RELATIONSHIPS BETWEEN SUCCESSION AND COMMUNITY STRUCTURE AND FUNCTION OF *ALNUS*-ASSOCIATED ECTOMYCORRHIZAL FUNGI IN ALASKAN BOREAL FORESTS

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Abstract

Rates of production and carbon cycling in northern ecosystems depend heavily on nitrogen (N) availability across the landscape. Since much of the available N enters these systems through biological N-fixation, *Alnus*, with its capacity to fix large amounts of N, plays a critical role in ecosystem response to environmental change. However, because of its high phosphorus (P) demands, the abundance, distribution, and N-fixing capacity of *Alnus* is tightly controlled by the availability of P and its ability to assimilate P by associating with ectomycorrhizal fungal (EMF) symbionts. We assessed the potential of *A. tenuifolia*-associated EMF to access organic P forms of varying complexities. More than half of the community on *A. tenuifolia* were individuals from the genera *Alnicola* and *Tomentella*, indicating that the community of EMF on *Alnus* is a relatively distinct group of host-specific ectomycorrhizal fungi. However, the aggregated acid phosphatase, phosphodiesterase and phytase activities of the *Alnus*-EMF community were not dramatically different from other boreal plant hosts on the root tip level. We detected variability in the activities of the two *Alnus* dominants to mobilize acid phosphatase and phosphodiesterase. However, it appears that contrary to the hypothesis that nitrogen-fixing species would associate with EMF types well suited to P acquisition, the potential acid phosphatase activity of *Alnicola luteolofibrillosa* was significantly below the community mean. Our finding that enzyme activities of *Alnus*-EMF are not substantially greater than those found on other plant hosts suggests that if host specific EMF on *Alnus* facilitates P mobilization and uptake, the steps between P hydrolysis and assimilation into plant tissue as well as other pathways of P acquisition may be of greater importance in determining P provisioning to *Alnus by EMF*.

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1. Introduction

Studies on nutrient controls over net primary production (NPP) in boreal forests have focused almost entirely on nitrogen (N). There is good reason for this, due to the close coupling between plant N content, photosynthesis and growth rates (Chapin III et al. 1986), and the slow rates of soil organic matter turnover in boreal forest soils (Flanagan and Van Cleve 1983, Rineau and Garbaye 2009). We know comparatively little about terrestrial phosphorus (P) cycling in boreal forests (but see Marion et al. 1993; Giesler et al. 2002) despite the tight stoichiometric coupling between N and P in terrestrial and aquatic ecosystems (Sterner and Elser 2002). Phosphorus is the second most common element limiting growth in terrestrial ecosystems globally, and some have suggested that it is as limiting to productivity as N across terrestrial, freshwater and marine and ecosystems (Elser et al. 2007). P limitation is likely to become even more prevalent with continued deposition of atmospheric N and under future increases of atmospheric CO_2 concentration, which have the potential to increase plant growth rates (Cairney 2011).

Globally, most soil P is of limited bioavailability. During the first phases of soil development, P is primarily found in highly reduced inorganic forms of insoluble calcium minerals, such as apatite, which are not directly available to plants (Marion et al. 1993). As soil development progresses, labile inorganic P increases and, for a time, is more available to plants and microbes than in the earliest stages of succession. However, over time mineral forms become progressively more occluded as Fe and Al oxides (Walker and Syers 1976, Crews et al. 1995), and alternately biological activity transforms mineral P into organic forms (Tedersoo et al. 2008). Thus, organic P content increases with successional time (Allison et al. 2007), and typically comprises between 30 - 65% of total P in developed soils, but can exceed 90% in some soils (Harrison 1982). Dominant soil organic P compounds include phosphate monoesters (such

as phosphorylated sugars, mononucleotides, and other low molecular weight compounds), complex phosphate diesters (predominantly DNA and phospholipids), and more recalcitrant inositol phosphates such as phytic acid (Turner et al. 2007). In mid to late soil development, phytates and other large, positively charged P molecules can be tightly bound to soil particles and only available to plants through the release of specialized hydrolyzing enzymes or organic acids. Because of the complex dynamics involved with P solubility and the variability of organic P in soils, traditional approaches to fractionating soil P (Lajtha et al. 1999) may not accurately reflect the pools of organic P available to plants and soil microbes (Quiquampoix and Mousain 2005, Doolette et al. 2010).

N-fixing plants play a unique role in P biogeochemical cycling because of the high P demand associated with nitrogenase activity and production of root nodules. This explains the high rates of both N and P cycling in litter of N-fixing plants (Ruess et al. 2013). How these plants acquire sufficient P to maintain high rates of N fixation is somewhat of a puzzle, particularly early in primary succession when N fixation rates are stimulated by low N and high light availabilities. In old, highly-weathered tropical forests where P availability is often chronically low, it has been suggested that N fixing plants are able to persist because of their ability to access low-solubility inorganic or recalcitrant organic P forms using extracellular phosphatases (Houlton et al. 2008). In general, extracellular hydrolytic enzymes produced by mycorrhizal associations are a primary mechanism by which plants acquire organic P in soils (Smith and Reed 2008).

Throughout interior Alaska, thin-leaf alder (*Alnus incana spp tenuifolia*, hereafter, *A. tenuifolia)* forms dense riparian stands where it can contribute >140 kg N ha^{-1} $year^{-1}$ to early successional floodplain soils through N fixation (Uliassi and Ruess 2002, Ruess et al. 2009,

2013). As stands develop, *A. tenuifolia* remains an important understory species in both mid-successional hardwood forests dominated by balsam poplar (*Populus balsamifera*), and in late-successional conifer stands dominated by white spruce (*Picea glauca*). Field experiments have shown that ecosystem N inputs by *A. tenuifolia* are dramatically increased by P fertilization via increases in both nodule biomass and nitrogenase activity (Uliassi and Ruess 2002, Ruess et al. 2013), suggesting that N fixation rates are strongly P-limited in these ecosystems. However, the fact that *A. tenuifolia* persists and continues to maintain high nodule biomass and N fixation rates even into late succession (Anderson et al. 2004) suggests this long-lived species potentially changes strategies to access P across this successional sequence.

Perhaps more than other non-N-fixing species, *Alnus* is under strong pressure to access all available forms of P. The dynamics of this process are complicated in boreal systems, as *Alnus sp.* has root systems that are almost entirely infected by ectomycorrhizal fungi (J.W. McFarland, unpublished data). The presence of red alder has also been shown to increase inorganic soil P availability as compared to nearby plots of Douglas fir (Giardina et al. 1995) illustrating their potential to directly influence nutrient availably for both themselves and other adjacent plants and the resultant effects on other soil biogeochemical processes. Given *Alnus*' strong P demands and high growth rates, it stands to reason that the EMF community associated with alder may be specialized for mobilizing much of the P taken up by *Alnus* (Dilly et al. 2000, Baar et al. 2000, Yamanaka et al. 2003).

Enzyme activity in individual ectomycorrhizal root tips can be measured with high throughput microplate assays (Pritsch et al. 1997, Courty et al. 2005). Theses assays have shown that many species of ectomycorrhizal fungi (EMF) produce phosphohydrolase enzymes when associated with both *Alnus* (Walker et al. 2014) and other plant hosts (Plassard et al. 2011). In

general, EMF community composition is affected by a number of factors including: soil chemistry (Courty et al. 2005), seasonality (Koide et al. 2007), and successional stage (Tedersoo et al. 2008). Relative to EM trees, *Alnus* associates with a limited number of EMF partners and is often reciprocally selective (Tedersoo et al. 2009, Kennedy and Hill 2010, Põlme et al. 2013, Roy et al. 2013). This may suggest that the composition of *Alnus*-EMF communities is tied to the ability of individual fungal species to mobilize the forms of organic P that exist in the soil.

Whereas phosphatase activity is frequently measured on EMF root tips (Kroehler and Linkins 1988, Colpaert et al. 1997, Courty et al. 2006) there is little information as to whether these associations can mobilize P from the more complex forms of organic P in the field, or how this capacity varies among EMF species. Because different enzymes are required for the breakdown of various P forms, hydrolyzing phosphate from compounds such as orthophosphate monoesters, phosphodiesters, and phytate may represent a different level of investment. Just as certain non-mycorrhizal plant species may specialize in accessing P from one or more of these forms (Turner 2008), it is likely that certain EMF partners have different strategies for acquiring P and may target one source over another. For example, EMF species vary in their capacity to produce phytase (Colpaert et al. 1997), and some species are able to use phytate as their only source of P (Wallander et al. 1997).

The objective of this study was to understand how *A. tenuifolia* and its associated mycorrhizal partners mobilize organic forms of P across spatial (mineral and organic soil horizons) and temporal (a chronosequence of successional stages) scales. This will help to identify the controls over biological N-fixation and the coupling of N and P biogeochemical cycling. We hypothesized that: 1) *Alnus* associated EMF vary in their capacity to mobilize different sources of P, 2) the variability in EMF enzymatic potential, the community composition

of EMF associated with *Alnus*, and the total P-mobilizing potential of the *Alnus*-EMF community are coordinated with spatial and temporal variation in soil resources associated with different stages of boreal forest stand development. Related to the spatial and temporal variation in soil resources in our system, that we expected that soil P will be gradually transformed by biological processes from inorganic apatite to more recalcitrant organic forms creating a gradient of soil resources, as seen in successional sequences elsewhere, though we did not explicitly test this in this study.

The extent to which the production and growth of *Alnus* can respond to changing environmental conditions such as atmospheric N deposition and increases in atmospheric CO_2 concentration will in large part depend on its ability to access P. This may be driven in large part by the plasticity of strategies for P acquisition employed by *Alnus* and its EMF partners.

2. Methods

2.1 Site description

Field work was conducted in Interior Alaska at the Bonanza Creek Long-Term Ecological Research Site approximately 30-45 km SW of Fairbanks. Sites were located across a 200-year old forest successional sequence along the Tanana River floodplain, that has been described extensively elsewhere (Ruess et al. 2013). *A. tenuifolia* is the dominant canopy woody species early in succession, and persists in the understory in older stands dominated by balsam poplar and then white spruce. Mean annual air temperature ranges between -2˚C and -5˚C, but daily temperatures are extremely variable, with average highs of 16.3˚C in July and lows of -45˚C from November through February. Soils at these sites are cryofluvents and frequently contain buried organic layers resulting from silt deposits during flooding. Annual precipitation averages 287mm, 35% of which falls as snow between October and April.

2.2. Root tip sampling

In June 2009 20 x 20 m plots were established in stands across a chronosequence including early successional thin-leaf alder, mid-successional balsam poplar, and late successional white spruce stands (hereafter referred to as stages). These plots were adjacent to pre-established plots monitored annually by the LTER. Samples were also collected at a wetland site just north of Fairbanks, representative of sub-hygric locations with deep organic layers where thin-leaf alder is often found in high abundance. During late August of 2009 one soil core (15 x 20 cm) was taken approximately 20 cm from the base of 10 randomly chosen *Alnus* genets at each of the 4 sites. Cores were immediately stored on ice, and transported to the lab. Each core was split into mineral and organic horizons. Root systems were then carefully shaken, removed from the core, and washed in cool water.

2.3 Root tip selection

Washed fine roots were further cleaned of debris under 10-40x magnification. *Alnus* fine roots were identified by their reddish coloring and the presence of nodules. EMF root tips, between 2-4 mm in length, were excised from *Alnus* roots using a stainless steel surgical forceps under, and stored in 0.5M $CaCl_2$. We collected fifty healthy tips per horizon, and from these, a subsample of seven tips was randomly selected to be assayed for enzymatic activity.

Digital pictures of individual root tips were taken on a 4.5 X 4.5 mm plastic grid through a microscope under a consistent magnification. Estimates of root tip surface area were determined using root image analysis software Rootfly (Version 1.8.31, 2005-2009, Clemson University). All measurements of enzyme activity are reported per square mm root surface area.

2.4 Enzyme assays

Within one hour of root tip selection, tips were rinsed of $CaCl_2$ using 50mM sodium acetate buffer (pH= 5.5) and run sequentially through a series of enzyme assays to assess their potential to mobilize phosphorus from organic sources in the following order: acid phosphatase, phosphodiesterase, and phytase.

Individual root tips were placed in sieve strips (Pritsch et al. 2004). In brief, strips of 8 200µl tubes were cut off and nylon mesh (250 micron) was fitted over the bottom of the tube and adhered with electrical heat shrink tubing. Individual root tips were placed in seven of the wells, with the eighth well left blank as a control. Sieve strips fit snugly into 96-well black-bottomed microplates (Corning Life Sciences, Tewksbury, Massachusetts, USA) and were moved among different microplates for multiple enzyme assays without damage to the root tip.

The potential activities of acid phosphatase (AP) and phostodiesterase (PD) were measured with MU-linked substrates: 6,8-difluoro-4-methylumbelliferyl phosphate (diFMUP, Invitrogen D6567) and Bis(4-methylumbelliferyl)phosphate (bisMUP, BioSynth B-3500), respectively. Substrates were dissolved in dimethylformamide. Root tips were assayed with two concentrations of each enzyme, diFMUP at 100µM and 250µM, and bisMUP at 100µM and 500µM. Tips were incubated in diFMUP for 10 minutes per concentration and in bisMUP for 30 minutes per concentration based on prior testing to optimize enzyme assays.

Between incubations in the same substrate, sieve strips were taken out of microplates, blotted on tissue to remove excess substrate, and rinsed for 3 minutes in wells of sodium acetate buffer. A similar procedure was followed between incubations in different enzyme substrates; however, the rinse incubation time was increased to 10 minutes. Additionally, sieve strips were gently rinsed under flowing sodium acetate buffer. At the end of the incubation period for each

enzyme, 90µl of the solution in each well was transferred into new a well containing 10µl 0.5M NaOH to stop the reaction and fluorescence was read immediately.

Wells containing substrate without root tips were included in microplates to control for autofluorescence of the substrate. Three reference standards were included in each plate using 0, 0.5, 1, 1.5, 2.5, 3.5, 4.5, 5.5mM concentrations of 6,8-difluoro-7- hydroxy-4-methylcoumarin (MU). Standards were used to generate calibration curves that related fluorescence values to the quantity of MU released by the enzyme.

Phytase activity was assayed using the EnzCheck Ultra Phytase Assay Kit (Molecular Probes Inc., Eugene, Oregon, USA). Root tips were placed into a reaction mixture containing 50mM sodium acetate buffer (pH= 5.5), maltose phosphorylase, maltose, glucose oxidase, horseradish peroxidase, Amplex UltraRed dissolved in dimethylsulfoxide, and 2.0mM phytic acid. Inorganic phosphorus (P_i) released from the hydrolysis of phytic acid during the assay reacts with enzymes in the reaction mixture to form H_2O_2 which reacts with Amplex UltraRed to create a florescent product that is proportional to the amount of P_i released. Wells containing reaction mixture without phytic acid were also included to adjust for autofluorescence of the reaction mixture. Root tips, in sieve strips, were placed in 100µl of the reaction mixture for one hour. At the end of the incubation, sieve strips were removed and 65µl solution was transferred from each well into a new read plate. Three reference standards were included in each plate. Standards contained the reaction mixture, with 0, 25, 50, 100, 200, 400, 800, 1600µM potassium phosphate replacing the phytic acid in the mixture.

Fluorescence was read with a wavelength of 360nm Ex/460nm Em for both diFMUP and bisMUP and at 540nm Ex/590nm Em for phytase on a Bio-Tek FL600 plate reader (Winooski, Vermont, USA).

Incubations were conducted at 20ºC in an environmental chamber. All incubations were completed within 14 hours of soil core collection. After incubations, root tips were freeze-dried and stored frozen for molecular analysis.

2.5 Molecular analysis

To match the enzyme activity with a specific fungal type, EMF species on individual root tips were characterized using molecular methods. DNA was extracted from individual root tips using the DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California USA). Prior to extraction, the root tips were hydrated in lysis buffer and disrupted using a Kontes pellet pestle attached to a drill. Samples were incubated at 65ºC for 10 min and then for 16 hours at 20ºC to improve the extraction success. The remainder of the extraction followed the standard protocol for the DNeasy Kit.

The EMF species on individual roots tips were identified using Sanger sequencing of the fungal nuclear ribosomal internal transcribed spacer (ITS). This region was amplified using the fungal specific primers ITS1-F and ITS4 (Gardes and Bruns 1993). Stock genomic DNA from extracted samples was diluted tenfold. Diluted template (5µl) was added to a PCR reaction mixture of 10X PCR Buffer (Sigma-Aldrich), 10mM dNTPs, 25mM $MgCl_2$, a 50mM concentration of each primer and 2.5KU of Taq polymerase (Sigma-Aldrich). The PCR cycling conditions for those reactions included an initial melt at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 35 s. The program terminated after a final extension at 72°C for 10 min.

After amplification, PCR products were sent to Functional Biosciences (Madison, WI) for sequencing in both directions (5' and 3') with ITS1-F and ITS4 primers. We used the methods of Taylor and Houston (2011) to assemble sequences, remove ambiguous and poor

quality sequences, and to group sequences into phylobins that were then assigned a fungal identity. In brief, reads were first trimmed and aligned using CodonCode Aligner 3.7 (Dedham, MA) and then assessed for base quality. Low quality bases, with a phred score below 20, were trimmed from ends, low quality bases within sequences were changed to Ns to remove ambiguous sequences, and sequences were oriented. We grouped sequences into phylogenetic clusters at approximately the species level using a script that carries out the following steps (Taylor and Houston 2011). First, sequences are clustered into broad groups using the genome assembly program TGICL (Pertea et al. 2003), which is based on Cap3 (Huang and Madan 1999), with default settings except a percent identity threshold of 90%. All sequences falling into a Cap3 'contig' are then subject to BLAST searches (Altschul et al. 1997) against the database in GenBank. All unique BLAST matches are added to the file of input sequences for that cluster. Next the query sequences plus BLAST matches within a cluster are aligned using default parameters in MUSCLE (Edgar 2004). Then the best maximum-likelihood phylogenetic tree is sought using the rapid bootstrap protocol in RAxML (Stamatakis et al. 2008). Lastly, the script explores the best tree and defines 'phylobins' based on a combination of branch length (defaults: minimum 0.001, maximum = 0.03) and bootstrap support (default: minimum = 70%, maximum = 95%). Representative sequences from each phylobin and singletons were submitted to the GenBank and UNITE databases. Phylobins were identified to species level at higher than 97% similarity; to the genus level at between 93% and 97% and to the family level at between 83% and 93% (Timling et al. 2014).

2.6 Plant host identification

After initial analysis of the fungal community revealed a wide array of fungi not characteristically detected on *Alnu*s, we used restriction length fragment polymorphism (RFLP)

to identify the plant host of all samples. As standard references, DNA from all abundant woody plant species found in our plot was extracted from fresh tissue using the DNeasy Plant Mini Kit (Qiagen Inc.). Prior to extraction, plant tissue was ground by hand using liquid nitrogen. Samples were extracted as described above. The trnL region of the plant chloroplast was amplified using the trnC - trnD primer pair (Taberlet et al. 1991, Kennedy et al. 2011) using DNA from both the plant tissue controls and from all root tip DNA used in enzyme analysis. The PCR cycling conditions for those reactions included an initial melt at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 35 The program terminated after a final extension at 72°C for 10 min. PCR products were digested with the TaqI restriction enzyme at 65°C for 2 hours and then run out on 3% agarose gels. Sample fragments were matched to banding patterns of plant standards for host identification. This analysis revealed that our root tips belonged to four plant species within our plots: *Alnus tenuifolia, Populus balsamifera, Picea glauca,* and *Salix spp.*

2.7 Statistical analysis

All statistical analyses were carried out in SAS (SAS Institute Inc., version 9.1). Data were tested for deviations from normality and homogeneity of variance by viewing plot fits and residuals; when non-normal, the data were log transformed. We used separate one-way ANOVAs to test for the effects of stage and horizon on potential enzyme activity of all root tips using PROC GLM. Using root tips for which we could identify a plant host, we tested again for the effects of stage, horizon, and plant host identity on potential enzyme activity. Finally, using root tips for which we could determine both a plant and fungal identity we tested the effect of fungal identity on potential enzyme activity. Least square means were estimated by the model and used in analysis. Multiple linear regression was used to test whether enzyme activities were

correlated across stages and horizons. Differences in enzyme activity among dominant EMF taxa were assessed with t-tests. We transformed our data into a distance matrix using a Bray-Curtis distance measure and then graphed the variance into an ordination plot using nonmetric multidimensional scaling (NMS) to visualize differences in fungal community structure by stage, horizon and plant host identity. We then tested the significance of these groupings using multi-response permutation procedures (MRPP). Generally, the threshold for statistical significance was set at $p < 0.05$. Because our root sampling techniques limited the statistical power of these tests, size constrained by root sampling techniques, we also discuss marginally significant values ($P < 0.10$) that are ecologically meaningful.

3. Results

3.1 Stage and horizon effects on enzyme activities

To examine how P-mobilizing enzymes vary through forest succession and between mineral and organic soil horizons, activities were first compared without regard to plant host or colonizing fungal species. Root tip acid phosphatase activity and phosphodiesterase activity showed strong responses to substrate concentration ($t=9.42$, 74, $p<0.001$; $t= 7.47$, 74, $p<0.001$, respectively), with rates being approximately double at the higher substrate concentration (Figure 1).

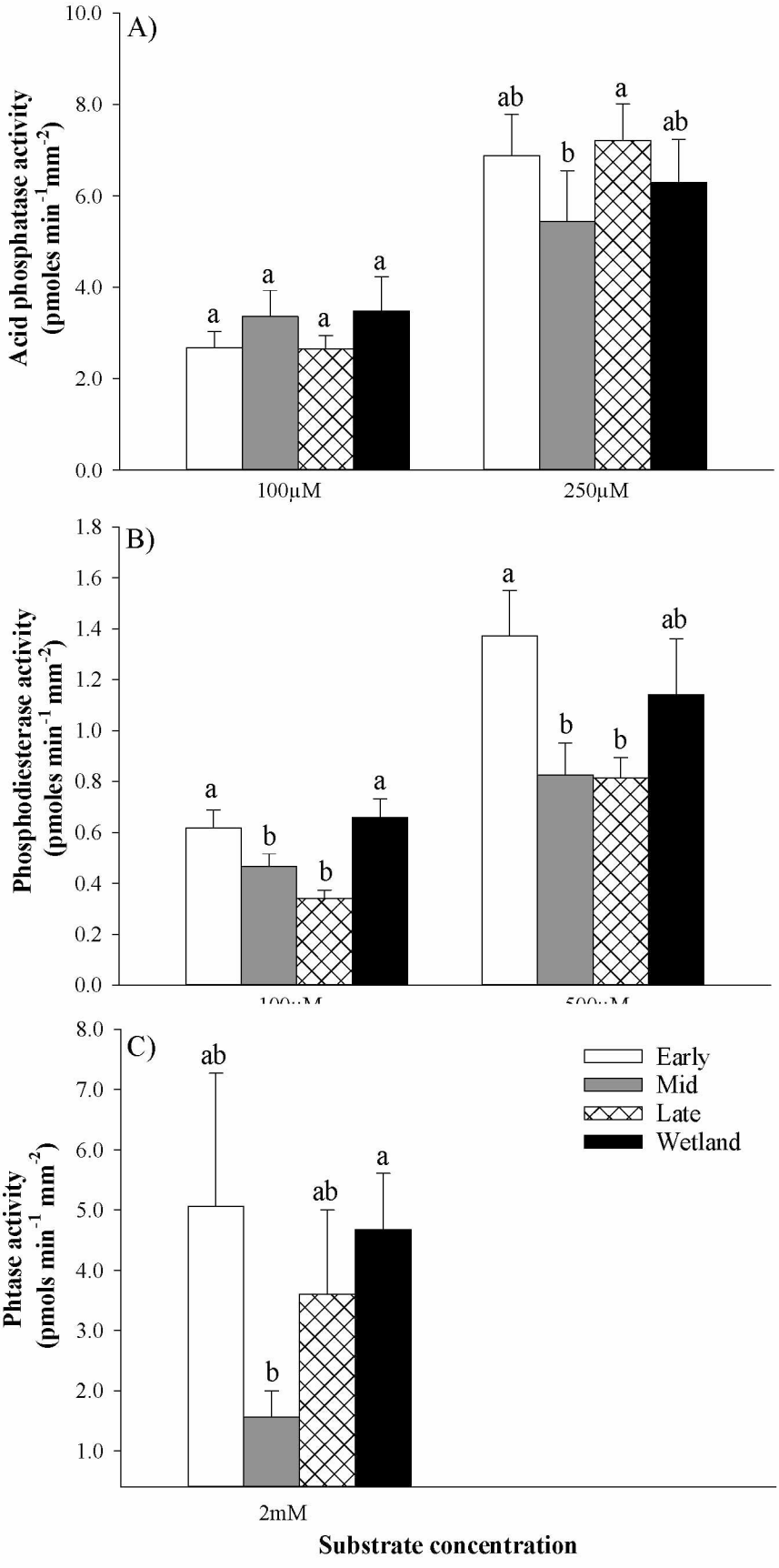
A)
Acid phosphatase activity (pmoles min-1 mm-2)
10.0
8.0
6.0
4.0
2.0
0.0
a
a
a
a
ab
b
a
ab
100µM
250µM
B)
Phosphodiesterase activity (pmoles min-1 mm-2)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
a
a
b
b
ab
C)
Phtase activity (pmols min-1 mm-2)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ab
b
ab
a
2mM
Substrate concentration
Early
Mid
Late
Wetland

Figure 1. Potential activities of A) acid phosphatase, B) phosphodiesterase and C) phytase enzymes on the surfaces of individual ectomycorrhizal root tips (n=524) collected from mineral and organic soil horizons at early, mid, late, and wetland successional sites near Fairbanks, AK. Bars represent the mean root tip enzyme activity from 10 replicate cores at each stage ± 1 SE. Different letters indicate significant differences ($p<0.05$) between stages within a substrate concentration.

Stage effects were significant for phosphodiesterase at both substrate concentrations (100μM, $F_{3,67} = 8.46$, $p<0.001$; 500μM, $F_{3,67}=2.87$, $p=0.042$), driven by higher activities in early succession ($p<0.05$) and at wetland sites ($p<0.05$), relative to mid and late successional stages. Root tip phytase activity was especially variable in early succession and did not differ significantly across successional stages ($p>0.10$) (Figure 1).

Activities of P-mobilizing enzymes did not differ consistently between mineral and organic soils across successional stages (Figure 2).

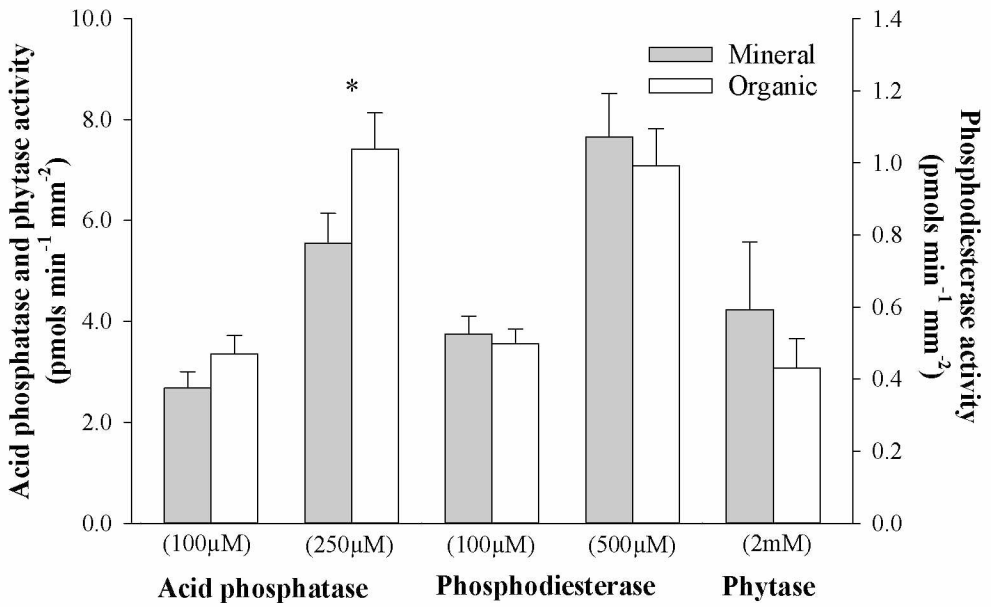

Figure 2. Potential activities of phosphorus mobilizing enzymes on the surfaces of individual ectomycorrhizal root tips (n=524) collected from mineral and organic soil horizons at early, mid, late, and wetland successional sites near Fairbanks, AK. Bars represent the mean activity of tips per core from mineral and organic horizons across all stages ± 1 SE. Significant differences between horizons are indicated with an *(*= $p<0.05$).

Acid phosphatase tended to have higher rates in organic compared with mineral horizons, but this effect was only significant at the higher substrate concentration ($F_{1,67}$=4.97, p=0.029), and was driven by large differences found in wetland soils (data not shown). In contrast, phosphodiesterase activity tended to be higher in mineral than in organic soils when measured at high substrate concentration, but again, this was significant only for wetland soils ($p<0.05$). Wetland soils had higher root-tip phytase activity in organic vs. mineral layers (6.91 ± 1.15 pmoles mm^{-2} min^{-1} vs. 2.72 ± 1.02 pmoles mm^{-2} min^{-1}), $p< 0.001$). However, when averaged across all sites, phytase activity tended to be higher in root tips from mineral compared to organic soils, although at these sites differences between horizons were not significant.

Across all samples, enzyme activities were positively inter-correlated (all pair-wise comparisons $p<0.001$). Yet, activities at the low substrate concentration for both acid phosphatase and phosphodiesterase explained less than half of the variation in activities at the high concentration. There were significant positive relationships between activities at the low substrate concentrations for acid phosphatase and phosphodiesterase at early (r^2= 0.167, $p<0.0001$), mid (r^2= 0.129, $p<0.0001$) late (r^2= 0.171, $p< 0.001$) stages, but not at wetland (r^2= 0.004, p=0.243) sites. There were similar positive relationships for activities at both the high concentrations of acid phosphatase and phosphodiesterase at all sites (all $p<0.05$). While relationships between enzyme activities within stages were highly significant, none were particularly strong (all r^2 values below 0.320); although there was a tendency for relationships to be stronger in organic than mineral soils. Notably, acid phosphatase and phosphodiesterase activity were both unable to predict phytase activity (all r^2 values below 0.10).

3.2 Enzyme activity differences across hosts

We unequivocally identified the plant host for 366 of the 524 root tips for which we assayed enzyme activities. Across all stages and horizons, activity differed by plant host for acid phosphatase (100μM) ($F_{3,345}$= 5.31, p=0.001), phosphodiesterase (100μM) ($F_{3,345}$= 25.48, p<0.001), phosphodiesterase (500μM) ($F_{3,345}$= 21.18, p<0.001), and phytase (2mM) ($F_{3,345}$= 4.38, p=0.005), but not for acid phosphatase at high substrate concentrations (250μM) ($F_{3,345}$=1.12, p=0.342) (Figure 3).

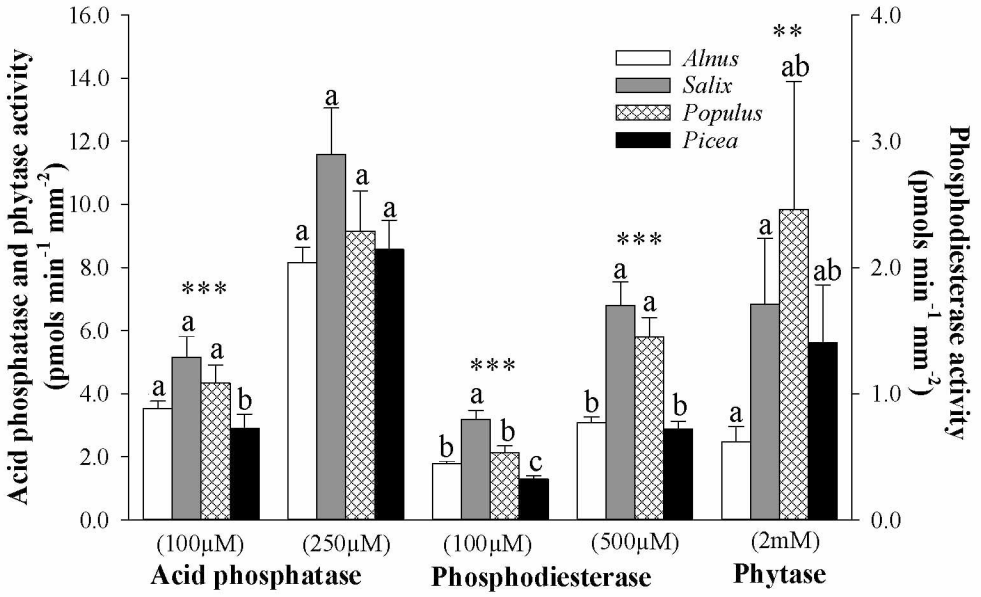

Figure 3. Potential activities of acid phosphatase (100µM), acid phosphatase (250µM), phostodiesterase (100µM), phostodiesterase (500µM), and phytase (2mM) enzymes by plant host on the surfaces of individual ectomycorrhizal root tips (n=366) collected from plots at early, mid, late, and wetland successional sites near Fairbanks, AK. Plant host species was identified to genus with RFLP, and Sanger sequencing where needed. Bars represent the mean activity of tips from mineral and organic horizons combined, across all stages. Significant differences between host for each enzyme are indicated with an *(*= $p<0.10$, **= $p<0.05$, ***= $p<0.001$). Different letters indicate significant differences ($p<0.05$) between hosts for each enzyme.

These differences were driven primarily by higher acid phosphatase and phosphodiesterase activities on *Salix* root tips, and lower acid phosphatase and phosphodiesterase activities on *Picea* root tips relative to the other species. Data for *Populus* do not appear robust enough for comparisons with other species due to low sample sizes, but limited data for *Salix* and *Populus* combined suggest that *Alnus'* root tip enzyme activities are not exceptionally high. Moreover, the data also fail to reveal strong differences in the enzyme activities on the root tips of gymnosperms vs. angiosperms across successional stages.

3.3 Individualistic responses within hosts across stages

Patterns of enzyme activity across successional stages and between horizons within host species were, in general, similar to overall patterns discussed above when data were averaged across host species. However, we note a few significant differences in enzyme activity across stages for particular hosts and enzymes. For example, AP activities on *Alnus* root tips varied significantly by stage (100μM, $F_{3,181} = 3.40$, $p=0.019$; 250μM, $F_{3,67}=3.181$, $p<0.001$), due to higher activities in late succession relative to early sites. Overall phosphodiesterase activity did vary across successional stages, peaking in early and wetland sites (Figure 1). This pattern generally held when activities were analyzed by host and was significant for *Salix* (100μM, $F_{3,181} = 6.12$, $p=0.004$; 500μM, $F_{3,67}=4.83$, $p=0.013$), *Alnus* (100μM, $F_{3,181} = 2.47$, $p=0.063$; 500μM, $F_{3,67}=6.88$, $p<0.001$) and the lower substrate concentration for *Picea* (100μM, $F_{3,181} = 5.94$, $p=0.001$) (Figure 4).

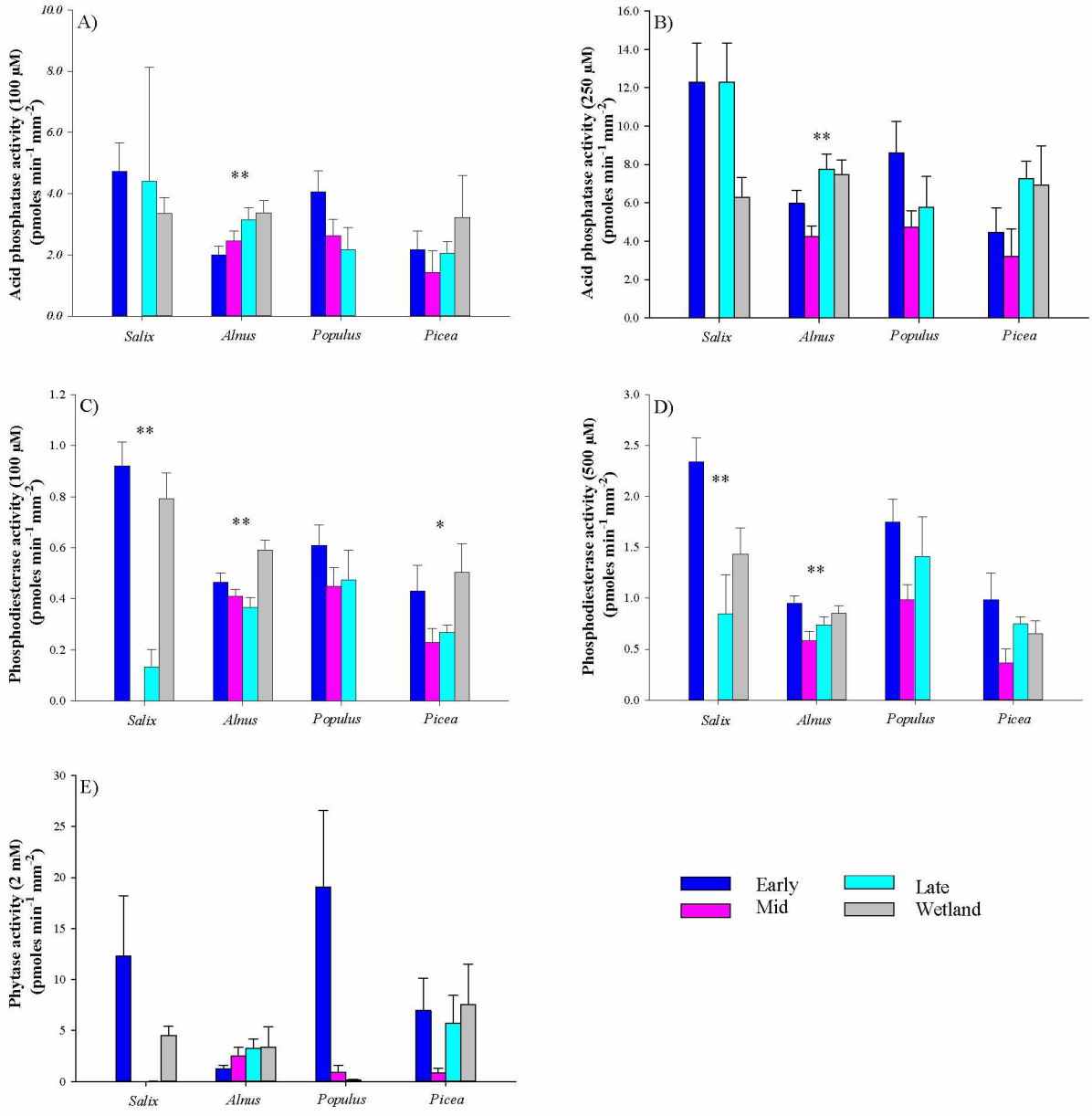

Figure 4. Potential activities of A) acid phosphatase (100µM), B) acid phosphatase (250µM), C) phostodiesterase (100µM), D) phostodiesterase (500µM), and E) phytase (2mM) enzymes on the surfaces of individual ectomycorrhizal root tips (n=366) collected from plots at early, mid, late, and wetland successional sites near Fairbanks, AK. Plant host species was identified to genus with RFLP, and Sanger sequencing where needed. Bars represent the mean activity of tips from mineral and organic horizons combined, across all stages ± 1 SE. Significant differences between horizons are indicated with an *(*= $p<0.01$, **=$p<0.05$).

Similar to our previous findings, phytase activity did not differ significantly across successional stage for any host and was highly variable in the early successional stage (Figure 4).

Whereas patterns of enzyme activities across successional stages were not consistently affected by plant host identity, analysis of activities within stages suggested important differences in the potential capacity of host species to mobilize different forms of P. For example, *Almus* phosphodiesterase did not vary by successional stage, but tended to be highest in early successional forests and wetlands (Figure 4). Significant stage affects for *Salix* phosphodiesterase activity were due to high activities in early succession. Enzyme activities on *Populus* root tips showed no significant stage effects, but tended to have highest activities of all enzymes in early successional stands (Figure 4). *Picea* tended to have lowest activities in mid succession, and higher activities of most enzymes in late-successional forests. When *Almus* and *Picea* were compared directly, phosphodiesterase (100μM) rates were significantly higher on *Almus* in late succession ($p=0.037$). Phytase activity did not vary significantly among stand types for any host; however, activity on *Almus* tended to increase across the successional sequence.

Almus exhibited greater variation in enzyme activity among stages compared to other host species (Figure 4). However, there were no consistent patterns across stages, across the three enzymes studied, or in comparison to other host species (Figure 4).

3.4 Host by horizon effects

Almus was the only host species that showed distinct differences in enzyme activities between organic and mineral horizons. Averaged across stages, acid phosphatase activities in mineral soils were 27% lower (100μM) and 26% lower (250μM) than in organic soils (both $P < 0.05$). Similarly, *Almus* phytase activity was 24% lower in mineral soils relative to organic soils, as was phosphodiesterase at 100μM (-4%, NS) and at 500μM (-2% NS).

Acid phosphatase activities (100μM) were lower in mineral soils than in organic soils ($F_{1,181}$ = 5.98, p=0.016), but this pattern was not seen for other enzymes. *Alnus* roots tips had 27% lower acid phosphatase (100μM) activity, 26% lower acid phosphatase (250μM) activity, 4% lower phosphodiesterase (100μM) activity, 2% lower phosphodiesterase (500μM) activity, and 24% lower for phytase (2mM) activity in mineral soils compared with organic soils.

Relationships between hosts were similar to those described above for enzyme activities across stages. The addition of host as a factor generally improved the percent of variability explained by the regression model. Again there were strong relationships between the same enzyme at both concentrations and between acid phosphatase and phosphodiesterase activity. However, neither acid phosphatase nor phosphodiesterase was a strong predictor of phytase activity.

3.5 Fungal community identification

After removal of potential fungal pathogens and known non-EMF types, we identified 112 unique fungal types, 60 basidiomycetes and 52 ascomycetes on 246 roots tips across all plant hosts (Appendix). The overall fungal community was dominated by basidiomycetes (69%) over ascomycetes (31%). This trend was similar on the 135 *Alnus* roots tips (73% basidiomycetes vs. 27% ascomycetes) as well as for *Salix sp.* tips (70% basidiomycetes vs. 30% ascomycetes). However, on *Picea* there were similar proportions of basidiomycetes (46%) to ascomycetes (54%). No ascomycetes were identified on balsam *Populus* root tips.

Acid phosphatase activities were not significantly different on ascomycetes vs. basidiomycete fungi when averaged across host species; however, basidiomycete fungi showed significantly higher phosphodiesterase (100μM) ($F_{1,297}$=12.75, p<0.001) and phytase activities ($F_{1,297}$=12.77, p<0.001) (Figure 5).

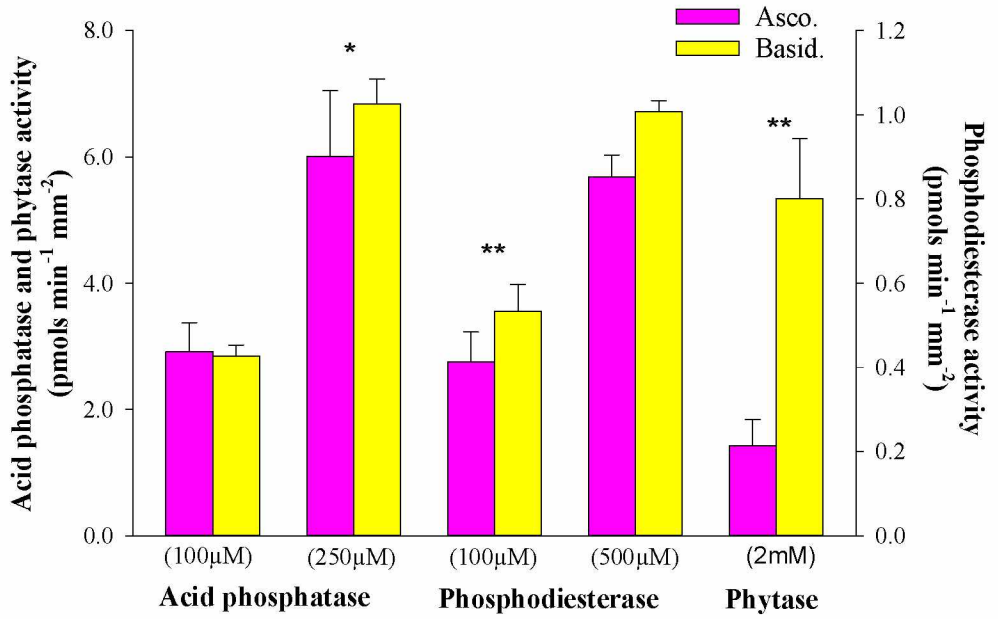

Figure 5. Potential activities of acid phosphatase (100µM), acid phosphatase (250µM), phostodiesterase (100µM), phostodiesterase (500µM), and phytase (2mM) enzymes on the surfaces of root tips infected with ascomycete and basidiomycete fungi (n=246) collected from plots at early, mid, late, and wetland successional sites near Fairbanks, AK. Bars represent the mean activity of tips from mineral and organic horizons combined, across all stages ± 1 SE. Significant differences between horizons are indicated with an * (*= $p<0.01$, **=$p<0.05$).

In general, there was little overlap of dominant fungal species among the EMF communities on different plant hosts (Figure 6).

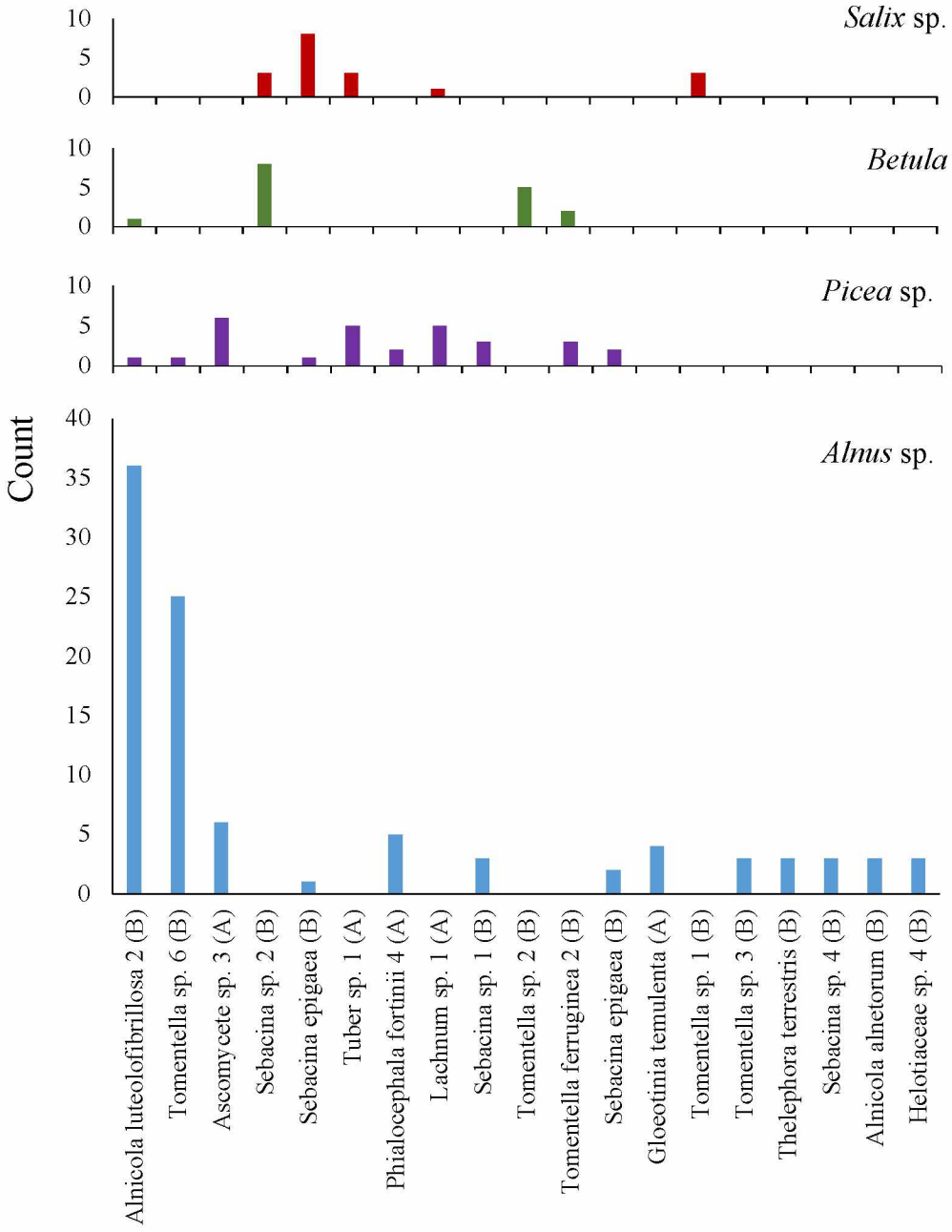

Figure 6. Frequency of the 19 most abundant EMF species on four host plant species collected across early-, mid-, and late-successional, and wetland stages near Fairbanks, AK. Fungal species were characterized with Sanger sequencing and plant host species was identified to genus with RFLP, and Sanger sequencing where needed. Bars represent total abundance across successional stages for each plant species. Sample sizes are unequal across plant hosts: *Salix sp.* (37), *Populus balsamifera* (20), *Picea sp.* (54) *and Almus sp.* (135). Ascomycete and Basidiomycetes are denoted with (A) and (B), respectively.

MRPP analyses of fungal communities indicated that fungal community composition varied significantly among hosts (A = 0.149, p<0.0001) and successional stages (A = 0.094, p< 0.001). The strongest grouping from the NMS ordination was for host, particularly for *Alnus*-associated fungi (Figure 7).

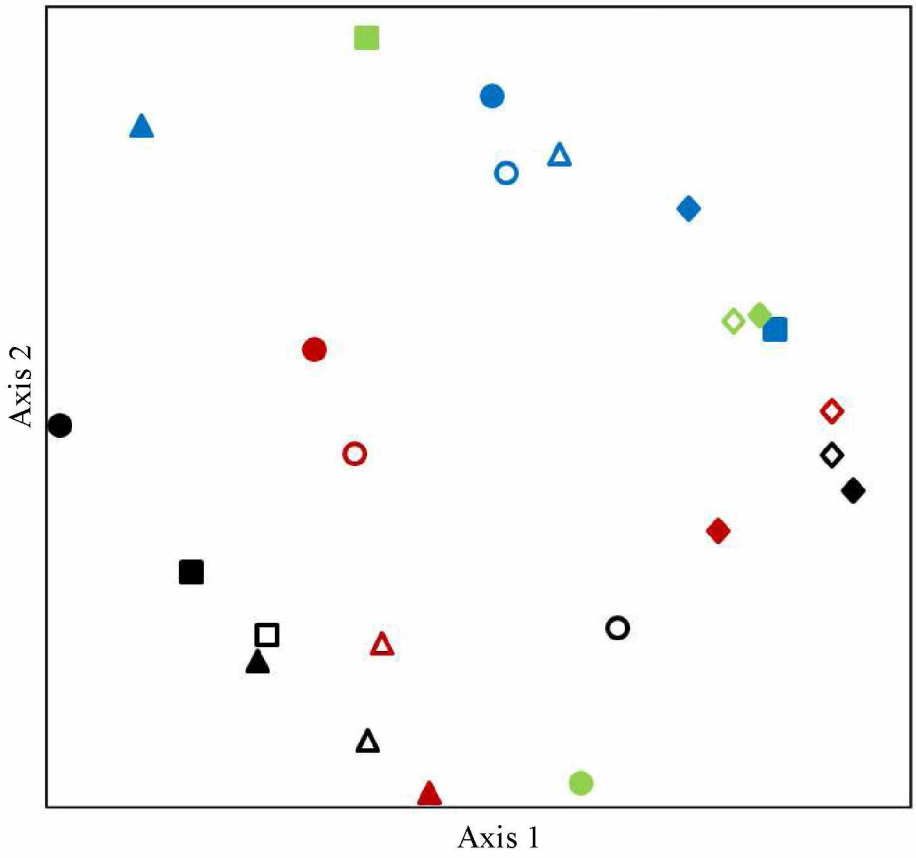

Figure 7. Non-metric multidimensional scaling (NMS) ordination of EMF species on root tips from mineral and organic soils across four successional stages at sites near Fairbanks, AK. Shapes indicate host plant species: *Alnus* (diamond), *Salix* (triangle), *Populus* (square), *Picea* (circle), and colors indicate successional stages: early (black), mid (green), late (blue) and wetland (red). Open shapes represent mineral soils and filled shapes represent organic soils.

Horizon did not explain any of the variation in fungal community structure; *Alnus* in particular showed strong similarities in fungal communities between horizons within successional stages. Also, there are relatively tight parings between mineral and organic horizons of a particular host.

3.6 EMF specific activities within and among hosts

Low sample sizes of several plant host species and minimal overlap of EMF species among hosts limited our ability to compare enzyme activities of specific EMF species among hosts or successional stages on a particular host. However, a few important examples were evident. Five EMF species occurred on one host across multiple stages or on several hosts within the same successional stage; two of these (*Ascomycete* sp. 3 and *Lachnum* sp. 1) were shared by *Alnus* and *Picea*. There was evidence that some enzyme activities from roots associated with these fungal species were higher on *Alnus* than on *Picea* across successional stages. Between *Salix* and *Populus* hosts, however, there was no significant difference in enzyme activities on root tips infected with *Sebacina* sp. 2. (both $p > 0.05$), an EMF type found on both hosts.

The two dominant *Alnus*-associated EMF taxa, *Alnicola luteolofibrillosa* and *Tomentella* sp 6, showed significant differences in their enzymatic capacity to break down different sources of organic phosphorus. *Tomentella* sp. 6 showed a greater potential acid phosphatase activity at both the low and high substrate concentration, whereas *Alnicola luteolofirbillosa 2* had higher potential phosphodiersterase activity at the higher substrate concentration (Figure 8).

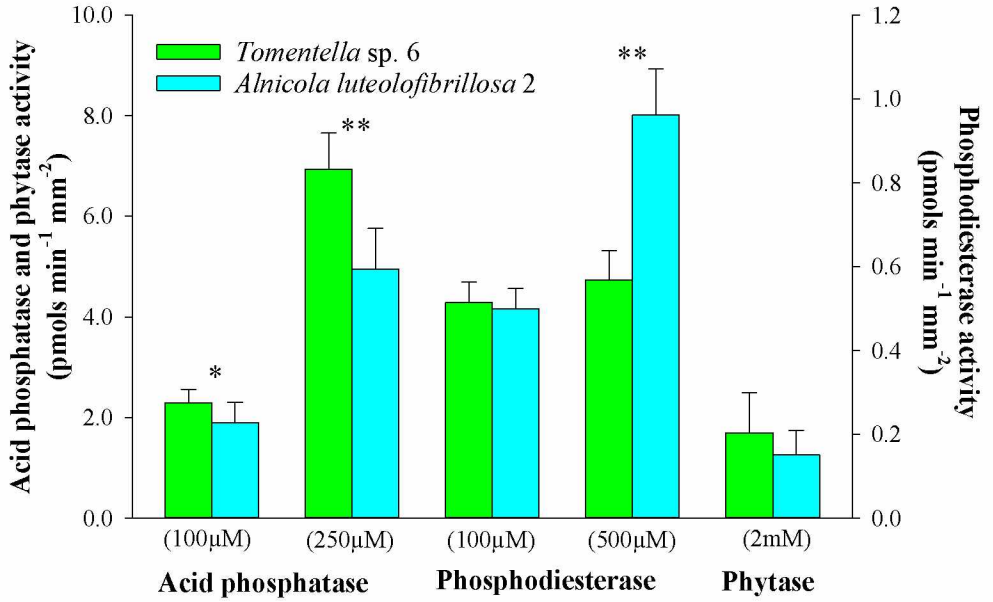

Figure 8. Potential activities of acid phosphatase (100µM), acid phosphatase (250µM), phostodiesterase (100µM), phostodiesterase (500µM) and phytase (2mM) enzymes on the surfaces of two dominant EMF on *Almus* root tips: *Alnicola luteolofirbillosa 2 and Tomentella* sp. 6. (n=135) collected from plots at early, mid, late, and wetland successional sites near Fairbanks, AK. Plant host species was identified to genus with RFLP, and Sanger sequencing where needed. Bars represent the mean activity across all stages ± 1 SE. Significant differences between horizons are indicated with an *(*= $p<0.01$, **=$p<0.05$).

Alnicola luteolofibrillosa 2 was much more abundant in early succession on *Almus*, but enzyme activities were similar between early successional stands and wetland sites for this species (data not shown). Phosphodiesterase activities of *Tomentella* sp 6. infected root tips, an *Almus* specialist more evenly distributed across stages, were significantly different across stages, due to higher activities in early and wetland stand types as compared to mid and late successional sites, but this was only significant at higher substrate concentrations (500μM) ($F_{1=3,21}$ =12.77, p=0.021).

4. Discussion

4.1 Successional patterns of root-tip P mobilizing enzymes

Soil development during primary succession along interior Alaskan floodplains involves vegetation-driven transformations in soil C, N, pH, and organic matter quality over an approximately 200-year vegetation sequence from deciduous shrubs, to overstory hardwoods, and eventually conifer-dominated stands (Van Cleve et al. 1993). We predicted that over the course of succession, soil P would be gradually transformed by the biota from inorganic apatite, which is the dominant form of P in alluvial loess soils (Marion et al. 1993), to progressively more recalcitrant organic forms, reminiscent of models of soil P transformations elsewhere (Walker and Syers 1976). We predicted that in response to these changes, activities of P-mobilizing enzymes on *Almus* root tips would shift towards increases in phosphodiesterase and phytase as soil organic matter accumulated and became more recalcitrant (Flanagan and Van Cleve 1983), particularly in white spruce and wetland stands. We found only partial support for this prediction; *Almus* root tips showed a progressive increase in phytase activity across stands, with lowest values recorded in early successional forests and highest values in wetlands, where soil organic layers are least and most developed, respectively. Furthermore, there were no

distinct successional patterns in enzyme activities consistent with this successional model when all host species were combined (Figure 1), or examined separately (Figure 4). However, recent ^{31}P-NMR data (K.D. Olson, unpublished data, 2010) indicate that while the amount of soil organic P increases during succession on an area basis as soil organic C accumulates, concentrations of organic P, and proportions of the dominant forms of inorganic (orthophosphate and pyrophosphate) and organic (primarily phosphomonoesters and phosphodiesters) P within organic horizons are actually quite similar across successional stages, once a dense *Alnus* canopy develops in early succession. This is somewhat similar to the increase in area-based total free amino acids across succession despite relatively similar proportions of individual amino acids across successional stages (K.D. Olson, unpublished data, 2010). Thus, the lack of an increase in enzymes specifically targeting organic forms of P may not necessarily be unexpected. The fact that rates of P-mobilizing enzymes were positively intercorrelated across all root tips sampled, suggests that ectomycorrhizae are using a coordinated suite of enzymes to meet P demands, or perhaps simply that healthier root tips/mycorrhizae have more intact enzymes. Within the early successional stands we sampled, organic horizons are poorly developed, with an approximate thickness of 1 cm. Nevertheless, there is a high root density of early successional woody species in surface soils (Ruess et al. 1998), and likely a rapid turnover of organic P from decaying microbes and plant tissues, which may explain why all three enzymes appear to be up-regulated in a coordinated manner to meet plant P demands. For this reason, it would make sense for gene transcription for P-mobilizing enzymes to be linked. This could be tested by comparing activities of these same enzymes during very early stages of floodplain succession prior to the establishment of a dense *Alnus* canopy, where apatite is the dominant form of P in soils (K.D.

Olson, unpublished data 2010), or in controlled experiments where *Alnus* is supplied only a recalcitrant form of P such as apatite.

4.2 Differences in enzyme activities among host species

A motivation behind this study was the strong growth and physiological responses of *A. tenuifolia* to field P-fertilization (Uliassi and Ruess 2002, Ruess et al. 2013), and the suggestion that dominance of N-fixing plants in early successional environments is due in part to their unique capacity to access low-solubility inorganic or recalcitrant organic P forms using extracellular enzymes (Houlton et al. 2008). Although our original intent was to focus on successional patterns of ectomycorrhizal community composition and P-mobilizing enzyme activities in *Alnus*, root tips of other host species among our samples offer additional insight into whether and how *Alnus* contributes uniquely to P cycling in these ecosystems. In this regard, it is notable that activities of P-mobilizing enzymes were not greater on *Alnus* root tips relative to other hosts, contrary to what would be predicted by the high P demands of *Alnus* (Huss-Danell et al. 2002, Gentili et al. 2006, Ruess et al. 2013), and known capacity of *Alnus* to increase soil P availability (Giardina et al. 1995, Mitchell and Ruess 2009). Moreover, other than lower acid phosphatase and phosphodiesterase activities in spruce (100µM substrate concentration), and a tendency for higher enzyme activities in *Salix* and *Populus* root tips in early successional soils, we found statistically similar activities across the four host species for all three enzymes (Figure 3). We suspect low sample size for the non-*Alnus* hosts contributed to our inability to detect greater interspecific differences (Figure 4). Physiological functions, including nutrient uptake rate, and morphological characteristics, including root architecture and demography, are closely coupled with soil nutrient availability, as well as growth rates and other functional traits of boreal and arctic species (Chapin III et al. 1986, Reich et al. 1998, Pregitzer et al. 2002, Burton et al.

2002, Ruess et al. 2006, Kielland and Chapin III 2014). Our early successional stands (~25 years old) were a closed canopy of dense, mature *Alnus*, with scattered *Populus* and *Salix* extending into and above the canopy, but with seedlings and small saplings of spruce growing in dense shade. Whereas these growth conditions for the four host species appear to parallel trends in P-mobilizing enzyme activities in these early successional stands, these inferences are clearly speculative. Nevertheless, it speaks for the need to measure root-tip enzyme activities of younger *Alnus* (and other species) growing earlier in stand development (0-15 years) under high light conditions, in order to definitively test whether *Alnus* has greater capacity to utilize apatite, phosphodiesters and inositols than other woody species.

3.3 Structure of Alnus ectomycorrhizal communities

Our results support a growing literature indicating that *Alnus* associates with a relatively low diversity of host-specific ectomycorrhizal fungi of approximately 50-60 species (Põlme et al. 2013). We identified forty-six fungal phylobins on *Alnus*, the majority (73%) in the Phylum Basidiomycota. Of the *Alnus* phylobins, five were found more than four times: *Alnicola luteolofibrillosa* 2 (297), *Tomentella* sp. 6 (22), Ascomycete sp. 3 (28), *Phialocephala fortinii* 4 (339), and *Lactarius lilacinus* (89) (Figure 6, Appendix). Twelve rarer types were found between one and three times; five out of these twelve were found in more than one stage. The remaining twenty-nine phylobins were detected only once across all sites combined. *Alnicola* and *Tomentella*, the two dominant genera in our study, have been recognized as some of the most common *Alnus*-specific EMF taxa across multiple biomes throughout Europe, North and South America (Pritsch et al. 1997, Moreau et al. 2006, Tedersoo et al. 2009). Individuals from these two genera made up 55% of the total community on *Alnus* across our sites. We detected species from other EMF genera known to be selective towards *Alnus*, including *Alpova* (Tedersoo et al.

2009, Rochet et al. 2011, Bogar and Kennedy 2013), *Inocybe* (Kennedy and Hill 2010), *Tuber* (Tedersoo et al. 2009), *Lactarius* (Rochet et al. 2011) and *Cortinarius* (Walker et al. 2014), but did not find other common *Alnus*-specific EMF lineages including *Peziza Michelii–Peziza Succosa, Genea–Humaria, Pachyphloeus–Aamylascus,* and *Tarzetta–Geopyxis* (Tedersoo et al. 2009, Rochet et al. 2011).

The composition and low diversity of the *Alnus*-EMF communities we detected across stand types are not surprising given the strong fidelity of *Alnus*-EMF relationships (Tedersoo et al. 2009), particularly for basidiomycetes (Molina 1981, Kennedy et al. 2011). However, similar to our data, some of the most common EMF genera containing species compatible with Alnus such as *Alnicola* and *Tomentella* have been found capable of colonizing other plant hosts including *Betula paperifera* (Bogar and Kennedy 2013). Nevertheless, we found little overlap of the dominant fungal species among plant hosts, and results of our MRPP analyses support the notion that *Alnus* EMF communities are highly distinct relative to other host species. However, we do not have the sample size to adequately characterize EMF communities for other plant hosts at these sites.

Although the strong host specificity of EMF on *Alnus* has been repeatedly confirmed, reasons behind this are less clear. The specificity of host-EMF relationships may result from geographic isolation; however, some argue that such specialization derives from spatial heterogeneity in soil physical/chemical parameters, and specialization to those conditions (Roy et al. 2013). For instance, Huggins et al.(2014) showed that the high acidity and nitrate levels characteristic of soils under *Alnus* may select for specific EMF types that can tolerate these conditions. It is also possible that the unique physiology of N-fixing species require EMF partners well suited to facilitating their growth. This could include specialization for P

mobilization and uptake, as mentioned above, given the capacity of EMF to aide in P acquisition (Bolan 1991, Cairney 1999, 2011). But specialization could also be tied to micronutrient acquisition, e.g. Mo, which is required by *Alnus* as a co-factor in the protein complex that reduces atmospheric N_2. To our knowledge studies have not investigated the ability *Alnus*-EMF to access micronutrients, but their distribution and richness has been tied to soil macronutrient levels, including calcium (Põlme et al. 2013) and potassium (Roy et al. 2013). Finally, given that *Alnus* appears to shift *Frankia* partners across successional stages based on their carbon cost relative to N-fixing capacity (Ruess et al. 2013, Anderson et al. 2013), similar market economies may be at play in *Alnus*-EMF communities. Ruess et al. (2013) suggested that the up-regulation of nodulation and N-fixation by *Alnus* in P-fertilized plots was enabled by lower carbon partitioning to ectomycorrhizae. The combined costs of having to support both N-fixing bacterial partners and mycorrhizal partners place unique carbon demands on N-fixing plants, and suggest that hosts may closely regulate carbon partitioning to EMF mutualists relative to the benefits they afford. Yamanaka et al. (2003) found that *Alnus tenuifolia* grown on a mix of perlite and crushed basalt, and inoculated with both *Frankia* and *Alpova diplophloeus*, a known *Alnus* partner, had higher growth rates than seedlings grown with either partner separately, suggesting a strong cross-dependency for this tripartite relationship. Whereas some studies have indicated that *Alnus* appears to be unique among N-fixers in its EMF specificity (Scheublin et al. 2004), a detailed assessment of cost:benefit tradeoffs across a suite of potential EMF and Frankia partners using multiple carbon and element isotope probes may help resolve explanations for the low diversity and high degree of specialization found in Alnus-EMF communities.

4.4 Patterns of EMF enzyme activities

Our analysis of enzyme activities on *Alnus* root tips suggests pronounced physiological differences in the capacity of the two EMF dominants, *A. luteolofirbillosa* 2 and *Tomentella sp. 6*, to mobilize P from various P compounds across the successional gradient. *A. luteolofirbillosa 2* had significantly higher acid phosphatase activities than *Tomentella sp. 6* (Figure 8), particularly in early successional stands, where *A. luteolofibrillosa* was the most abundant EMF partner identified (data not shown). In contrast, *Tomentella* sp. 6 had significantly higher phosphodiesterase activity than *A. luteolofibrillosa* when measured at high substrate concentration, suggesting that an enhanced ability to hydrolyze phosphate from diesters is associated with a higher abundance of phosphodiesterase enzymes on the root tip surface. This difference in the enzyme activity between EMF dominants suggests that these mycorrhizal species differ in their ability to access P from different substrates and will thus have differing benefits to *Alnus*. While there is clear distinction in the enzyme activities of these two dominants, neither appeared to be greatly superior in their capacity to mobilize P from organic sources relative to the *Alnus*-EMF community as a whole. In fact, *A. luteolofibrillosa* had significantly lower than average acid phosphatase activity at both the low ($p<0.001$) and high substrate ($p<0.01$) concentration relative to all other species combined, excluding *Tomentella* sp. 6.

There have been few other studies that have tested the functionality of *Alnus*-EMF communities as they relate to phosphorus mobilization. However, Walker et al. (2014) found that the potential acid phosphatase activity of the EMF community on *Alnus rubra* was significantly higher than that of Douglas fir (*Pseudotsuga menziesii)* at two sites in central Oregon. Interestingly, they also found that some of the most dominant fungi in the *Alnus*-EMF

community did not demonstrate superior enzymatic activity relative to the community average, similar to our finding that *Alnicola* sp. has reduced capacity to mobilize P from phosphomonoesters.

Colonization by EMF is known to increase P concentration in plant tissues (Smith and Reed 2008). For example, Yamanaka et al. (2003) demonstrated that *Alnus* tissues had higher P concentrations when colonized by *A. diplophloeus*. This would support the suggestion that there should be selection pressure for *Alnus* to associate with fungi that are P specialists (Houlton et al. 2008). Given mixed results across the few studies that have investigated the functionality of *Alnus*-EMF communities, it remains unresolved how *Alnus* meets its phosphorus demands and achieves high foliar P concentrations. Our finding that the enzyme activities of *Alnus*-EMF dominants and the *Alnus*-EMF community as a whole are not substantially greater than those found on other plant hosts suggests that if the community of host specific EMF on *Alnus* is related to P mobilization other factors such as a unique EMF foraging strategy, extent of hyphal penetration in the soil, density of first-order root tips colonized by EMF along a branch, EMF growth rate, or P uptake and transport (Bolan 1991), which all affect P assimilation over the whole plant-fungal interface, are essential to the benefit EMF play in providing their host with P. Exploration type of the EMF assemblage is likely an important factor for determining P acquisition. There are significant differences in the mantles and in the exploration types of EMF fungi (Agerer 2001), and EMF types vary in the amount of mantle as a proportion of total structure (Alvarez et al. 2012). A given EMF species may have lower enzyme efficiency or infection frequency across root tips, but a more extensive hyphal system would facilitate access to P at greater distances from the root resulting in greater overall P uptake. Additionally, much of the P assimilated by the EMF is retained by the fungus (Colpaert et al. 1999, Cairney 2011)

which limits P transfer to the plant host, despite a high potential for P hydrolysis at the root surface. Differences among EMF species in the proportion of P transferred to the host would not be detected by measuring root tip enzyme activities, but may explain some of the specialization we detected in EMF. In boreal forest soils of interior Alaska, we observed the greatest proliferation of EMF-infected root tips late in the growing season, suggesting that host carbon partitioning to EMF may facilitate nutrient acquisition for storage that is then used for growth the following spring. Future studies are needed to consider the steps between P hydrolysis and assimilation into plant tissue, and the time and scale at which these processes take place to fully understand EMF-host specificity.

4.5 Phytase activity

Although several studies have assessed the potential of EMF to mobilize P from organic sources (Bolan 1991, Plassard et al. 2011, Cairney 2011) most have focused on acid phosphatase activity (Courty et al. 2006, Rineau and Garbaye 2009, van Aarle and Plassard 2010, Alvarez et al. 2012, Walker et al. 2014) and to a lesser extent phosphodiesterase activity (Allison et al. 2007). Phytase activity has rarely been measured on EMF root tips (with the exception of Colpaert et al., 1997) and never before to our knowledge on *Alnus*, despite the reputedly large proportion of phytate in total soil P pools (Turner et al. 2002, 2007). Reasons behind this may relate to the importance of simple phosphomonoesters as a component of soil organic P. However, this phenomenon may also be methodological; assessing phytase activity on root tips and in soils is difficult. The method we used for measuring phytase activity on root tips has potential to further our understanding of the role of EMF in breaking down complex recalcitrant P molecules, that often make up very large proportions of the organic P pool (Dalal 1977, Turner

et al. 2002, 2007), and may become increasingly important where P is limiting. Recently, other methods have also been developed for assessing phytase activity in soils (Berry et al. 2009).

We were unable to detect major differences in phytase activity across succession, between horizons, or hosts; however, we note the high variability of this enzyme activity at all levels. This may suggest large differences among EMF species in the capacity to release phytase. Doolette et al. (2010) studied phytate decomposition using repeated sampling and ^{31}P-NMR analysis following phytate additions to calcareous soils, and reported relatively rapid turnover rates, suggesting this complex organic P compound is highly available biologically. Understanding how host-EMF mutualisms respond to, and influence organic soil P fractions is clearly important to broader questions of P biogeochemical cycling in boreal forests, which are now approachable given refined methods for the analyses of soil P fractions, enzyme activities, EMF identities, and functional genes.

5. Conclusions

Given the strong differences in enzyme activities between the two dominant *Alnus*-associated fungi in our study, it is puzzling that we didn't find larger shifts in EMF community composition or aggregated enzyme rates at the whole plant or ecosystem level. Many other studies document variation in the enzyme activities of individual EMF, but assessing the enzyme potential at the plant or system level is more difficult. This work points to the importance of such knowledge as the benefit to the plant will not only be dependent on the activities of individuals but their abundance in the community.

The similarity in activity rates of P-mobilizing enzymes measured under controlled conditions for *Alnus* growing across very different stand types, and in *Alnus* relative to other dominant woody plant species may not necessarily translate to *in situ* differences in the effects

that these host species have on P biogeochemical cycling at the stand level. In the field, rates of whole plant P hydrolysis and acquisition are impacted by soil temperature and moisture, root density, plant growth and biomass partitioning, and whole-plant P demands. Average growing season soil temperatures decline from early to mid to late-successional sites, and soil moisture across stand types can be highly variable (Ruess et al. 2013). In early successional stands where *Alnus* dominates, *in situ* rates of root-tip P-mobilizing enzyme activity rates may be higher than where *Alnus* grows in later successional soils where soil temperature is reduced by mosses, litter, and canopy cover, and soil moisture is often lower due to higher terrace heights. Moreover, in both mid-succession and late-succession, where *Alnus* grows in the sub-canopy of balsam poplar and white spruce, respectively, light levels are lower, and *Alnus* growth rates and N-fixation rates are reduced relative to early successional stands (Ruess et al. 2013). Consistent with greater growth and N-fixation, whole plant P demand, as indexed by higher leaf P concentrations and greater N-fixation responses to P fertilization, is higher in these early-successional, dense stands dominated by *Alnus* (Ruess et al. 2013).

Appendix.

Phy-lobin	Assigned study name	Best Match	Phylum
278	*Alnicola alnetorum*	*Naucoria alnetorum*	Basid
292	*Alnicola luteolofibrillosa 1*	*Alnicola luteolofibrillosa*	Basid
297	*Alnicola luteolofibrillosa 2*	*Alnicola luteolofibrillosa*	Basid
293	*Alnicola silvaenovae*	*Alnicola silvaenovae*	Basid
301	*Alnicola tantilla*	*Alnicola tantilla*	Basid
274	*Alpova alpestris*	*Alpova alpestris*	Basid
162	*Amphinema byssoides*	*Amphinema byssoides*	Basid
167	*Amphinema* sp. 1	*Amphinema* sp.	Basid
68	*Articulospora* sp.	uncultured Articulospora	Asco
26	Ascomycete sp. 1	Ascomycete sp.	Asco
27	Ascomycete sp. 2	Ascomycete sp.	Asco
28	Ascomycete sp. 3	Ascomycete sp.	Asco
30	*Cadophora finlandica 1*	*Cadophora finlandica*	Asco
31	*Cadophora finlandica 2*	*Cadophora finlandica*	Asco
364	*Cadophora luteo-olivacea*	*Cadophora luteo-olivacea*	Asco
376	*Cenococcum geophilum*	*Cenococcum geophilum*	Asco
139	*Chalara holubovae*	*Chalara holubovae*	Asco

GenBank	UNITE ID	Match	Align-ment Length (bp)	Score
	SH190731.06FU	98.54	615	1088
JN943976		97.90	714	1234
	SH190719.06FU	99.00	599	1072
JN943941		97.37	685	1162
	SH190747.06FU	99.67	605	1107
	SH241746.06FU	97.24	688	1162
	SH029314.06FU	99.79	471	865
	SH229867.06FU	99.80	507	931
	SH234748.06FU	96.55	464	767
	SH037210.06FU	91.01	367	488
	SH037210.06FU	90.44	366	473
	SH037210.06FU	93.44	673	590
	SH207167.06FU	98.92	465	830
	SH207167.06FU	98.07	466	809
	SH204718.06FU	97.24	544	918
JN943893		97.61	964	1648
	SH209190.06FU	97.78	451	773

Appendix cont.

Phylobin	Assigned study name	Best Match	Phylum	GenBank	UNITE ID	Match	Alignment Length (bp)	Score
141	*Chalara microchona*	*Chalara microchona*	Asco	DQ093752		98.90	451	778
64	*Cladophialophora* sp. 1	uncultured *Cladophialophora*	Asco		SH025802.06FU	98.22	506	885
103	Cortinariaceae sp. 1	*Alnicola luteolofibrillosa*	Basid		SH190719.06FU	89.93	606	767
104	Cortinariaceae sp. 2	*Alnicola luteolofibrillosa*	Basid	JN943976		90.26	688	878
238	Cortinariaceae sp. 3	Cortinariaceae sp.	Basid		SH232566.06FU	99.02	612	1096
352	Cortinariaceae sp. 4	*Hebeloma testaceum*	Basid		SH190721.06FU	84.04	614	564
207	*Cortinarius casimiri 1*	*Cortinarius casimiri*	Basid		SH232912.06FU	96.08	510	830
208	*Cortinarius casimiri 2*	*Cortinarius casimiri*	Basid		SH232830.06FU	99.41	507	920
237	*Cortinarius cephalixu*	*Cortinarius cephalixu*	Basid	AY174784		99.29	708	1279
199	*Cortinarius* sp. 1	*Cortinarius subsertipes*	Basid	HQ604709		96.75	615	1020
183	*Cyathicula microspora*	*Cyathicula microspora*	Asco		SH014066.06FU	99.36	469	850
182	*Cyathicula* sp. 1	*Cyathicula microspora*	Asco		SH014066.06FU	93.63	471	702
34	*Geomyces* sp. 1	*Geomyces destructans*	Asco	EU854569		96.73	888	1476
4	*Geopora* sp. 1	*Geopora* sp.	Asco		SH232047.06FU	98.72	546	968
129	*Gloeotinia temulenta*	*Gloeotinia temulenta*	Asco		SH235673.06FU	99.80	506	928
349	*Gyoerffyella* sp. 1	*Gyoerffyella* sp.	Asco		SH234755.06FU	97.35	452	765
353	*Hebeloma kuehneri*	*Hebeloma kuehneri*	Basid	GU234097		97.52	685	1188

Appendix cont.

Phylobin	Assigned study name	Best Match	Phylum	GenBank	UNITE ID	Match	Alignment Length (bp)	Score
1	Helotiaceae sp. 1	*Leohumicola minima*	**Asco**	AY706329		91.95	559	765
92	Helotiaceae sp. 2	Helotiaceae sp.	Asco	HQ157915		93.11	537	784
180	Helotiaceae sp. 3	uncultured Helotiaceae	Asco		SH030055.06FU	97.38	458	774
377	Helotiaceae sp. 4	Helotiales sp.	Asco		SH444216.06FU	87.31	536	592
329	Helotiales sp. 1	*Leptodontidium* sp.	Asco		SH034136.06FU	99.06	531	953
347	Hyaloscyphaceae sp. 1	*Calycina languida*	Asco		SH208263.06FU	90.74	475	638
175	*Hygrophorus pustulatus*	*Hygrophorus pustulatus*	Basid	FJ845412		99.80	501	920
282	*Inocybaceae* sp. 1	*Inocybaceae* sp.	Basid		SH240210.06FU	99.31	583	1051
6	*Lachnum* sp. 1	uncultured *Lachnum*	Asco		SH189775.06FU	97.44	468	795
89	*Lactarius lilacinus*	*Lactarius lilacinus*	Basid		SH238120.06FU	99.21	630	1136
63	*Leccinum rotundifoliae 1*	*Leccinum rotundifoliae*	Basid	GU234155		98.62	1088	1925
385	*Leccinum rotundifoliae 2*	*Leccinum rotundifoliae*	Basid	GU234155		98.98	1177	2102
284	*Leohumicola minima*	*Leohumicola minima*	Asco	AY706329		97.27	549	928
357	*Leptodontidium orchidicola 1*	*Leptodontidium orchidicola*	Asco	AF486133		98.93	652	1168
359	*Leptodontidium orchidicola 2*	*Leptodontidium orchidicola*	Asco	AF486133		97.85	650	1118
360	*Leptodontidium orchidicola 3*	*Leptodontidium orchidicola*	Asco	AF486133		98.29	644	1125
361	*Leptodontidium orchidicola 4*	*Leptodontidium orchidicola*	Asco	AF486133		98.59	638	1127

Phy-lobin	Assigned study name	Best Match
363	*Leptodontidium orchidicola 5*	*Leptodontidium orchidicola*
14	*Lycoperdon foetidum*	*Lycoperdon foetidum*
76	*Meliniomyces bicolor*	*Meliniomyces bicolor*
316	*Mycosphaerella tassiana*	*Mycosphaerella tassiana*
96	*Penicillium* sp. 1	*Penicillium polonicum*
46	*Phialocephala fortinii 1*	*Phialocephala fortinii*
47	*Phialocephala fortinii 2*	*Phialocephala fortinii*
49	*Phialocephala fortinii 3*	*Phialocephala fortinii*
339	*Phialocephala fortinii 4*	*Phialocephala fortinii*
366	*Phialocephala lagerbergii*	*Phialocephala lagerbergii*
320	*Piloderma* sp. 1	*Piloderma* sp.
128	Plectosphaerellaceae sp.	*Verticillium leptobactrum*
3	Pyronemataceae sp. 1	Pyronemataceae sp.
172	Pyronemataceae sp. 2	Pyronemataceae sp.
61	*Russula cessans*	*Russula cessans*
59	*Russula* sp. 1	*Russula cessans*

Appendix cont.

Phylum	GenBank	UNITE ID	Match	Align-ment Length (bp)	Score
Asco	AF486133		98.90	637	1138
Basid		SH244736.06FU	97.42	658	1120
Asco		SH207170.06FU	99.79	475	872
Asco		SH196750.06FU	98.26	461	819
Asco		SH231333.06FU	98.82	507	902
Asco		SH213468.06FU	98.32	476	833
Asco		SH213468.06FU	98.82	592	1055
Asco		SH213468.06FU	98.95	476	850
Asco		SH213468.06FU	97.69	477	824
Asco		SH204727.06FU	99.20	500	900
Basid	UDB001733		100.00	621	1147
Asco		SH235676.06FU	94.11	475	715
Asco		SH019273.06FU	97.37	533	905
Asco		SH216337.06FU	98.37	552	966
Basid	FJ845437		98.92	646	1151
Basid	FJ845437		93.32	704	1044

Phylobin	Assigned study name	Best Match
60	*Russula* sp. 2	*Russula cessans*
380	*Russula* sp. 3	*Russula cessans*
262	*Russula versicolor*	*Russula versicolor*
343	*Sebacina dimitica*	*Sebacina dimitica*
85	*Sebacina epigaea*	*Sebacina epigaea*
57	*Sebacina* sp. 1	*Sebacina* sp.
119	*Sebacina* sp. 2	*Sebacina* sp.
159	*Sebacina* sp. 3	*Sebacina* sp.
161	*Sebacina* sp. 4	*Sebacina* sp.
344	*Sebacina* sp. 5	*Sebacina* sp.
328	*Sebacinaceae* sp. 1	uncultured Sebacinaceae
24	*Tetracladium maxilliforme*	*Tetracladium maxilliforme*
25	*Tetracladium* sp. 1	*Tetracladium apiense*
12	*Thelephora* sp. 1	uncultured *Thelephora*
100	*Thelephora terrestris*	*Thelephora terrestris*
20	Thelephoraceae sp. 1	Thelephoraceae sp.
21	Thelephoraceae sp. 2	Thelephoraceae sp.
99	Thelephoraceae sp. 3	Thelephoraceae sp.
197	*Tomentella ellisii*	*Tomentella ellisii*
132	*Tomentella ferruginea* 1	*Tomentella ferruginea*

Appendix cont.

Phylum	GenBank	UNITE ID	Match	Align-ment Length (bp)	Score
Basid	FJ845437		94.64	634	976
Basid	FJ845437		95.04	605	941
Basid		SH224391.06FU	98.69	610	1081
Basid		SH231629.06FU	98.49	530	931
Basid	EU819519		95.69	532	907
Basid	HQ215803		99.82	542	996
Basid		SH305078.06FU	98.30	529	926
Basid		SH231609.06FU	99.25	534	963
Basid		SH231664.06FU	98.71	541	957
Basid		SH231595.06FU	99.26	541	977
Basid		SH008994.06FU	92.15	522	721
Asco	EU883429		97.13	593	1000
Asco		SH216431.06FU	94.43	485	741
Basid		SH016849.06FU	96.30	595	976
Basid	HM189958		93.22	708	1031
Basid		SH195974.06FU	94.75	590	917
Basid		SH195974.06FU	93.00	586	848
Basid		SH219843.06FU	95.52	536	856
Basid		SH222919.06FU	98.88	536	957
Basid	AF272909		98.74	555	985

Appendix cont.

Phylobin	Assigned study name	Best Match	Phylum	GenBank	UNITE ID	Match	Alignment Length (bp)	Score
134	*Tomentella ferruginea* 2	*Tomentella ferruginea*	Basid	AF272909		98.17	546	952
7	*Tomentella* sp. 1	*Tomentella* sp.	Basid		SH213382.06FU	99.65	576	1055
239	*Tomentella* sp. 10	*Tomentella ellisii*	Basid		SH222911.06FU	96.61	590	977
245	*Tomentella* sp. 11	uncultured Tomentella	Basid		SH202465.06FU	100.00	579	1070
264	*Tomentella* sp. 12	uncultured *Tomentella*	Basid		SH202475.06FU	97.74	576	992
269	*Tomentella* sp. 13	*Tomentella* sp.	Basid	U83482		96.05	683	1110
379	*Tomentella* sp. 14	*Tomentella sp.*	Basid		SH202538.06FU	99.15	587	1055
8	*Tomentella* sp. 2	uncultured *Tomentella*	Basid		SH199023.06FU	98.78	572	1016
9	*Tomentella* sp. 3	uncultured *Tomentella*	Basid		SH009486.06FU	93.14	583	848
16	*Tomentella* sp. 5	*Tomentella sublilacina*	Basid	HM189986		93.77	658	979
22	*Tomentella* sp. 6	*Tomentella sublilacina*	Basid	HM189994		95.83	695	1120
98	*Tomentella* sp. 7	uncultured Tomentella	Basid		SH021829.06FU	94.67	582	896
221	*Tomentella* sp. 8	*uncultured Tomentella*	Basid		SH202719.06FU	96.89	578	963
223	*Tomentella* sp. 9	uncultured *Tomentella*	Basid		SH219870.06FU	98.14	590	1027
224	*Tomentella viridula*	*Tomentella viridula*	Basid	AF272914		99.66	581	1061
280	*Tomentellopsis* sp. 1	*Tomentellopsis submollis*	Basid		SH199523.06FU	96.98	597	1009

Appendix cont.

Phy-lobin	Assigned study name	Best Match	Phylum	GenBank	UNITE ID	Match	Align-ment Length (bp)	Score
192	*Trichophaea* sp. 1	*Trichophaea* cf *hybrida*	Asco		SH227980.06FU	100.00	522	965
10	*Tuber* sp. 1	uncultured *Tuber*	Asco		SH204354.06FU	99.66	592	1081
62	*Tuber* sp. 2	*Tuber rapaeodorum*	Asco	EU784430		95.88	582	937
384	*Tuber* sp. 3	uncultured *Tuber*	Asco		SH204354.06FU	99.66	592	1081
41	Vibrisseaceae sp. 1	*Phialocephala fortinii*	Asco		SH016932.06FU	84.11	151	147
149	*Wilcoxina rehmii* 1	*Wilcoxina rehmii*	Asco		SH023929.06FU	98.24	455	795
150	*Wilcoxina rehmii* 2	*Wilcoxina rehmii*	Asco		SH023929.06FU	97.58	455	778
151	*Wilcoxina rehmii* 3	*Wilcoxina rehmii*	Asco		SH023929.06FU	98.46	455	800
153	*Wilcoxina rehmii* 4	*Wilcoxina rehmii*	Asco		SH023929.06FU	97.80	455	785

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