Bray–Curtis distances. We used the metaMDS function, default settings, which include a rotation of the major axis of variation to align with axis 1. For the unfertilized seedlings, we ran NMDS+envfit analyses with rare species excluded. The “envfit” function fits vectors of continuous variables to the ordination, with the length of the arrow proportional to the correlation with the ordination. Biplots of species and envfit vectors were generated to visualize the results. As each envfit analysis included multiple tests, the false discovery rate (FDR) of each analysis was controlled for using the Benjamini–Hochberg procedure provided in the Handbook of Biological Statistics (McDonald 2014). We reported significance at FDR of 0.1, 0.2 and 0.25. Note that FDR is not equivalent to α , but is rather an approach to control the proportion of false positives under multiple comparisons, with an FDR of 0.1 equivalent to one false positive out of 10 tests deemed significant under the FDR criteria. Results from the more relaxed criteria should be interpreted with caution.

To test the relationship between individual fungal species and seedling nutrient and biomass metrics, we used a number of approaches. First, we used PERMANOVA in the Primer 6 package. PERMANOVA is a permutational equivalent of MANOVA commonly used in community analyses (Anderson 2001; McArdle and Anderson 2001). In order to test for the effects of fungal species on host plant parameters, we reversed the normal relationship of predictor and response variables commonly used in PERMANOVA, i.e., fertilization level and abundance of EcMF species were predictors, and response variables were univariate or multivariate combinations of seedling nutrition or growth parameters. In multivariate analyses, response variables were normalized before analysis, and distance matrices were Euclidean. As we had unequal sample size and continuous predictors, we followed the advice of Anderson et al. (2008) and used type I sums of squares. As we were interested in the species effects after fertilization had been accounted for, we put fertilization first in the model, treating it as equivalent to a covariate (in the sense of controlling for its effect). We ran a separate test for each of the three most abundant taxa as well as the nonmycorrhizal root tips, including fertilization, taxon percentage abundance, and their interaction. In the univariate

case, the interaction term is essentially a test for homogeneity of slopes between the species effects at the two fertilization levels (Anderson et al. 2008). To constrain the number of tests, we only ran these analyses for response variables with a $p < 0.1$ in the NMDS–envfit correlations. As above, we report significance at FDR from 0.1 to 0.25, where the BH FDR was calculated separately for each class of response variables, i.e., element concentration, element content, stoichiometry, and biomass metrics.

Second, we ran and plotted linear and nonlinear regression analyses, as appropriate, to understand and visualize the effect of species under different fertilization conditions. These were carried out in Sigmaplot 12.5. Additionally, Welch’s two-tailed t tests in the R VEGAN package were conducted in order to compare effects of fertilization on seedling traits. Results were considered significant with an $\alpha = 0.05$, and marginally significant with an $\alpha = 0.1$.

Third, to test whether the effects of fungal taxa on seedling response differed significantly, for the subset of nutrients that showed significant species effects in the initial regressions, slopes of regression lines were compared to those of other species using parallel line analysis in Sigmaplot 12.5. A difference in slope between taxa is interpreted to mean that their effect on the response variable differed as a function of percent of root colonized. Nonlinear relationships were linearized using logarithmic transformation of x or y data, as appropriate. Results were considered significant with an $\alpha = 0.05$, and marginally significant with an $\alpha = 0.1$.

Results

Fungal community composition

Five EcMF species were identified by sequencing. Four of the five species and all three dominant species were basidiomycetes (Supplemental Table 2). Two species were only found on single seedlings. Of the three dominant fungi, one was identified as *T. terrestris*, and two species were in the family Atheliaceae, both of which matched to as yet uncharacterized species. Therefore, we used the unique species hypothesis names applied in the UNITE database: *Amphinema* sp. SH029298.06FU, and Atheliaceae sp. SH029299.06FU (Supplemental Fig. 2). The latter is likely also an *Amphinema* species but was not designated as such in UNITE. See Supplemental results for more information on these taxa.

The two additional EcMF species each found only on single seedlings were *Boletus variipes*, a basidiomycete more commonly associated with the genus *Quercus*; and *Sphaerospora brunnea*, an ascomycete commonly found as EcMF in greenhouses and known to associate with coniferous species (Danielson 1984; Supplemental Table 1). We

focus on the three dominant species plus nonmycorrhizal root tips in the following analysis.

Fertilization effects on seedling nutrients, biomass, and allocation

The mean foliar nutrient concentration of fertilized seedlings was higher for all nutrients except for Na and K (Supplemental Table 3). However, N was the only element to show a statistically significant treatment effect ($p < 0.0001$) with almost three times higher N concentration under fertilization. P was marginally higher under fertilization ($p = 0.051$). In unfertilized seedlings, N was the common limiting nutrient, with an average concentration of 9 g/kg, below the estimated deficiency threshold of 10.5 g/kg (Fisher and Binkley 2000). Cu was also right at the deficiency threshold (Supplemental Table 3). In fertilized seedlings, all nutrients for which we had data were above published deficiency thresholds (Fisher and Binkley 2000; Lehto et al. 2010; Polle et al. 1992; Supplemental Table 2). Seedlings receiving fertilization also had higher mean aboveground biomass, higher mean belowground biomass, and a marginally higher mean root/shoot ratio than unfertilized seedlings (Supplemental Table 4).

EcMF community effects on foliar nutrient concentration, content, biomass, and allocation

The EcMF community was significantly related to the nutrient status of the seedlings. NMDS combined with envfit analysis revealed that, in the unfertilized seedlings, the EcMF community affected foliar concentrations of N (Fig. 1a, Table 1). For fertilized seedlings, there was a significant EcMF community effect on concentrations of Mn and B, with marginally significant effects on P, Ca, S, Mg and Zn (Fig. 1b, Table 1). Given the high number of significant or near-significant results, all of these, plus Cu, were significant at a FDR of 0.25 or lower (Table 1).

We observed fewer EcMF community effects on foliar nutrient content. In unfertilized seedlings, a significant effect was observed on P (Fig. 1c) content and a marginally significant effect for K that was not significant at a FDR=0.25 (Table 1). In fertilized seedlings, marginally significant effects for Mn and Cu (Fig. 1d) were not found significant with a FDR of 0.25 (Table 1).

A marginally significant EcMF community effect was observed in fertilized seedlings on root/shoot ratio ($R^2 = 0.51$, $p = 0.06$). For all other biomass metrics (total biomass, root biomass, stem biomass, leaf biomass, and shoot biomass),

Fig. 1 Nonmetric multidimensional scaling (NMDS) ordination analysis of EcMF communities with envfit correlations for **a** nutrient concentrations in unfertilized seedlings, **b** nutrient concentrations in fertilized seedlings, **c** nutrient content in unfertilized seedlings, and **d** nutrient content in fertilized seedlings. Solid circles represent seedlings; arrows represent envfit correlations, solid $p < 0.05$, dashed $p < 0.1$; and names are abbreviations for species used in the analysis: *Amp_sp.* *Amphinema* sp., *Ath_sp.* *Atheliaceae* sp., *NM* nonmycorrhizal, *The_ter* *Thelephora terrestris*

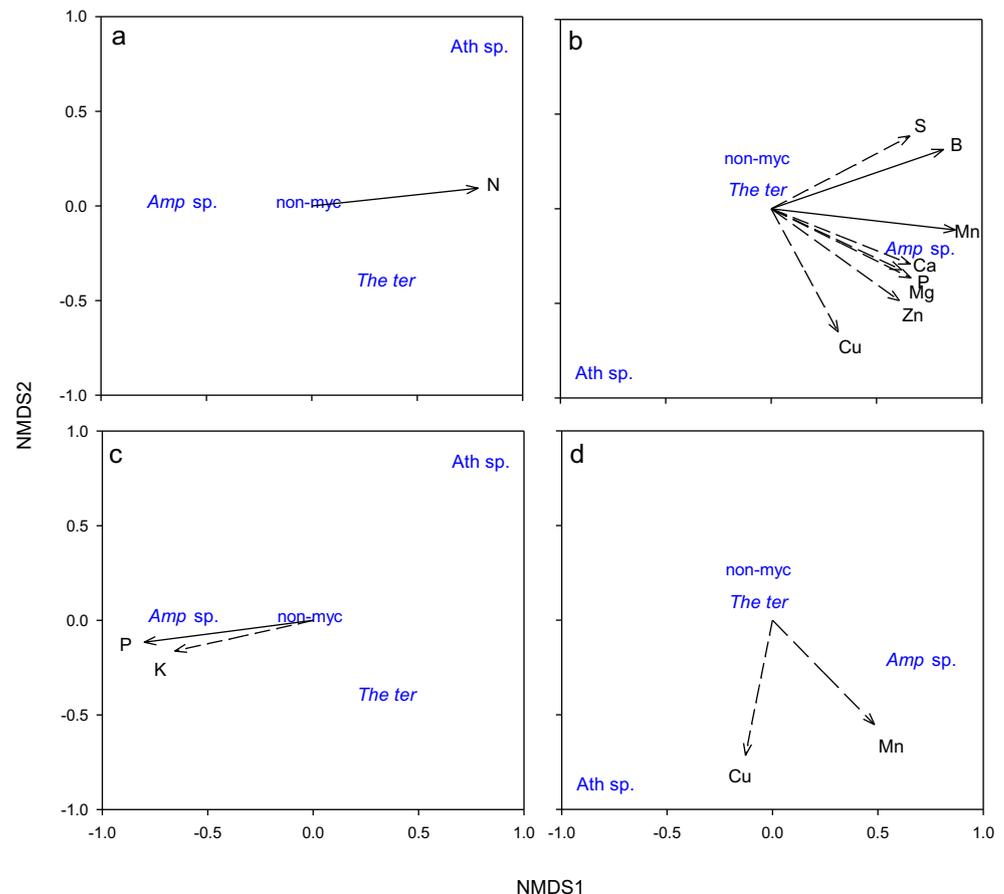


Table 1 NMDS–envfit analyses for community relationship with the concentration and content of 13 elements in white spruce seedling foliar tissues

Element	Concentration						Content					
	Unfertilized			Fertilized			Unfertilized			Fertilized		
	r^2	p value	BH	r^2	p value	BH	r^2	p value	BH	r^2	p value	BH
N	0.63	0.017	*	0.33	0.241	ns	0.17	0.525	ns	0.40	0.147	ns
P	0.22	0.384	ns	0.49	0.098	*	0.66	0.01	**	0.34	0.24	ns
K	0.33	0.196	ns	0.11	0.678	ns	0.46	0.085	ns	0.19	0.516	ns
Ca	0.06	0.782	ns	0.52	0.089	*	0.28	0.255	ns	0.23	0.397	ns
Mg	0.06	0.785	ns	0.57	0.051	**	0.20	0.378	ns	0.28	0.325	ns
S	0.38	0.157	ns	0.58	0.05	**	0.19	0.424	ns	0.14	0.599	ns
Mn	0.11	0.632	ns	0.78	0.007	***	0.24	0.326	ns	0.54	0.073	ns
B	0.16	0.496	ns	0.77	0.007	***	0.19	0.423	ns	0.41	0.186	ns
Cu	0.25	0.336	ns	0.53	0.109	*	0.28	0.265	ns	0.53	0.075	ns
Zn	0.09	0.704	ns	0.61	0.054	**	0.29	0.232	ns	0.43	0.145	ns
Al	0.39	0.143	ns	0.04	0.871	ns	0.36	0.164	ns	0.27	0.338	ns
Fe	0.08	0.724	ns	0.04	0.878	ns	0.18	0.446	ns	0.36	0.203	ns
Na	0.17	0.469	ns	0.04	0.856	ns	0.17	0.471	ns	0.04	0.871	ns

Rare species were excluded from the NMDS analysis. Analyses were run separately for each fertilization level. R^2 , p values, and the Benjamini–Hochberg (BH) significance are reported under three false discovery rates. BH significance was calculated for each envfit run (column) separately ($n=13$). As a result, because BH is sensitive to the number of low p values in the analysis, equivalent p values in different columns can have a different BH significance

Italics indicate significance under a Benjamini–Hochberg false discovery rate with an α set at 0.05, and a BH FDR less than or equal to 0.25. Asterisks indicate the different Benjamini–Hochberg false discovery rates (FDRs) under which the results were found significant

ns FDR>0.25

*FDR=0.25, **FDR=0.2, ***FDR=0.1

no significant EcMF community effects were observed in either unfertilized or fertilized seedlings.

Fungal species effects on foliar nutrient concentration, content, biomass, and allocation

To understand the source of these significant community effects, we analyzed species effects for those factors found significant at the community level (Table 1).

PERMANOVA analysis of species effects indicated that *Amphinema* had the greatest impact on patterns of seedling nutrition, with a significant main effect and interaction with fertilization in the multivariate test on the overall element composition (Table 2). Breaking this down to individual elements, *Amphinema* sp. had significant main effects on concentration for four elements (P, Ca, Mg, and Zn) and interactions with fertilization for four elements (N, Mg, B, and Mn; Table 2). When we examined this interaction using parallel lines analysis, we found positive slope in the fertilized treatment that differed significantly from negative slopes in the unfertilized treatment when percent abundance of *Amphinema* sp. was regressed against N ($p=0.0009$), Mn ($p=0.0005$), and B ($p=0.0024$) concentration (Supplemental Fig 4). While the trend was

similar for Mg, the slopes were not significantly different (data not shown).

Amphinema also had the most effects on element content, with significant main effects on P and Mn, and also a significant interaction with fertilization for Mn (Table 2), with a steeper positive slope when fertilized than when unfertilized. *Amphinema* also had a significant main effect and interaction term for N/P. *Thelephora* had only one significant main effect on P concentration. Atheliaceae sp. had no significant main effects, but did have a significant interaction with fertilization for B concentration and P and Cu content. We interpret Atheliaceae results with caution given the low number of seedlings colonized by this species. Nonmycorrhizal root tip percentage abundance had no significant effects on nutrient content or concentration, but did have a strong main effect and interaction with fertilization on root/shoot (Table 2).

Regression analyses within individual fertilization treatments generally supported the PERMANOVA results and elucidated difference among species in their relationship with nutrition. In unfertilized seedlings, N concentration was negatively related to percent abundance of *Amphinema* sp. ($R^2=0.79$, $p=0.0003$; Fig. 2a) and positively related to summed percent abundance of Atheliaceae sp. and *T. terrestris* ($R^2=0.90$, $p<0.0001$;

Table 2 PERMANOVA *p* values and Benjamini–Hochberg FDR (BH) for individual species percent abundance as predictors of a suite of seedling condition response variables

Response variables	<i>Amp</i>		Fert× <i>Amp</i>		<i>The</i>		Fert× <i>The</i>		<i>Ath</i>		Fert× <i>Ath</i>		Non		Fert×Non	
	<i>p</i> value	BH	<i>p</i> value	BH	<i>p</i> value	BH	<i>p</i> value	BH	<i>p</i> value	BH	<i>p</i> value	BH	<i>p</i> value	BH	<i>p</i> value	BH
Concentrations																
All ^a	0.027	*	0.011	**	0.127	ns	0.526	ns	0.695	ns	0.205	ns	0.803	ns	0.791	ns
N	0.876	ns	0.010	**	0.967	ns	0.182	ns	0.744	ns	0.290	ns	0.866	ns	0.991	ns
P	0.004	**	0.754	ns	0.024	*	0.188	ns	0.422	ns	0.617	ns	0.182	ns	0.608	ns
Ca	0.020	**	0.090	ns	0.321	ns	0.555	ns	0.454	ns	0.449	ns	0.790	ns	0.455	ns
Mg	0.033	*	0.009	**	0.404	ns	0.561	ns	0.842	ns	0.272	ns	0.555	ns	0.337	ns
B	0.328	ns	0.029	*	0.603	ns	0.541	ns	0.542	ns	0.007	**	0.347	ns	0.262	ns
Mn	0.101	ns	0.002	**	0.765	ns	0.308	ns	0.386	ns	0.072	ns	0.573	ns	0.568	ns
Zn	0.011	**	0.055	ns	0.201	ns	0.507	ns	0.461	ns	0.714	ns	0.921	ns	0.252	ns
Cu	0.519	ns	0.076	ns	0.164	ns	0.139	ns	0.139	ns	0.820	ns	0.264	ns	0.746	ns
S	0.080	ns	0.066	ns	0.068	ns	0.202	ns	0.902	ns	0.322	ns	0.378	ns	0.838	ns
Content																
All ^a	0.101	ns	0.318	ns	0.461	ns	0.964	ns	0.409	ns	0.097	ns	0.231	ns	0.128	ns
P	0.025	*	0.333	ns	0.228	ns	0.995	ns	0.541	ns	0.041	*	0.485	ns	0.098	ns
Mn	0.004	**	0.026	*	0.660	ns	0.800	ns	0.172	ns	0.994	ns	0.284	ns	0.048	*
Cu	0.860	ns	0.709	ns	0.347	ns	0.485	ns	0.896	ns	0.035	*	0.029	*	0.105	ns
Stoichiometry																
N/P (log)	0.018	**	0.029	**	0.134	ns	0.572	ns	0.267	ns	0.273	ns	0.409	ns	0.944	ns
Biomass																
All ‡	0.945	ns	0.452	ns	0.727	ns	0.441	ns	0.300	ns	0.181	ns	0.035	**	0.159	ns
Root:shoot	0.951	ns	0.688	ns	0.771	ns	0.219	ns	0.226	ns	0.145	ns	0.0001	***	0.004	**

Each test included one species (or nonmycorrhizal roots), fertilization, and their interaction, but the main effect of fertilization is not shown for clarity. *p* values in bold are significant with BH FDR≤0.25. BH significance at three different false discovery rates was estimated for each class of analyses, i.e., for all leaf nutrient concentration (*n*=80), leaf nutrient content (*n*=24), stoichiometry (*n*=8), and seedling biomass (*n*=16)

Asterisks indicate the different Benjamini–Hochberg false discovery rates (FDRs) under which the results were found significant

Numbers in italics are significant *p*<0.10

Amp *Amphinema* sp., *The* *Thelephora terrestris*, *Ath* *Atheliaceae* sp., *Non* nonmycorrhizal, *Fert* fertilization, *ns* FDR>0.25

*FDR=0.25, **FDR=0.2, ***FDR=0.1

^a N, P, K, Ca, Mg, S, Mn, B, Cu, Zn, Al, Fe, Na

^b Root biomass, stem biomass, leaf biomass, shoot biomass, total biomass, and root/shoot ratio

Fig. 2b). Parallel line analysis confirmed the inequality of these slopes (*p*=0.0002).

In fertilized seedlings, *Amphinema* sp. and *T. terrestris* had largely opposite relationships with foliar nutrient concentrations. Foliar P concentration was positively correlated with *Amphinema* sp. relative abundance ($R^2=0.51$, *p*=0.02; Fig. 3a) but negatively with *T. terrestris* ($R^2=0.54$, *p*=0.02; Fig. 3b), leading to significant difference in slopes (*p*=0.0099). *Amphinema* sp. also had a positive slope when regressed against foliar concentrations of N, B, Mn, and Zn (Fig. 4) and marginally with Mg ($R^2=0.31$, *p*=0.09). In contrast, *T. terrestris* had no positive effects on these nutrients.

As in the PERMANOVA analysis, there were fewer significant species effects on foliar nutrient content (Table 3). For unfertilized seedlings, *Amphinema* sp. had a strong positive

relationship with foliar P content ($R^2=0.75$, *p*=0.0006), whereas *Atheliaceae* sp. and the sum of *Atheliaceae* sp. and *T. terrestris* both had strong negative relationships with foliar P content ($R^2=0.40$, *p*=0.037, and $R^2=0.66$, *p*=0.002, respectively; Fig. 5), with slopes of all of these lines significantly different from that of *Amphinema* sp. (*p*=0.0001). On fertilized seedlings, *Amphinema* sp. showed a significant positive effect on foliar Mn content ($R^2=0.62$, *p*=0.01), whereas nonmycorrhizal seedlings had a negative relationship with foliar Cu content ($R^2=0.57$, *p*=0.01).

EcMF species differed in their effect on host N/P stoichiometry (Table 1). In unfertilized seedlings, *Amphinema* sp. had a strong negative relationship with N/P ($R^2=0.86$, *p*=0.0002; Fig. 6). In contrast, for *T. terrestris* and *Atheliaceae* sp., the marginally significant relationship

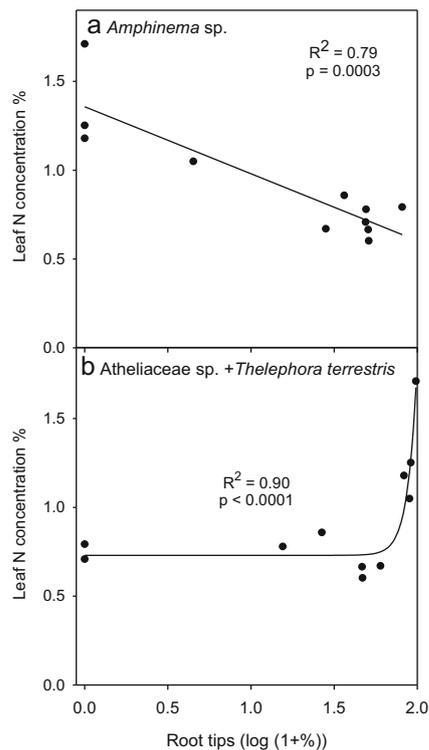


Fig. 2 Foliar nitrogen concentration of unfertilized seedlings vs. EcMF species root abundance [log (1+ % root tips)] for **a** *Amphinema* sp. and **b** Atheliaceae sp.+ *T. terrestris*

was positive ($R^2=0.28$, $p=0.09$ and $R^2=0.26$, $p=0.10$, respectively), and their combined percent abundances exhibited a stronger positive relationship ($R^2=0.76$,

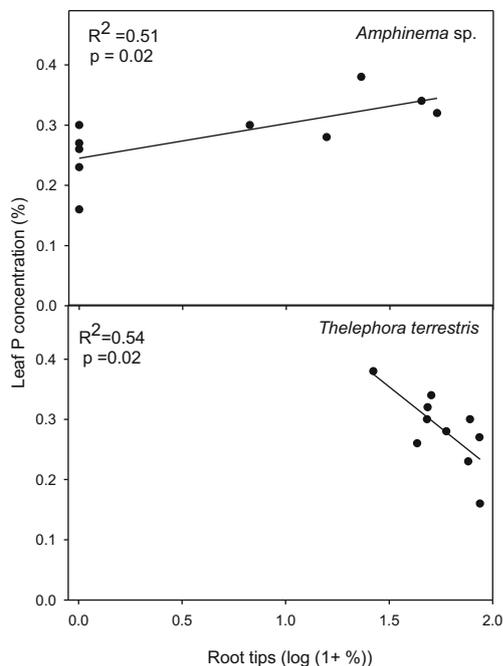


Fig. 3 Regressions of foliar P concentration of fertilized seedlings vs. species root abundance (log (1+ % root tips)) for **a** *Amphinema* sp. and **b** *Thelephora terrestris*

$p=0.0003$; Fig 6), with linearized slopes of all of these significantly different from *Amphinema* sp. ($p=0.0001$, 0.0002, and 0.0001, respectively).

There were no EcMF species effects on biomass or root/shoot ratios (Table 1), but there was a strong positive nonmycorrhizal percent abundance effect on root/shoot ratio in the fertilized treatment ($R^2=0.89$, $p<0.0001$) that was still marginally significant after removal of a high leverage point (Fig. 7).

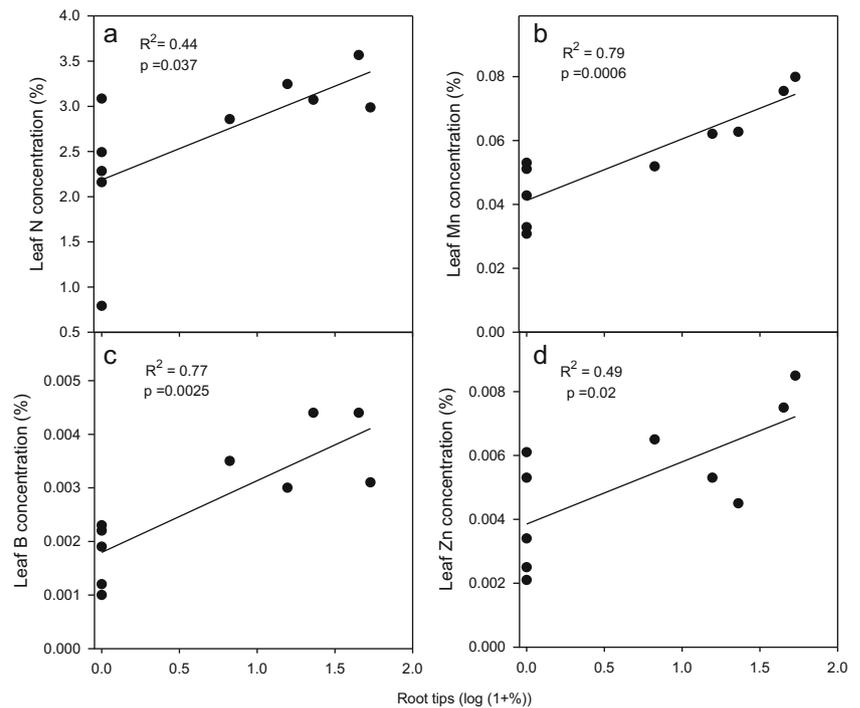
Discussion

Community effects on nutrient status

Demonstration of fertility-dependent EcMF community effects on a broad suite of seedling macro- and micronutrients is novel, supporting the hypothesis that the composition of the EcMF community can be important for regulating host nutrition under both high and low fertility conditions. The nature of community impacts differed with fertilization, with community impacts on N and P under low fertility strongly contrasting with effects on a broad suite of non-N nutrients under high fertilization. The effect of EcMF community structure on N concentration, P content, and N/P stoichiometry under low nutrient conditions is consistent with the hypothesis that there was a functional divergence for relative supply of N and P within EcMF communities colonizing these seedlings. Although we must be cautious not to overinterpret any single result given the large number of tests, our FDR should ensure that the large majority of our results are robust, and the overall pattern of many impacts on host nutrition should be robust to individual type I errors. These communities are admittedly very simple, making the task of detecting community effects possible even with our small sample size. However, the present approach should be viable as community complexity increases if accompanied by increased sample size.

Previous studies of EcMF community effects on seedlings have mostly involved artificially constructed communities used to explore effects of diversity on host function (e.g., Jonsson et al. 2001; Baxter and Dighton 2001; Velmala et al. 2014). These studies found that both EcMF diversity and sampling/species effects can affect host nutrition and growth, possibly mediated by variation in extracellular enzyme activity (Velmala et al. 2014). Studies examining seedling nutritional effects of naturally occurring communities have found effects of location of origin (e.g., Kranabetter 2004), diversity (e.g., Karst et al. 2011), and community composition (e.g., Poznanovic et al. 2014). The latter study employed a similar approach to the present study, finding significant EcMF community effects on seedling foliar nutrition (N, K) of yellow birch (*Betula alleghaniensis*) and eastern hemlock (*Tsuga canadensis*) growing on coarse woody debris in the forest understory. Thus, it appears that the composition of the simple

Fig. 4 Regressions of *Amphinema* sp. percent abundance vs. foliar concentrations of **a** N, **b** Mn, **c** B, and **d** Zn in fertilized seedlings



communities found under both field and greenhouse conditions may well have significant effects on host nutrition and thus should be considered in managing greenhouse seedling fungal communities as well as in conceptual models of factors regulating seedling dynamics, which are presently focused largely on density dependent factors (e.g., Packer and Clay 2000).

Individual species effects on nutrition and growth

EcMF species effects were contingent on fertilization treatment. This was especially noticeable for N in *Amphinema*, where the significant negative effect of this species on foliar N concentrations in unfertilized conditions switched to a significant positive effect under fertilized conditions (Supplemental Figure 4). Our finding of a negative relationship of *A. byssoides* complex with foliar N concentration under low N availability is consistent with the finding of Velmala et al. (2013) for *Picea abies* seedlings. However, the effect of this species on foliar N content was the reverse of its effects on concentration, suggesting that the concentration effects are mediated by changes in foliar mass, which tended to increase, although not significantly, with *Amphinema* sp. abundance.

Given the negative effect on N concentration, one might infer that *Amphinema* sp. is a poor mutualist in unfertilized plants. However, our content and stoichiometry data suggest that *Amphinema* sp. did not negatively affect host plant N uptake, but rather elevated the levels of other nutrients and perhaps growth, hence diluting the N pool. The combined data from low and high fertility treatments suggests that

Amphinema sp. supplies other nutrients at higher rates than N. Thus, we can expect that this species will have a predictable effect on plant stoichiometry and should be most beneficial to growth under at least moderate N availability. Species-specific effects on seedling nutrient stoichiometry provide insights into the role of EcMF taxa in relative supply rates of different nutrients, which could be as important as the absolute supply of any one nutrient in affecting plant function (Allen et al. 2003). For example, given the critical role of N and P in seedling growth, differing effects of *Amphinema* sp. vs. the other two EcMF dominants on N/P in unfertilized seedlings could structure these species' relative benefits under natural or anthropogenic gradients of N and P availability (Lilleskov 2005). The threefold range of N/P ratios found on these seedlings as a strong function of EcMF community (Fig. 6) was striking, and would likely have large ecological and fitness implications for hosts.

Additionally, reducing the supply of N or P relative to other nutrients could be an effective fungal strategy for maximizing C allocation to EcMF. Low foliar N or P concentrations can limit aboveground biomass production, which would be expected to stimulate belowground C allocation (Ericsson 1995). Thus, there could be selection under certain conditions, especially those of conditional parasitism, to constrain the stoichiometry of nutrients supplied such that seedlings are shifted toward N and/or P limitation.

The role of EcMF species in supply of nutrients other than N and P is much less studied, but supply of these nutrients could also have significant impacts on plant carbon balance. *Amphinema* colonization was associated with higher

Table 3 Summary of selected regression relationships between percent abundance of EcMF species and nutrient content (C_t), nutrient concentration (C_n), stoichiometry, and biomass allocation in both fertilized and unfertilized seedlings

Variable	Fertilized						Unfertilized									
	<i>Amphinema</i> sp.		Atheliaceae sp.		<i>Thelephora terrestris</i>		Non-mycorrhizal		<i>Amphinema</i> sp.		Atheliaceae sp.		<i>Thelephora terrestris</i>		Non-mycorrhizal	
Nutrients	Ct	Cn	Ct	Cn	Ct	Cn	Ct	Cn	Ct	Cn	Ct	Cn	Ct	Cn	Ct	Cn
N	(↑)	(↑)	(↓)	(↓)	(↓)	0	(↓)	(↓)	(↑)	(↓)	(↓)	(↓)	(↓)	(↓)	(↑)	(↓)
P	(↑)	(↑)	(↑)	0	(↓)	(↓)	(↓)	(↓)	(↑)	(↓)	(↓)	(↓)	(↓)	(↓)	(↓)	(↑)
Mg	(↑)	(↑)	(↑)	0	(↓)	(↓)	(↓)	(↓)	(↑)	(↓)	(↓)	(↓)	(↓)	(↓)	0	(↑)
Ca	(↑)	(↑)	(↑)	0	(↓)	(↓)	(↓)	(↓)	(↑)	(↓)	(↓)	(↓)	(↓)	(↓)	(↑)	(↑)
Mn	(↑)	(↑)	(↑)	0	(↓)	(↓)	(↓)	(↓)	(↑)	(↓)	(↓)	(↓)	0	(↑)	(↑)	0
Cu	(↑)	(↑)	(↑)	0	(↓)	(↓)	(↓)	(↓)	0	(↑)	(↓)	(↓)	(↓)	(↓)	(↑)	0
Zn	(↑)	(↑)	(↓)	(↓)	(↓)	(↓)	(↓)	(↓)	(↑)	(↑)	(↓)	(↓)	(↓)	(↓)	(↑)	(↑)
B	(↑)	(↑)	(↓)	(↓)	(↓)	0	(↓)	(↓)	(↑)	(↓)	(↓)	(↓)	(↓)	(↓)	(↑)	(↑)
Stoichiometry																
N/P	0	(↓)	(↓)	(↑)	(↓)	(↓)	(↓)	(↓)	(↓)	(↑)	(↑)	(↑)	(↑)	(↓)	(↓)	(↓)
Biomass allocation																
Root/shoot	0	(↓)	(↓)	(↓)	(↓)	(↓)	(↓)	(↓)	0	0	0	0	0	0	0	(↑)

Sign of the slope of the effect is represented by arrows, with significant effects ($p < 0.05$) without parentheses, and nonsignificant effects in parentheses. If no monotonic slope could be determined relationship is indicated with a 0

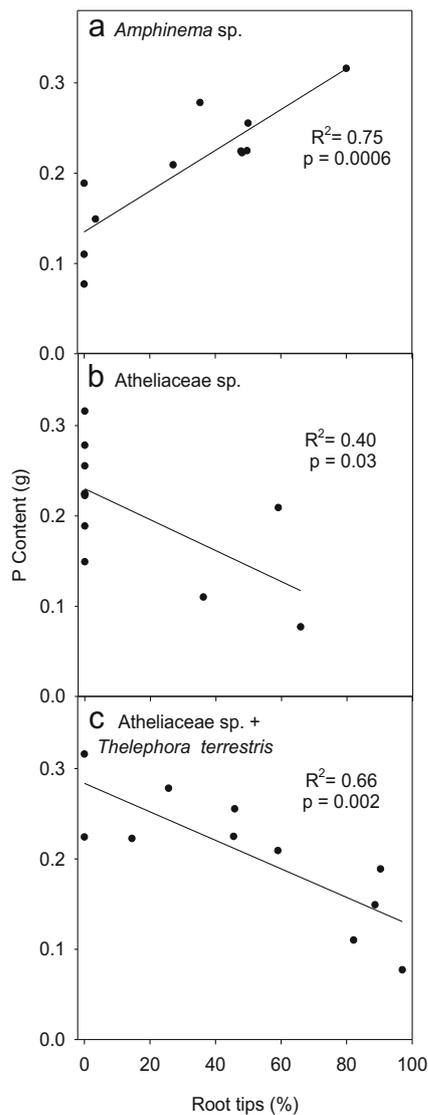


Fig. 5 Regression of foliar P content in unfertilized seedlings as a function of the percentage of roots colonized by different species: **a** *Amphinema* sp., **b** Atheliaceae sp., and **c** *Thelephora terrestris*+ Atheliaceae sp.

concentrations of Mg (marginally), Mn, Zn, and B under fertilization, all of which are thought to be critical to C gain or mycorrhization. As an essential component of chlorophyll, Mg has a stronger effect on C gain than on aboveground growth (Ericsson and Kähr 1995). Similarly, Mn is a critical component of chloroplasts, specifically benefiting photosystem II (Teichler-Zallen 1969), and so should also be critical in photosynthetic C gain. One of the major impacts of Zn deficiency is decline in chlorophyll production and photosynthetic rates (Hu and Sparks 1991). Although the role of B in plants is uncertain (Blevins and Lukaszewski 1994; Bolaños et al. 2004), it is likely that B plays a role in plant cell membranes (Blevins and Lukaszewski 1998; Lehto et al. 2010), and it appears that B fertilization leads to increased EcMF colonization (Mitchell et al. 1987, 1990; Lehto et al. 2004, 2010). Thus, the *Amphinema* sp.

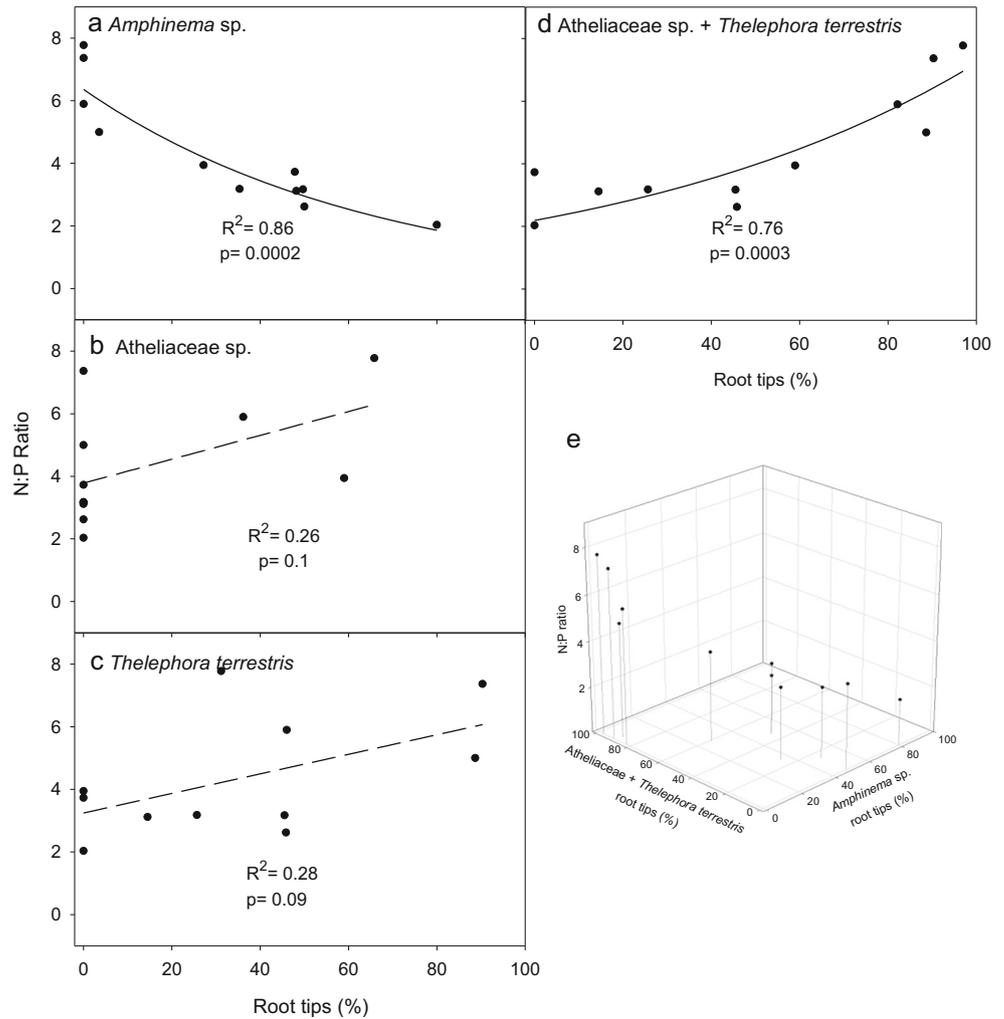
impact on nutrient supply patterns observed in our study might enhance C gain and supply from the host seedling to the fungus, which is clearly beneficial to the fungal partner. However, this nutritional effect only occurred under fertilized conditions when enhanced micronutrient supply is likely to be less beneficial. Further experiments are required to elucidate the range of conditions in which availability of these nutrients to the host plant is enhanced.

T. terrestris is considered a greenhouse parasite because it has been shown to inhibit growth in inoculated seedlings (relative to uninoculated controls) in both nursery and outplanted settings (Trappe 1977; Quoreschi and Timmer 1998). In the present study, we found no significant effect of *T. terrestris* relative abundance on plant growth. However, *T. terrestris* did negatively affect foliar P concentrations under fertilization, although concentrations were still above limiting thresholds. In unfertilized seedlings, no significant *T. terrestris* effects were observed. However, the positive relationship of combined Atheliaceae sp. and *T. terrestris* abundance on foliar N under low N conditions requires further investigation. *T. terrestris* has been associated with increased seedling N content, but not concentration in experimental communities (Velmalala et al. 2014). Its sister taxon, *Tomentella sublilacina*, was similarly correlated with higher foliar N concentrations in eastern hemlock and yellow birch seedlings growing on coarse woody debris (Poznanovic et al. 2014).

These findings are generally concordant with earlier single species inoculations, which indicate EcMF effects depend on species, at least for macronutrients (e.g., Rincón et al. 2005; Smith and Read 2008). There is much less synthesized information about EcMF effects on micronutrients (George et al. 1994). It appears that effects on micronutrients may be contingent on both host and symbiont (e.g., Mitchell et al. 1984, 1990; Dixon and Hiol-Hiol 1992; Walker et al. 2003).

Ultimately, we would like to understand the ecological strategies associated with species functional differences. One intriguing hypothesis is that adaptations that regulate the N shared with hosts affect EcMF partner's overall carbon budget differentially under high and low N supply. *Amphinema* sp. is closely related to, or conspecific with, *A. byssoides*, which responded negatively to N deposition over an anthropogenic N deposition (Lilleskov et al. 2002). Atheliaceae sp. is closely related to Basidiomycete 1 of Lilleskov et al. (2002) as represented by "AY010285 Basidiomycete EL100" in Supplemental Fig. 2. This species plus *T. terrestris* represented two of the handful of EcMF species on roots of mature *Picea glauca* that responded positively to high levels of atmospheric N deposition (Lilleskov et al. 2002), suggesting that these taxa are adapted to high N conditions on both seedlings and mature roots. The combination of their divergent response to N deposition, and what tentatively appears to be divergent effects on seedling nutrition, is consistent with the hypothesis that EcMF species

Fig. 6 Regressions for unfertilized white spruce seedlings of foliar N/P ratio vs percent abundance of **a** *Amphinema* sp. SH029298.06FU, **b** Atheliaceae sp. SH029299.06FU, **c** *T. terrestris*, **d** Atheliaceae+*T. terrestris*, and **e** 3D plot of both Atheliaceae+*T. terrestris* vs *Amphinema* sp. vs. N/P



regulation of costs associated with N supply to hosts could affect C balance of the fungus and success under high N conditions. Whereas *Amphinema* species enhanced seedling N nutrition under fertilized conditions,

the other two species did not. Somewhat counterintuitively, this could be advantageous to the latter from a carbon cost perspective under high N conditions because uptake and transfer of N to hosts entails a significant C cost (Wallander 1995) that, if avoided, could be used for other functions such as sporocarp production or colonization of new roots.

Carbon costs of EcMF may also affect the balance between root and shoot biomass production. The significant positive correlation between root/shoot and abundance of nonmycorrhizal root tips suggests a key role for EcMF in biomass allocation under fertilized conditions. Greater root growth could be compensating for reduced nutrient-absorbing surface area compared to mycorrhizal seedlings (Smith and Read 2008). It is also likely that, when not supporting EcMF, root-allocated C that was supporting the fungus is available for root production, leading to enhanced root growth. Our finding is consistent with a recent meta-analysis showing that EcMF colonization leads to a decrease in the root/shoot ratio, especially in *Picea* (Karst et al. 2008).

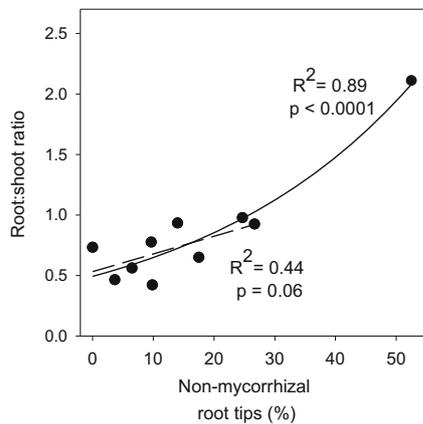


Fig. 7 Regression of nonmycorrhizal root tip percent abundance vs. root/shoot ratio in fertilized white spruce seedlings, with and without a high leverage point

Conclusions

Multivariate methods combined with regression permit exploration of EcMF effects on seedling status in mixed communities, revealing significant EcMF community effects on greenhouse-grown seedlings under high and low nutrient conditions. This approach holds promise for identifying targets for enhancement or suppression in the nursery or greenhouse environment, and for understanding EcMF species impacts on hosts in the field. Scaling up this approach to more complex communities could provide insights into the function of EcMF species that are difficult to culture.

In high fertility conditions, *Amphinema* sp. appears to be an efficient mutualist, elevating the concentration and/or content of many macro- and micronutrients. In unfertilized conditions, *Amphinema* sp. appears to have a positive impact on P nutrition and a negative impact on N/P, preferentially supplying P over N. In contrast, *T. terrestris* and Atheliaceae sp. were often associated with negative or neutral trends in concentration, content, and growth, all of which could be detrimental for seedling development. By not increasing available nutrients or growth in seedlings, especially in the unfertilized setting, *T. terrestris*, and perhaps Atheliaceae sp., demonstrate very little value as mutualists in low fertility conditions.

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